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

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Running title: **Blocking TLR-TICAM-1 pathway by RSV sG**

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Summary

Monocyte-derived dendritic cells (mDCs) recognize viral RNA extrinsically by TLR3 on the membrane and intrinsically RIG-I/MDA5 in the cytoplasm to induce type I interferons (IFNs) and mDC maturation. When mDCs were treated with live or UV-irradiated respiratory syncytial virus (RSV), early (~4 h) induction of IFN-β detected 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 Abs 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 protein remains undetermined, G proteins may neutralize the F protein function that promotes IFN-mediated mDC modulation via TLR4 and may cause insufficient raising cell-mediated immunity against RSV.

Key words: Toll-like receptor, TICAM-1 (TRIF), respiratory syncytial virus, type I interferons, dendritic cells

Introduction

Respiratory syncitial 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 cytotoxic T lymphocytes (CTL), natural killer (NK), and B cells, develops followed by maturation of mDCs (2). These lymphocytes produce gamma interferon (IFN-γ) which orchestrates the acquired immune response to eradicate viral infection (3). Toll-like receptors (TLRs), 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 anti-viral 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 dsRNA, whereas TLR3 captures only dsRNA. Their signaling pathways partially overlap in that they converge upon the IRF3-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-homology (TIR) domain of TLR3 recruits the adaptor molecule TICAM-1 (TRIF) (13,14), while lipopolysaccharide (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 MDA5 reside in the cytoplasm and interact with a mitochondrial protein, IPS-1/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 (p)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 through 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 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 proinflammatory cytokines (29). On the other hand, the G glycoprotein (G protein), that mediates attachment of the virus particle to the target cell (31), and SH protein is not functionally well-understood (32). Infected cells also produce a smaller secreted form of the G protein (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.

Materials and methods

Cell culture, viruses and reagents

HEp-2, Vero and HEK293 cells were maintained in DMEM (Invitrogen, San Diego, CA) supplemented with 10% heat-inactivated FCS (JRH Biosciences, Lenexa, KS) 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 $TCID_{50}$ 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. The mycoplasma lipopeptide MALP-2 was prepared as described (35). MALP-2 and poly I:C were treated with polymyxin B (10 μg/ml) (an LPS inhibitor) for 1 h at 37 ˚C before stimulation of cells (35). Usually, 50 or 10 μg/ml of poly I:C, 100 ng/ml of LPS and 100 nM of MALP-2 were utilized for TLR stimulation. Mouse IgG, mouse immunoglobulin G2b (IgG2b), and anti-Flag M2 monoclonal antibody (mAb), and anti-Flag polyclonal Abs 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); anti-CD40 mAb was from PharMingen (San Diego, CA); fluorescein isothiocyanate (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); and FITC-labeled and non-labeled goat anti-RSV polyclonal antibody was from Chemicon.

Preparation of dendritic cells (mDCs)

Human 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) (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 human GM-CSF, 100 IU/ml human IL-4 (Prepro Tech, London UK) and antibiotics for 6 days. Morphological changes were examined by phase contrast microscopy (Olympus IX-70, Tokyo Japan).

Flow-activated cell-sorting (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 Ab. Cells were washed, and their fluorescence intensities were measured by FACS.

Determination of human TNF-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 TNF-α or IFN-β in the culture medium was determined with enzyme-linked immunosorbent assay (ELISA) kits (Amersham Pharmacia and FUJIREBIO, Tokyo Japan). The detection limits of human TNF-α and IFN-β were < 5 pg/ml and < 2.5 U/ml, 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, WI) 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; 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, CA), pELAM-Luc reporter plasmid was constructed as referred in the ref (29). pRL-TK vector was from Promega, WI. 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 from Dr. K Miyake (the University of Tokyo, Tokyo), that for TBK1 expression from Dr. M Nakanishi (the Nagoya City University, Nagoya) and that for IKKε expression from Dr. T Maniatis (Harvard Medical School, Boston). Plasmids for constitutive active forms of RIG-I and MDA5 (ΔRIG-I and MDA5N) expression were kindly provided from Dr. 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, CA) 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, WI). 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 PMSF. 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, 1 % NP-40, or 50 mM sodium citrate (pH 5.0), 0.5 % SDS, and incubated at 37 ˚C for 14 h with Endoglycosidase F (EndoF) (Takara) or Endoglycosidase H (Endo 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 (mDC, HEK293 cells) were transfected with pGV-E2/huELAM (ELAM promoter-linked firefly luciferase) or pISRE-Luc (ISRE promoter-linked firefly luciferase), and phRL-TK (thymidin kinase with renilla luciferase). The last one is the internal control. 24 hours later, cells were washed and treated with live or UV-irradiated RSV (MOI = 0.5, otherwise indicated), LPS, or medium. In some experiments, Abs against RSV proteins (20

μg/ml) 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 soluble G 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. 6 h 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, WI). 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 Abs by FACS analysis (Fig. 1A). Thus, human mDCs are susceptible to RSV of this subgroup B isolate.

To examine DC maturation by RSV, mDCs were stimulated with live or UV-irradiated RSV, LPS (TLR4-ligand), MALP-2 (TLR2-ligand), or poly I: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. 24 h 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) 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 butfailed 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 (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,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). These activation was not due to contaminating LPS since the HEK293 cells did not express TLR4 (Fig. 3A,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,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 poly I: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 poly I: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 poly I: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 Abs 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. 4D, 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/poly I: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 of these proteins tended to form multimers. In particular, the SH and F proteins formed multimers, that 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 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 poly I:C and afer 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 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. Overexpressing RSV soluble G (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, poly I:C or media (presence or absence of the RSV sG protein for 6 h (Figs. 6B and C). ISRE activation by LPS-TLR4 (Fig. 6B) and poly I: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 preincubated with mDCs, partially suppressed the increase of the IFN-β message by live and UV-irradiated RSV up to 4 hours 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. 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). 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 measles virus (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 (Figs. 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 (Matsumoto M., and Seya T., 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. 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 dendritic cells. 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 (Matsumoto and 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, a recent report (41) suggested that the TIR-containing adaptor SARM exhibits such 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 (42). 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 critially 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 secret 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 (10,43), 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 of the TICAM-1 pathway may benefit RSV survival and happen to suppress mDC-derived cellular immune responses. Sever 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 (44-46). 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 (GCRR) of the RSV sG protein inhibits production of NF-κB-inducible inflammatory cytokines through TLR4 (47). Since MyD88 is not a target of the RSV G 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 (48). Vaccinia virus proteins also target

TLR adaptor proteins (49). 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.

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Abbreviations:

Ag, antigen; DC, dendritic cell; RSV F protein, fusion glycoprotein; G protein, RSV glycoprotein; IFN, interferon; IFNAR, type I IFN-alpha receptor; IKKε, IκB kinase-related kinase ε; IPS-1, interferon-β promoter stimulator 1; IRF, IFN regulatory factor; LPS, lipopolysaccharide; MALP, macrophage-activating lipopeptide; MAVS, Mitochondria antiviral signaling; MDA5, melanoma differentiation associated gene 5; mDC, monocyte-derived DC; moi, multiplicity of infection; MV, measles virus; NAP, NAK-associated protein 1; pDC, plasmacytoid DC; p.i., post infection; RIG-I, retinoic acid-inducible gene I; RSV, respiratory syncytial virus; sG proteins, soluble G proteins; TBK1, TANK-binding kinase; TCID50, 50% tissue culture infective dose; TLR, Toll-like receptor.

Figure legends

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 Abs 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). 24 h later, mDCs were allowed to react with the indicated Abs 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". Panel 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) or medium were used as controls. 24 h later, the supernatants were collected, and the levels of IFN-β were measured by ELISA. Asterisk, not detected.

Fig. 2 Early induction of minute amounts of IFN-β through RSV-mDC interaction

Panels A,B: Early induction of TNF-α and IFN-β by 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. 1E. 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. 1E was used. 24 h later, mRNA levels of the indicated genes were assessed by RT-PCR. GAPDH is a control.

Fig. 3. Replication-dependent promoter activation by live RSV

Panel A, 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. 1E. At timed intervals, Luciferase reporter activity was determined for ELAM (A) and ISRE (B) activation. Panel C,D: HEK293 cells with the ELAM or ISRE reporters were transfected with the plasmid set for TLR4 expression. 24 h later, cells were washed and treated with RSV (MOI = 0.5), LPS, or medium. 6 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.

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 pEF-Bos TLR4 plasmid sets (TLR4, MD-2 and CD14) (13) or TLR3 plasmid. 24 h 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 (panel A) or poly I:C (panel B). 6 h 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. 24 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 panel B). Under the conditions where the RSV-mediated inhibition was observed, pAbs against RSV were added to the cells (RSV-live, anti-RSV pAbs). 6 h 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 RSVenvelope 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 Panel A). 24 h later, cell lysates were immunoprecipitated with anti-Flag Ab 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 panel B were treated with endo H or endo F, and analyzed on SDS-PAGE followed by immunoblotting. The conditions of the analyzed samples are shown in the panel.

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, pEF-Bos TLR3, and indicated plasmids encoding RSV proteins. 24 hours later, cells were incubated with 10 μg/ml of poly I:C or buffer only. After 6 h, dual luciferase reporters were assayed as in Fig. 4A. Panel B, C: sG protein inhibits ISRE activation by LPS or poly I: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 pEF-Bos TLR4 expression plasmids or TLR3 plasmid. 24 h later, cells were stimulated with 100 ng/ml of LPS (B) or 10 μg/ml of poly I: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. 4A. 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

Panels 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. 24 h later, the supernatants were harvested to measure the IFN-β content. LPS was used as control as in Fig. 2D. 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. 20 h later, the IFN-β levels released in the supernatant of mDCs were determined by ELISA.

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. 6 h later, cells were washed, lysed with lysis buffer and the reporter assay was performed as in Fig. 4A. 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.

References

1. Ogra, P. L. 2004. Respiratory syncytial virus: the virus, the disease and the immune response. Paediatr Respir Rev 5:119-26.

2. Steinman, R. M., and Hemmi, H. 2006. Dendritic cells: translating innate to adaptive immunity. Curr Top Microbiol Immunol 311:17-58.

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 186:421-432.

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 27:352-357.

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 176:1733-1740.

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 81:1401-1411.

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 79:3350-3357.

8. Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., Poeck, H., Akira, S., Conzelmann, K. K., Schlee, M., Endres, S., and Hartmann, G. 2006. 5'-Triphosphate RNA is the ligand for RIG-I. Science 314:994-997.

9. Pichlmair, A., Schulz, O., Tan, C. P., Naslund, T. I., Liljestrom, P., Weber, F., and Reis e Sousa, C. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. Science 314:997-1001.

10. Sasai, M., Shingai, M., Funami, K., Yoneyama, M., Fujita, T., Matsumoto, M., and Seya, T. 2006. NAK-assiciated protein 1 participates in both the TLR3 and the cytoplasmic pathways in Type I IFN induction. J Immunol 177:8676-8683.

11. Akazawa, T., Ebihara, T., Okuno, M., Okuda, Y., Shingai, M., Tsujimura, K., Takahashi, T., Ikawa, M., Okabe, M., Inoue, N., Okamoto-Tanaka, M., Ishizaki, H., Miyoshi, J., Matsumoto, M., and Seya, T. 2007. Antitumor NK activation induced by

the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. Proc Natl Acad Sci U S A. 104:252-257.

12. Schulz, O., Diebold, S. S., Chen, M., Naslund, T. I., Nolte, M. A., Alexopoulou, L., Azuma Y. T., Flavell, R. A., Liljestrom, P., and Reis e Sousa, C. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature 433:887-892.

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 4:161-167.

14. Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K., and Akira, S. 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 169:6668-6672.

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 278:49751-49762.

16. Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., and Akira, S. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat Immunol 4:1144-1150.

17. Matsumoto, M., and Seya, T. 2008. TLR3: Interferon induction by double-stranded RNA including poly(I:C). Adv Drug Deliv Rev. 60: 805-812.

18. Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K. J., Takeuchi, O., and Akira, S. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol 6:981-988.

19. Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R., and Tschopp, J. 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 437:1167-1172.

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 122:669-682.

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 19:727-740.

22. Takeuchi, O., and Akira, S. 2008. MDA5/RIG-I and virus recognition. Curr Opin

Immunol. 20: 17-22.

23. Honda, K., Yanai, H., Negishi, H., Asagiri, M., Sato, M., Mizutani, T., Shimada, N., Ohba, Y., Takaoka, A., Yoshida, N., and Taniguchi, T. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature. 434: 772-777.

24. Uematsu, S., Sato, S., Yamamoto, M., Hirotani, T., Kato, H., Takeshita, F., Matsuda, M., Coban, C., Ishii, K. J., Kawai, T., Takeuchi, O., and Akira, S. 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: 915-923.

25. Hoshino, K., Sugiyama, T., Matsumoto, M., Tanaka, T., Saito, M., Hemmi, H., Ohara, O., Akira, S., and Kaisho, T. 2006. IkappaB kinase-alpha is critical for interferon-alpha production induced by Toll-like receptors 7 and 9. Nature. 440: 949-953.

26. Gotoh, B., Komatsu, T., Takeuchi, K., and Yokoo, J. 2001. Paramyxovirus accessory proteins as interferon antagonists. Microbiol Immunol 45:787-800.

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: 328-339.

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 72:3095-3101.

29. Kurt-Jones, E. A., Popova, L., Kwinn, L., Haynes, L. M., Jones, L. P., Tripp, R. A., Walsh, E. E., Freeman, M. W., Golenbock, D. T., Anderson, L. J., and Finberg, R. W. 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol 1:398-401.

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. 20:531-540.

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 68:2521-2524.

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 71:8973-8982.

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 68:1705-1714.

34. Tripp, R. A. 2004. Pathogenesis of respiratory syncytial virus infection. Viral Immunol 17:165-181.

35. Nishiguchi, M., Matsumoto, M., Takao, T., Hoshino, M., Shimonishi, Y., Tsuji, S., Begum, N. A., Takeuchi, O., Akira, S., Toyoshima, K., and Seya, T. 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 166:2610-2616.

36. Ebihara, T., Masuda, H., Akazawa, T., Shingai, M., Kikuta, H., Ariga, T., Matsumoto, M., and Seya, T. 2007. NKG2D ligands are induced on human dendritic cells by TLR ligand stimulation and RNA virus infection. Int. Immunol. 19: 1145-1155.

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 79:2221-2229.

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 (Pt 2): 493-499.

39. Yoneyama, M., and Fujita, T. 2007. Function of RIG-I-like receptors in antiviral innate immunity. J Biol Chem. 282:15315-15318.

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: 1049-1055.

41. 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: 1074-1081.

42. Takaoka, A., and Taniguchi, T. 2003. New aspects of IFN-alpha/beta signalling in immunity, oncogenesis and bone metabolism. Cancer Sci. 94:405-411.

43. 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: 3180-3190.

44. Olszewska-Pazdrak, B., Casola, A., Saito, T., Alam, R., Crowe, S. E., Mei, F., Ogra, P. L., and Garofalo, R. P. 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 72:4756-4764.

45. Panuska, J. R., Merolla, R., Rebert, N. A., Hoffmann, S. P., Tsivitse, P., Cirino, N. M., Silverman, R. H., and Rankin, J. A. 1995. Respiratory syncytial virus induces interleukin-10 by human alveolar macrophages. Suppression of early cytokine production and implications for incomplete immunity. J Clin Invest 96:2445-2453.

46. Tsutsumi, H., Matsuda, K., Sone, S., Takeuchi, R., and Chiba, S. 1996. Respiratory syncytial virus-induced cytokine production by neonatal macrophages. Clin Exp Immunol 106:442-446.

47. Polack, F. P., Irusta, P. M., Hoffman, S. J., Schiatti, M. P., Melendi, G. A., Delgado, M. F., Laham, F. R., Thumar, B., Hendry, R. M., Melero, J. A., Karron, R. A., Collins, P. L., and Kleeberger, S. R. 2005. The cysteine-rich region of respiratory syncytial virus attachment protein inhibits innate immunity elicited by the virus and endotoxin. Proc Natl Acad Sci U S A 102:8996-9001.

48. Li, K., Foy, E., Ferreon, J. C., Nakamura, M., Ferreon, A. C., Ikeda, M., Ray, S. C., Gale, M., Jr., and Lemon, S. M. 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 U S A 102:2992-2997.

49. 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: 353-364.

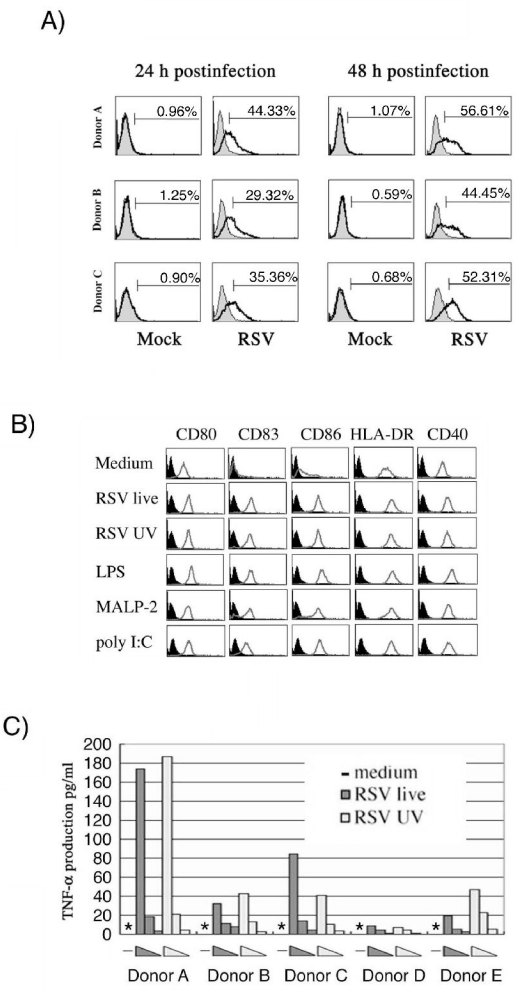

Fig. 1ABC

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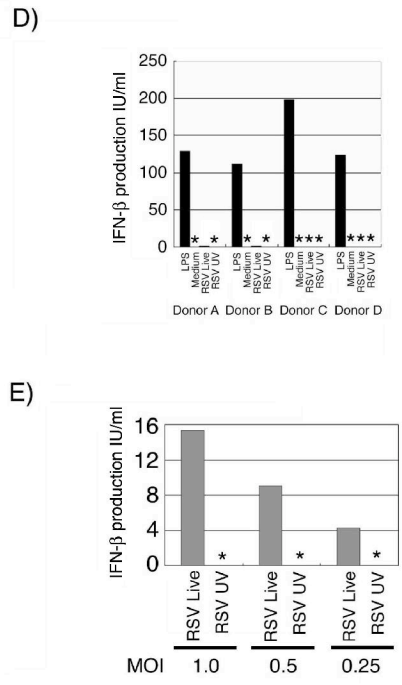

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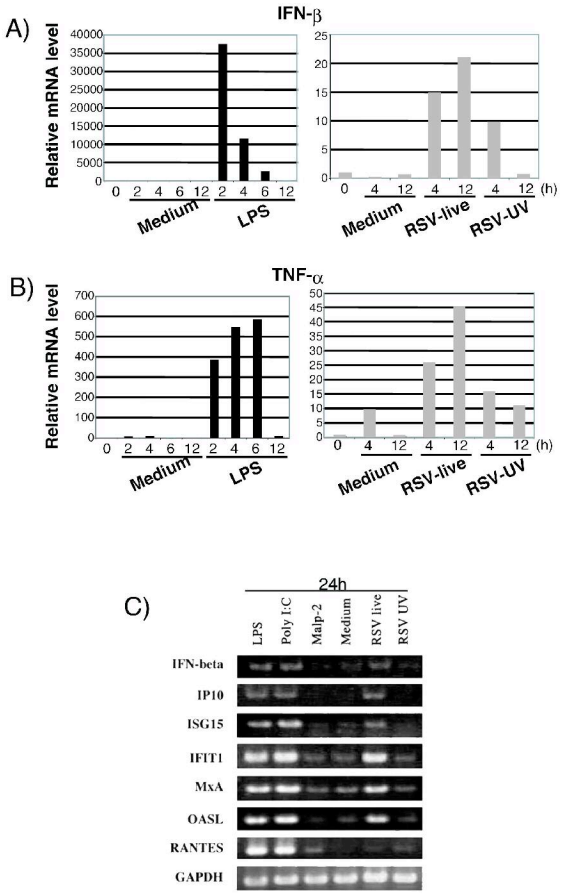

Fig.2

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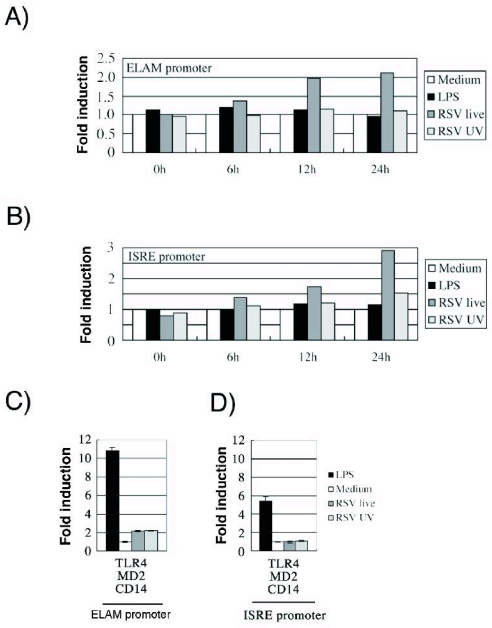

Fig.3

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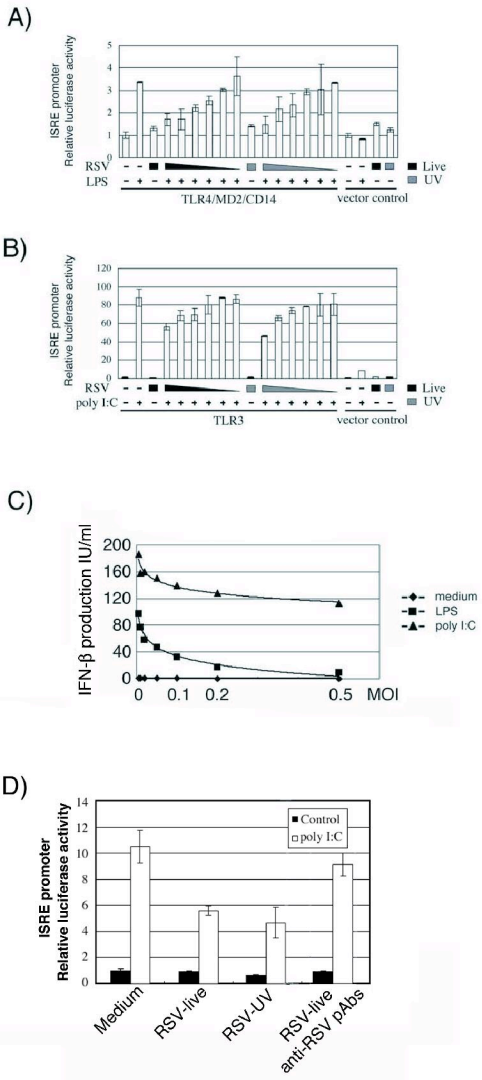

Fig.4

209x297mm (300 x 300 DPI)

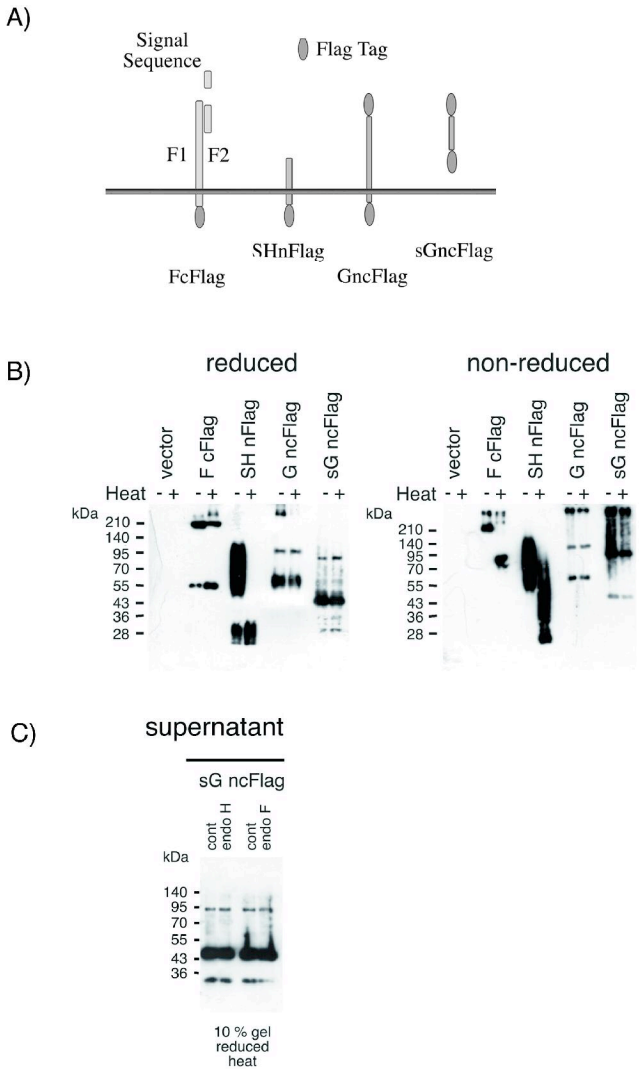

Fig.5

209x297mm (300 x 300 DPI)

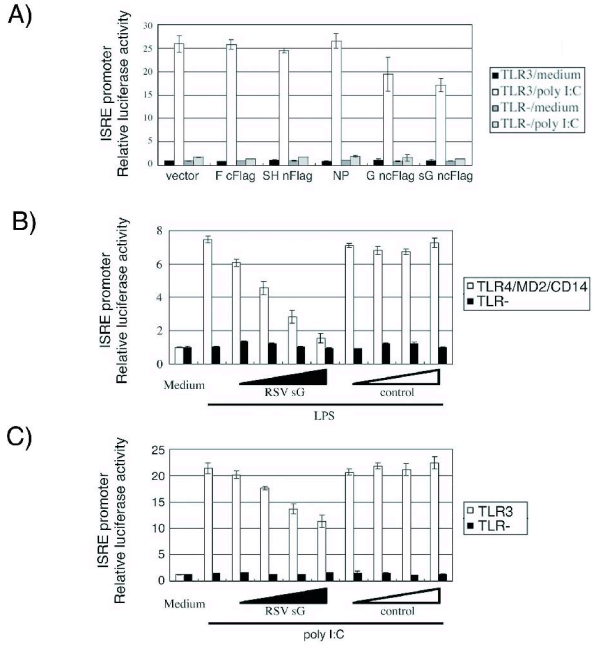

Fig.6

209x297mm (300 x 300 DPI)

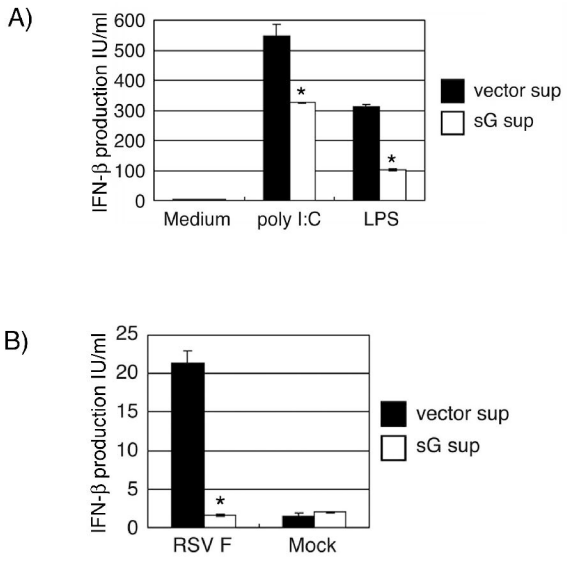

Fig.7

209x297mm (300 x 300 DPI)

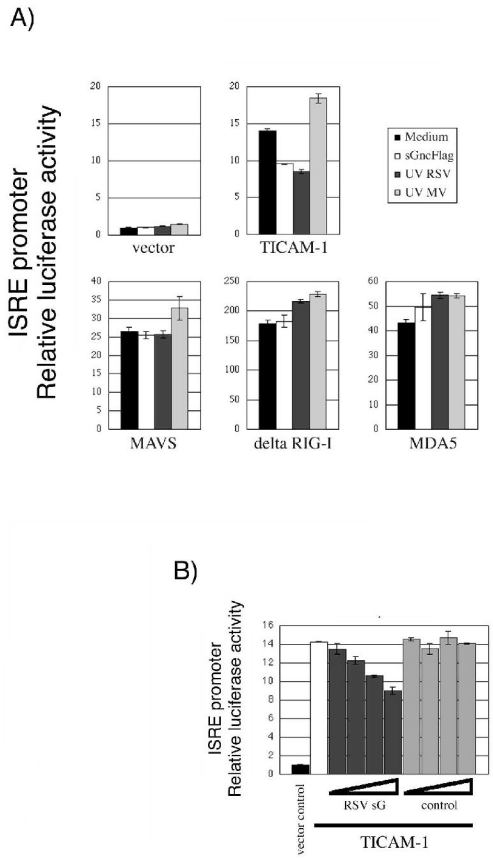
A)
ISRE promoter
Relative luciferase activity
vector
TICAM-1
MAVS
delta RIG-I
MDA5
Medium
sGncFlag
UV RSV
UV MV
B)
ISRE promoter
Relative luciferase activity
vector control
RSV sG
control
TICAM-1

Fig.8