Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus

Evelyn A. Kurt-Jones[1,2], Lana Popova[2], Laura Kwinn[2], Lia M. Haynes[3], Les P. Jones[3], Ralph A. Tripp[3], Edward E. Walsh[4], Mason W. Freeman[5], Douglas T. Golenbock[6], Larry J. Anderson[3] and Robert W. Finberg[1,2]

The innate immune system contributes to the earliest phase of the host defense against foreign organisms and has both soluble and cellular pattern recognition receptors for microbial products. Two important members of this receptor group, CD14 and the Toll-like receptor (TLR) pattern recognition receptors, are essential for the innate immune response to components of Gram-negative and Gram-positive bacteria, mycobacteria, spirochetes and yeast. We now find that these receptors function in an antiviral response as well. The innate immune response to the fusion protein of an important respiratory pathogen of humans, respiratory syncytial virus (RSV), was mediated by TLR4 and CD14. RSV persisted longer in the lungs of infected TLR4-deficient mice compared to normal mice. Thus, a common receptor activation pathway can initiate innate immune responses to both bacterial and viral pathogens.

CD14 and Toll-like receptors (TLRs) are essential for initiating the innate response to lipopolysaccharide (LPS) from Gram-negative bacteria as well as other microbial components of Gram-negative and Gram-positive bacteria, mycoplasmas, spirochetes and fungi[1–17]. The role of innate immune receptors in the response to viruses has been largely unexplored. We have examined the interactions of several respiratory syncytial virus (RSV) proteins with human monocytes and demonstrate that the fusion protein of RSV induces proinflammatory cytokines, and that this response is dependent on expression of CD14 and TLR4. We also

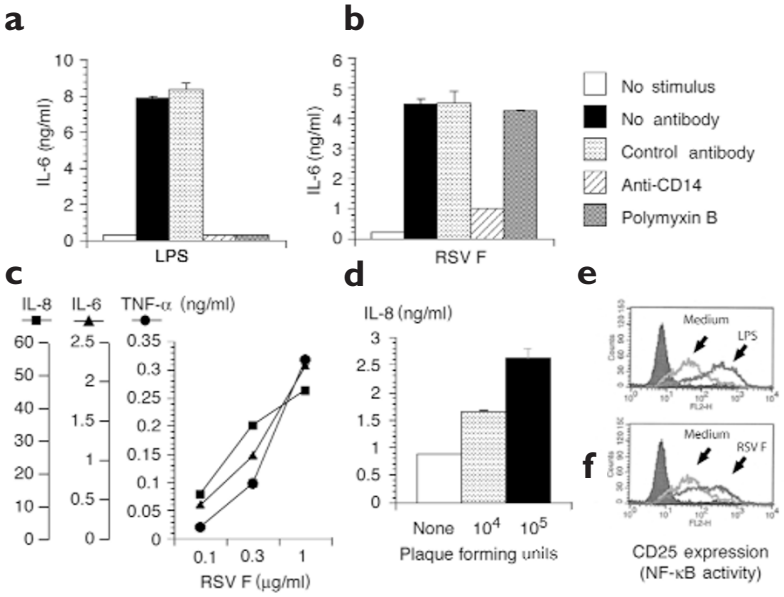


Figure 1. Viral proteins stimulate cytokine secretion from PBMC and monocytes. Purified human monocytes were stimulated with either (**a**) LPS (10 ng/ml) or (**b**) RSV fusion (F) protein (1 μg/ml). Where indicated the cells were treated with 10 μg/ml of anti-CD2 (control antibody), 10 μg/ml of MY4 (anti-CD14) or 1 μg/ml of polymyxin B (PMB). Control cultures were stimulated with medium alone or with the elution buffer used to isolate the viral proteins. Supernatants of PBMC cultures were collected 18 h after addition of the stimulants for analysis of cytokine secretion. IL-6 concentration was determined by enzyme-linked immunosorbent assay (ELISA). (**c**) Purified human monocytes were stimulated with various concentrations of RSV F. IL-8, IL-6 and TNF-α concentrations in 18 h culture supernatants were determined by ELISA. (**d**) Purified human monocytes were stimulated with various levels of sucrose gradient purified and UV-inactivated RSV. Virus levels are expressed as plaque forming units of live virus before inactivation. Culture supernatants were collected 16 h after the addition of virus and IL-8 concentrations determined by ELISA. (**e,f**) CHO cells stably transfected with human CD14 and an NF-κB promoter driven CD25 reporter gene were incubated with either (**e**) LPS (100 ng/ml) or (**f**) RSV F (1 μg/ml). CD25 expression was measured by flow cytometry of cells stained using a control PE-labeled mAb (solid figure) or PE-labeled CD25-specific mAb. CD25 staining of cells incubated in medium alone are shown as a gray lines. CD25 staining of cells incubated with (**e**) LPS or (**f**) RSV F are shown as black lines.

[1]*Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA.*
[2]*Infectious Disease Program, Department of Adult Oncology, Dana Farber Cancer Institute and Harvard Medical School, Boston, MA 02115, USA.*
[3]*Division of Viral and Rickettsial Diseases, National Center of Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA.*
[4]*University of Rochester School of Medicine and Dentistry, Rochester, NY, USA.* [5]*Massachusetts General Hospital and Harvard Medical School, Boston, MA 02115, USA.* [6]*The Maxwell Finland Laboratory for Infectious Diseases, Boston University School of Medicine, Boston, MA 02118, USA.*
Correspondence should be addressed to E. A. K.-J. (evelyn.kurt-jones@umassmed.edu).

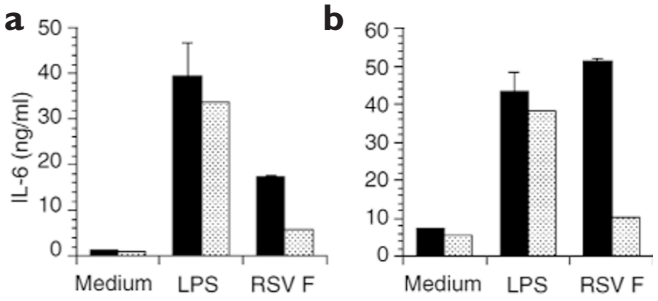


Figure 2. Cytokine-stimulating activity of RSV F protein is trypsin-sensitive. LPS or RSV F protein were incubated with trypsin immobilized on agarose beads for 15 min. Trypsin-digested (shaded bars) or mock-treated (filled bars) LPS and RSV F protein were then added to cultures of purified (**a**) human monocytes or (**b**) mouse peritoneal macrophages. Incubation of LPS or RSV F protein with immobilized agarose alone did not affect their cytokine stimulating activity. IL-6 secretion was determined by ELISA.

show that TLR4 expression has an important role in the control of viral replication *in vivo*; RSV replicated to a higher concentration and persisted longer in TLR4-deficient mice than in normal control mice.

RSV, a single-stranded negative-sense RNA virus of the *Paramyxovirus* family, is the single most common cause of serious lower respiratory tract disease of infants and young children worldwide and is a high priority for vaccine development[18,19]. Developing a safe and effective vaccine has been difficult, and it is likely that a better understanding of the pathogenesis of disease will assist vaccine development. The ability of different RSV proteins to alter the early, innate immune response is probably important to the pathogenesis of this disease[20–24]. In this study, we examined the ability of three RSV proteins—the attachment protein (G), the fusion protein (F) and the nucleocapsid protein (N)—to induce proinflammatory cytokines. Both the G and F proteins have been shown to be important in inducing protective immunity against RSV infection. Our studies reveal that RSV F protein induced interleukin 6 (IL-6) production by monocytic cells expressing CD14 and TLR4. RSV G and N proteins induced weaker cytokine responses than F. Thus, the F protein is capable of stimulating an innate immune response.

Results

Viral stimulation of inflammatory cytokines

We examined the ability of several purified RSV viral proteins to stimulate proinflammatory cytokine secretion from human mononuclear cells. We first incubated purified RSV F, G and N proteins with human peripheral blood mononuclear cells (PBMC) and found that the F but not the G or N proteins of RSV induced a marked increase in IL-6 secretion (**Fig. 1** and data not shown). This response was blocked by monoclonal antibody to CD14 (MY4) suggesting that RSV F protein induced IL-6 through its interaction with this monocyte innate immune receptor (**Fig. 1b**). Both purified human monocytes (>90% $CD14^+$) and thioglycollate-induced mouse peritoneal macrophages responded to incubation with the F protein with a marked increase in IL-6 production (**Fig. 2**) as well as IL-8, tumor necrosis factor α (TNF-α) (**Fig. 1c**) and IL-1β (data not shown), confirming the central role of monocytic cells as the source of the cytokine production. Monocytes responded in a dose-dependent manner to RSV F (**Fig. 1c**) and to UV-inactivated whole RSV virus (**Fig. 1d**).

The role of NF-κB in the response to RSV F was examined using an NF-κB reporter cell line[9]. Chinese hamster ovary (CHO) cells expressing human CD14 and a stably integrated NF-κB–driven CD25 reporter gene were incubated with RSV F or medium. RSV F-induced CD25 expression on the CHO reporter cells and the expression levels were similar to those induced by LPS treatment (**Fig. 1e,f**). CHO cells express TLR4 but not TLR2[9]; transfection with TLR2 enhanced the NF-κB–dependent reporter gene expression in response to RSV F (data not shown).

The possibility that the cytokine-stimulating activity of the RSV F protein was due to endotoxin contamination was ruled out with the use of two approaches. First, polymyxin B (PMB), a polycationic antibiotic which neutralizes LPS[25], was added to the RSV F protein preparations. PMB did not alter the ability of RSV F protein to stimulate IL-6 secretion, but did prevent the response to LPS (**Fig. 1a,b**). Second, treatment of the RSV F protein with trypsin before addition to the macrophage cultures abolished the cytokine-stimulating activity of RSV F, but had no significant effect on the cytokine-stimulating activity of LPS for human monocytes or murine macrophages (**Fig. 2**). These studies suggest that the cytokine-stimulating activity of the RSV F preparation was due to the protein component. RSV F protein isolated by either immunoaffinity chromatography or by lectin affinity chromatography was equally effective in stimulating IL-6 secretion from monocytes. In contrast, RSV G and N proteins, which were isolated by immunoaffinity chromatography under conditions similar to those used to purify RSV F, only weakly stimulated IL-6 secretion (data not shown). Further, elution buffer did not induce IL-6 secretion.

Monoclonal antibody inhibition of RSV F

RSV F–containing fractions from lectin affinity purification were further studied by monoclonal antibody (mAb) blocking, to confirm that the active component of the isolated material was the F protein itself. RSV F was incubated with mAbs to F, control mAbs to RSV G or CD3 bound to protein A–sepharose beads. The cytokine-stimulating activity of the lectin-purified RSV F was depleted by incubation with either of two mAbs to F but not with control antibodies (**Fig. 3**). In contrast, the cytokine-stimulating activity of LPS was not affected by incubation with anti-F or control antibodies.

Role of CD14 and TLR4

We further examined the role of CD14 in the response to RSV F protein using $CD14^{-/-}$ (knockout) mice. IL-6 (and IL-1β) was produced by macrophages from $CD14^{+/-}$ hemizygous mice, but not by macrophages

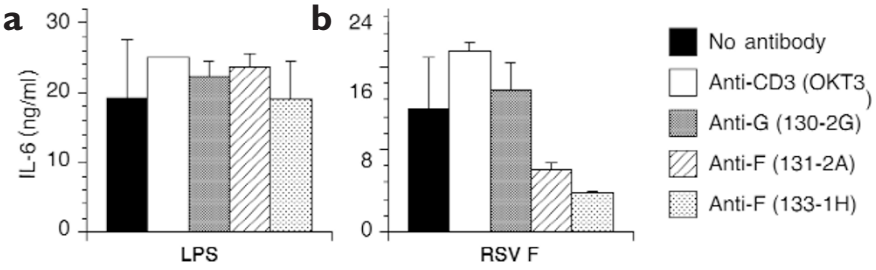


Figure 3. Monoclonal antibodies specifically block RSV F cytokine stimulating activity. (**a**) LPS or (**b**) RSV F were incubated with monoclonal antibodies to CD3 (OKT3), RSV G (130-2G) or RSV F protein (131-2A and 133-1H) immobilized on protein A sepharose beads. After removal of the beads, the LPS and RSV F preparations were added to human monocyte cultures and IL-6 secretion was measured as described above.

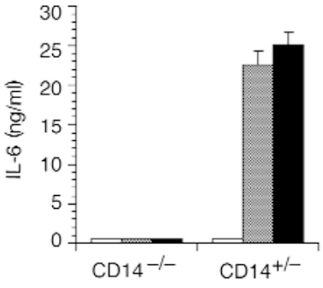


Figure 4. CD14 knockout mice do not respond to RSV F protein. Macrophages were collected from CD14$^{-/-}$ knockout mice or their CD14$^{+/-}$ hemizygous, control littermates and either unstimulated (open bars) or stimulated with 10 ng/ml of LPS (shaded bars) or 1 μg/ml of RSV F protein (filled bars). Responses to LPS and to RSV F protein were dependent on CD14. Macrophages from CD14 knockout mice responded normally to heat killed *Staphylococcus aureus* (data not shown).

from CD14$^{-/-}$ mice, when stimulated by RSV F protein (**Fig. 4**). Thus, F protein induction of IL-6 is dependent on the presence of CD14. This result suggested that RSV F protein stimulated monocytes by using receptors that shared components with the macrophage LPS receptor system.

We next examined the role of TLR4, a major LPS signal transducing receptor, in the F protein–induced IL-6 production of macrophages, using mice with two types of TLR4 deficiency. C57BL10/ScCr mice have a deletion of the gene encoding TLR4, whereas C3H/HeJ mice have a spontaneous point mutation in the intracellular domain of TLR4 that blocks LPS signaling[26–28]. These mouse strains respond poorly to LPS, suggesting that TLR4 is an important coreceptor for endotoxin responses. In both strains of TLR4-mutant mice, neither LPS nor the F protein induced IL-6 secretion (**Fig. 5**). These data suggest that the presence of TLR4 is a requirement for RSV F protein–induced IL-6 responses in monocytic cells.

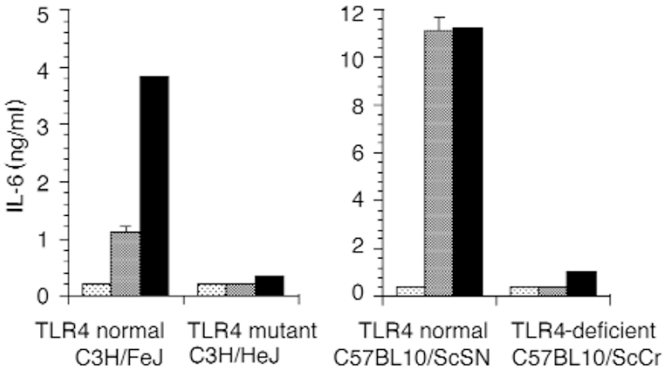
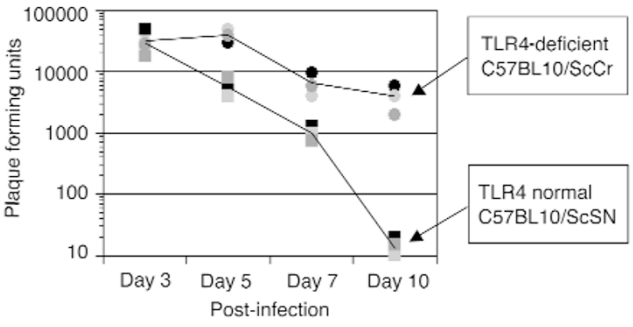


Figure 5. TLR4 is required for macrophage responses to LPS and RSV F protein. Macrophages were isolated from mice expressing normal TLR4, mutant TLR4, or mice lacking the gene encoding TLR4. The cells were either unstimulated (lightly shaded bars) or stimulated with 10 ng/ml of LPS (darkly shaded bars) or 1 μg/ml of RSV F protein (filled bars) for 18 h. IL-6 secretion into the culture supernatants was determined by ELISA.

RSV infection of TLR4-deficient mice

Our experiments suggest that TLR4 is an important receptor for innate immune recognition of RSV F protein. The immune response to the F protein plays a critical role in the severity of RSV infection[20–24]. We hypothesized that TLR4-deficient mice would be unable to mount an innate immune response to RSV F protein and, therefore, their ability to respond to RSV infection would be compromised. We tested this hypothesis using a murine model of intranasal infection with live RSV in TLR4-deficient C57BL10/ScCr mice and normal control mice (**Fig. 6**). TLR4-deficient mice have higher levels of infectious virus in the lungs than control mice. Further, the TLR4-deficient mice are either unable to clear RSV from their lungs or clear the virus several days later than control mice. (Detailed studies of RSV infection and immune function in large groups of TLR4-deficient mice have already been performed[29]. These studies confirm that TLR4-deficient mice mount a weak innate immune response to RSV compared to wild type mice.) These results suggest that TLR4 is important for clearing RSV infection. In the absence of TLR4, an inadequate response is mounted and RSV replicates to high levels in the lungs.



Discussion

Our studies suggest that RSV F protein stimulated innate immunity through shared components of the innate immune activation pathway, TLR4 and CD14. The importance of TLR proteins in the innate immune responses is suggested by their conservation in plants, insects and vertebrates[30–33]. In *Drosophila*, the immune response to fungal infection is dependent on Toll, a type I transmembrane receptor[33–35]. The *Drosophila* immune response to bacterial infection is dependent on *18 wheeler*, a Toll-like gene[36]. Toll, similar to CD14, has an extracellular domain containing leucine-rich repeats[33]. The cytoplasmic domains of Toll proteins are homologous to the cytoplasmic domain of mammalian type I interleukin 1 receptors (IL-1R) and to plant proteins involved in disease resistance[30–33]. Activation of Toll receptors leads to signal transduction and gene expression *via* the NF-κB pathway or its equivalent[33].

A family of Toll-like receptors (TLRs) has been identified in human cells[30–32]. At least two TLRs have been recently implicated in LPS-induced signal transduction, TLR2 and TLR4[5–8]. TLR4 is the major LPS receptor of murine and hamster cells, and is probably important for LPS stimulation of human cells as well[9,26–28,37]. It has also been shown that TLR2 is an LPS receptor in transfected cell lines, but unlike TLR4, its expression is not required for LPS activation[7–9]. Similarly, we find that TLR2 expression is not required for responses to RSV F protein but that TLR2 can serve as a receptor for RSV F responses (data not shown). TLR2 expression is, however, essential for responses to Gram-positive bacteria, mycobacteria and spirochetes[10–17].

We have shown that a viral protein can stimulate an innate immune response through TLRs and CD14 proteins. It is likely that activation of the innate antiviral immune response *via* TLR4 and CD14 is important in the pathogenesis of RSV disease, as well as other viral infections. Use of this common CD14-TLR receptor pathway in response to viral, bacterial and fungal components may have important implica-

Figure 6. Live RSV persists longer in TLR4-deficient than in control mice. TLR4-normal C57BL10/ScSN and TLR4-deficient C57BL10/ScCr mice were infected by intranasal inoculation with RSV A2 strain (10^6 plaque forming units per mouse). At the indicated time points, three mice of each strain were killed and the amounts of infectious virus in individual lung homogenates determined with a Vero cell plaque assay. The data shown are the mean number of plaque-forming units of RSV in individual lungs of infected mice from one of four separate experiments with similar results.

tions for control of the inflammatory response to infection. Thus, our data suggest that therapies that target the expression or signaling of TLRs or CD14 may be useful in altering the course of viral infections as well as bacterial and fungal infections.

Methods

Cell purification and stimulation. Human PBMC were isolated from leukopaks (discarded leukocytes from platelet donations) by density gradient centrifugation using Histopaque (Sigma, St. Louis, MO). Monocytes were isolated from PBMC by depletion of T and NK cells[38]. Briefly, PBMC were incubated with mAbs to CD3 (OKT3, ATCC) and CD2 (PharMingen, San Diego, CA), and $CD3^+$ $CD2^+$ cells were removed by magnetic bead depletion using goat anti-mouse immunoglobulin–conjugated magnetic beads (Advanced Magnetics Inc. Cambridge, MA) at a ratio of 10:1 bead:cell. The remaining cells were >90% monocytes, as determined by anti-CD14 staining and forward- and side-light scatter analysis using a FACScan (Becton-Dickenson, Elmhurst, IL). Monocytes were maintained in Ham's F-12 10% fetal calf serum, L-glutamine, and penicillin and streptomycin at 37 °C in 5% CO_2.

Monocytes or macrophages were stimulated with LPS (*E. coli* O111:B4, Sigma), RSV proteins or medium alone for 18 h at 37 °C. IL-6, IL-8, TNF-α and IL-1β secretion into the culture supernatants were determined by sandwich ELISA (Endogen, Cambridge, MA). Data are expressed as the mean ± s.d. of duplicate determinations. Where indicated, cells were incubated with 10 μg/ml of mAb to CD14 (MY4, Coulter-Immunotech, Westbrook, ME), control mAb to CD2 (PharMingen) or polymyxin B (1 μg/ml, Sigma) before the addition of F protein or LPS.

Peritoneal exudate macrophages were isolated from mice by intraperitoneal injection of 1 ml of a 3% w/v thioglycollate solution three days before peritoneal lavage. All cells were cultured in 24-well tissue culture plates at a concentration of 1×10^6 to 5×10^6 per well in 1 ml of medium.

RSV protein and virus preparations. RSV proteins were purified by affinity chromatography using mAbs to the F, G and N proteins according to the published method[39]. Briefly, The A2 strain of RSV was grown in Vero cell monolayers until the cytopathic effect was extensive. The culture medium was discarded and the cells lysed with detergent buffer containing 1 % Triton X-100 and 1% deoxycholic acid. After clarification by centrifugation, the lysate was passed over an affinity column containing the mAb. After washing extensively, the column was eluted with 0.1 M glycine, 0.1% Triton X-100, pH 2.5. For elution of the N protein, Triton was not included. The eluted fractions were buffered to pH 7 with Tris HCl, and the purity of the proteins assessed by PAGE and western blotting, as well as by solid-phase enzyme immunoassay using a panel of mAbs. Lectin-purified F protein was obtained from RSV A2–infected Vero cells. Briefly, RSV-infected Vero cells were frozen and thawed, sonicated and treated with 500 mM NaCl, 20 mM Tris and 0.5% Triton X-100, pH 8.3 for 1 h on ice in the presence of DNase, RNase, and a protease inhibitor (PMSF). The treated cell lysate was centrifuged for 1 h at 27,000*g* to remove cell components and virus. The clarified supernatant was recirculated over a concanavalin A lectin column for 16 h. After washing the column with PBS, the lectin-bound F protein was eluted by competition with free mannose and GlcNAc in a PBS-based elution buffer. RSV F was also enriched by two passages over a wheatgerm agglutinin column to remove G protein (see Web Figure 1 on the supplementary information page of *Nature Immunology* on line). The eluted material was highly enriched for F protein and made free of G protein by western blotting. Where indicated, 100 X stocks of RSV F protein (100 μg/ml) or LPS (1 μg/ml) were incubated with immobilized trypsin (Sigma) for 15 mins at 37 °C. Trypsin beads were removed by centrifugation before addition of the stimulants to cultured monocytic cells. Monoclonal antibodies to RSV F, G and CD3 (100 μg) bound to protein A sepharose beads (100 μl) were added to 2X stocks of RSV F (2 μg/ml) or LPS (20 ng/ml) in 600 μl of culture medium. After an 18-h incubation at 4 °C, the beads were removed by centrifugation and the stimulants were added to cultured monocytes.

RSV infection. Mice were infected with RSV strain A2 grown in Vero cells and titered as previously described[22,40]. Mice were anesthetized and inoculated intranasally with 10^6 plaque forming units of RSV. At various time points after infection, mice were killed and lung viral titers were determined by incubating Vero cells with serial dilutions of lung homogenates or a virus standard[41]. Viral plaques were enumerated 24 h later in methanol fixed and hematoxylin-eosin stained Vero cell cultures[22,40].

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