Respiratory Syncytial Virus–Induced Activation of Nuclear Factor–κB in the Lung Involves Alveolar Macrophages and Toll-Like Receptor 4–Dependent Pathways

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The transcription factor nuclear factor (NF)–κB controls the expression of numerous respiratory syncytial virus (RSV)–inducible inflammatory and immunomodulatory genes. Using a BALB/c mouse model, the present article shows that RSV potently and specifically activates NF-κB in vivo, a process that involves nuclear translocation of the subunits RelA, p50, and c-Rel in the lung. By depletion of alveolar macrophages (AMs) in BALB/c mice and use of C3H/HeJ mice lacking a functional Toll-like receptor (TLR)–4 signaling pathway, we demonstrate the existence of distinct but sequentially integrated RSV-inducible early NF-κB responses in the lung. The first response occurs early after RSV inoculation, is AM and TLR4 dependent, and is viral replication independent, whereas the second response involves epithelial cells and/or inflammatory cells, is TLR4 independent, and requires viral replication. NF-κB may be considered a central activator of not only inflammatory but also innate immune responses to RSV.

Respiratory syncytial virus (RSV), the major cause of serious lower respiratory tract infections in infancy and early childhood [1], can be considered among the most potent biological stimuli inducing the expression and/or secretion of proinflammatory and immunomodulatory mediators [2–4]. These events have been extensively demonstrated in airway epithelial cells, the primary site of RSV replication, and in other cell types that are targets of abortive viral replication or viral attachment only (such as monocytes/macrophages, eosinophils, and neutrophils). In vitro, RSV has been shown to induce expression of a number of genes in epithelial cells and macrophages, including the cytokines interleukin (IL)–1 [5], tumor necrosis factor (TNF)–α, IL-6 [6], and IL-10 [7]; the CXC chemokines IL-8, growth-related oncogene (GRO)–α, epithelial-derived neutrophil–activating protein (ENA)–78, interferon (IFN)–inducible protein (IP)–10, and I-TAC (IFN-inducible T cell α-chemoattractant) [4, 6, 8]; the CC chemokines RANTES, macrophage inflammatory protein (MIP)–1α, macrophage chemoattractant protein (MCP)–1, and TARC (thymus- and activation-regulated chemokine) [4, 9, 10]; the CX_3C chemokine fractalkine [4]; and IFN-β [11]. The expression of the membrane-associated adhesion and antigen-processing molecules intercellular adhesion molecule–1 [5, 12], major histocompatibility complex–I [11], and transporter associated with antigen presentation 1 (TAP-1)/low-molecular-mass protein (LMP)–2/LMP-7 [13] are also strongly induced after RSV infection in epithelial cells. Studies in experimental animal models and in human infants with naturally acquired infection have confirmed that production of many of these gene products, and others not yet described in vitro, is strongly induced or up-regulated in the airway mucosa during RSV infection, and some of them seem to play a crucial role in the pathogenesis of lung pathologic disorders and in the severity of clinical disease [14–18].

A number of highly inducible genes encoding cytokines, chemokines, and acute-phase reactant and adhesion molecules contain NF-κB–binding sites in their proximal promoters [19]. NF-κB constitutes a family of inducible transcription factors that include the potent RelA (p65) transactivator, RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52) subunits. The prototypical NF-κB complex, composed of p50–p65 heterodimers, is regulated by its association with a family of cytoplasmic inhibitors, IκBs, whose members bind and specifically inactivate NF-κB members by masking their nuclear localization sequence (NLS) and preventing nuclear translocation [20]. Extracellular stimuli initiate a signaling cascade that leads to rapid phosphorylation

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of IκBα by 2 IκB kinases (IKK), IKKα and IKKβ, at specific N-terminal serine residues. This event is coupled to the rapid ubiquitination and proteolytic degradation of phosphorylated IκBα through the 26S proteasome, thereby exposing the NLS and freeing NF-κB to translocate to the nucleus, where it binds and activates target genes [21]. We and others have shown that RSV induces NF-κB activation (translocation) in epithelial cells [22–24] through a mechanism that involves proteolysis of RelA-associated IκBα [25, 26] and a dual pathway that involves the 26S proteasome [25] and activation of the IKK complex (authors' unpublished data). Mutations of the NF-κB–binding site or inhibition of its translocation have been shown to completely abrogate RSV-inducible expression of the IL-8 and RANTES genes [22, 27, 28].

Here, using an experimental mouse model, we demonstrate that RSV infection activates the NF-κB pathway in vivo. Of interest, we show that, at the earliest time points after inoculation, RSV is able to induce NF-κB–binding activity in the lung independently from its ability to replicate. Finally, we demonstrate the crucial role that alveolar macrophages (AMs) and the Toll-like receptor (TLR)–4 play in the process of NF-κB activation, which suggests a link between the process of viral pathogen recognition and NF-κB–mediated initiation of innate immune and inflammatory responses.

Materials and Methods

RSV preparations. The human long strain of RSV (A2) was grown in Hep-2 cells (American Type Culture Collection) and purified by polyethylene glycol precipitation, followed by centrifugation on 35%–65% discontinuous sucrose gradients, as described elsewhere [10]. The virus was aliquoted, quick frozen, and stored at −70°C until used. Virus titer was determined by a methylcellulose plaque assay [29]. Purified RSV (pRSV) was UV-inactivated (UV-pRSV), as described elsewhere [10], and lack of viral replication was confirmed by the methylcellulose plaque assay. pRSV and UV-pRSV preparations were tested and found to be free of contaminating cytokines, including IL-1, IL-6, IL-8, TNF-α, granulocyte-macrophage colony-stimulating factor, and IFNs [30]. Crude, nonpurified RSV (cRSV) was grown in confluent monolayers of HEp-2 cells, harvested, clarified by centrifugation, and stored at −70°C until used [11]. Uninfected control HEp-2 cell lysates were obtained from confluent cell monolayers, clarified by centrifugation, and stored at −70°C. Viral and cellular preparations were routinely tested for mycoplasma contamination by polymerase chain reaction analysis and were used if they had <0.125 EU/mL endotoxin (by Limulus assay; BioWhittaker).

Mice and infection protocol. BALB/c (Harlan) and C3H/HeJ and C3H/HeSnJ mice (Jackson Laboratory) were housed in pathogen-free conditions in the animal research facility of the University of Texas Medical Branch, Galveston. Under light anesthesia, mice were inoculated intranasally (inl) with either 10 or 50 μL of pRSV (final inoculum, 10^7 pfu) diluted in PBS. In preliminary experiments, these 2 volumes of RSV inoculum gave similar patterns of response; therefore, the 50-μL volume was selected in all subsequent experiments [31]. Control mice were sham-inoculated with 50 μL of PBS. At the indicated time points after infection, mice were anesthetized with an intraperitoneal injection of ketamine and xylazine before the thoracic cavity was opened. Mice were exsanguinated via heart puncture, and their tracheas were opened by incision of the cricothyroid membrane. To collect bronchoalveolar lavage (BAL) fluid, the lungs were flushed twice with ice-cold sterile PBS (0.8 mL). Lungs were then removed for the extraction of nuclear proteins.

Extraction of lung nuclear proteins and electrophoretic mobility shift assay (EMSA). Lung tissue was quick-frozen in liquid nitrogen immediately after removal from the thoracic cavity. Nuclear proteins were isolated from the lung tissue using a modification of the method described by Böhrer et al. [32]. Lung tissue was homogenized in 5 mL of ice-cold buffer A (10 m*M* Hepes-KOH [pH 7.9], 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.5 m*M* dithiothreitol [DTT], 0.2 m*M* phenylmethylsulfonyl fluoride [PMSF], and 0.6% NP-40) for 1 min. After removal of cell debris by centrifugation (350 *g* at 4°C for 30 s), the supernatant was kept on ice for 5 min and centrifuged for 5 min at 6000 *g* at 4°C. Cytoplasmic proteins in the supernatant were collected and quick-frozen, and the pellet was resuspended in 200 μL of buffer B (10 m*M* Hepes-KOH [pH 7.9], 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 1.2 *M* sucrose, 0.5 m*M* DTT, and 0.2 m*M* PMSF). After centrifugation (13,000 *g* at 4°C for 30 min), the supernatant was discarded, and the pellet was resuspended in 100 μL of buffer C (20 m*M* Hepes-KOH [pH 7.9], 1.5 m*M* $MgCl_2$, 420 m*M* NaCl, 0.2 m*M* EDTA, 0.5 m*M* DTT, 0.2 m*M* PMSF, 2 m*M* benzamidine, 5 mg/mL leupeptin, and 25% glycerol). After incubation on ice for 20 min, the cellular debris was removed by centrifugation (6000 *g* at 4°C for 2 min), and the supernatant was quick frozen in aliquots at −80°C. Nuclear proteins were normalized by protein assay (Protein Reagent; Bio-Rad) and used to bind to duplex oligonucleotides corresponding to the RANTES NF-κB–1 binding site (wt) or mutated (Mut) under conditions described elsewhere [22]. The sequence of the wt oligonucleotide was 5′-GATCCATTTTGGAAACTCCCCTTAT-3′ and 3′-TAAAACCTTTGAGGGGAATATCTAG-5′. The sequence of the Mut oligonucleotide was 5′-GATCCATTTTGGcAcCTtaaCgTAT-3′ and 3′-TAAAACCgTgGAattGcATATCTAG-5′ (mutations are indicated by lowercase letters). In brief, DNA-binding reactions contained 10 μg of nuclear proteins, 6% glycerol, 5 m*M* Hepes-KOH, 100 m*M* NaCl, 0.4 m*M* MgCl, 0.05 m*M* EDTA, 1 μg of poly dA-dT, and 40,000 cpm of ^{32}P-labeled double-stranded oligonucleotide in a total volume of 40 μL. The nuclear proteins were incubated with the probe for 20 min at room temperature before they were fractionated on a 6% nondenaturing PAGE in TBE buffer (22 m*M* Tris-HCl, 22 m*M* boric acid, and 0.25 m*M* EDTA [pH 8.0]). Gels were dried and exposed for autoradiography using Kodak XAR films at −70°C. In competition assays, 2 pmol of unlabeled wt or Mut RANTES NF-κB–1 oligonucleotides were added at the same time the probe was added. For supershift assay, 1 μL of preimmune serum (PI), rabbit IgG anti-p50 (NF-κB1), anti-p65 (RelA), anti-p52 (NF-κB2), anti-RelB, or anti-cRel (Santa Cruz Biotechnology) was added to the binding-reaction mixtures, and the reactions were continued for an additional 12 h at 4°C prior to fractionation by PAGE. Gels were dried and exposed for autoradiography at −70°C overnight or for 24 h (for supershift assay). As indicated, NF-κB–binding complexes were quantified by optical densitometry

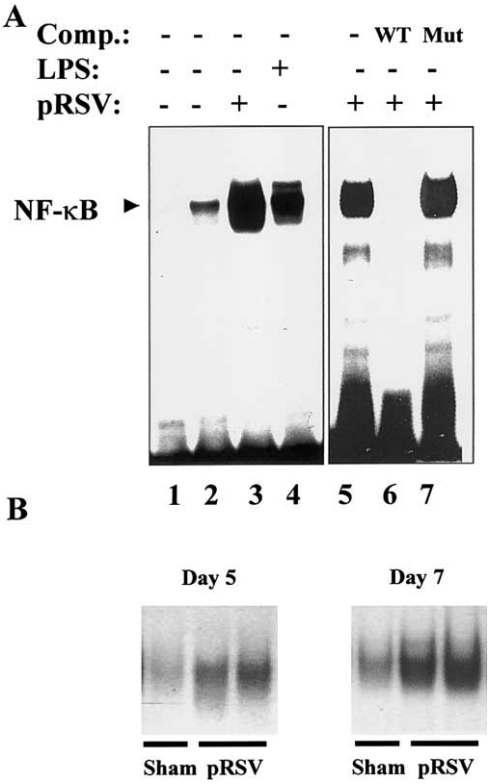

Figure 1. NF-κB activation in the lungs of respiratory syncytial virus (RSV)–infected mice. *A,* BALB/c mice were sham-infected, infected with purified RSV (pRSV), or inoculated with lipopolysaccharide (LPS; 1 μg/50 μL). Mice were killed 24 h after treatment, and nuclear proteins extracted from the lungs were used for electrophoretic mobility shift assay (EMSA; with double-stranded RANTES NF-κB–1 oligonucleotide probe). The figure shows NF-κB binding complexes of representative sham-infected *(lane 2),* RSV-infected *(lanes 3, 5, 6,* and *7),* or LPS-inoculated mice *(lane 4)* ($n = 4$ mice/group). *Lane 1,* Free probe (no nuclear proteins added). Sequence specificity of the detected complexes was determined by competition assays (Comp.), in which 2 pmol of unlabeled wild-type (WT; *lane 6*) or mutated (Mut; *lane 7*) oligonucleotides were included in the binding reaction with lung nuclear proteins from RSV-infected mice (the lower bands in lanes 5 and 7 are due to partial degradation during the isolation of nuclear proteins and indicate fragmented NF-κB proteins bound by the RANTES probe). +, Positive; −, negative. *B,* EMSA showing representative NF-κB binding complexes of lung nuclear extracts from mice ($n = 4$ mice/group) infected for 5 and 7 days with pRSV or sham-inoculated (Sham).

(measured with an AlphaImager 2200 optical densitometer; Alpha Innotech).

Depletion of AMs. AM depletion in mice was achieved by intratracheal and inl administration of 120 μL total of a liposome-encapsulated clodronate suspension (dichloromethylene-bisphosphonate [Cl_2MBP]; a generous gift of Roche Diagnostics), a procedure that results in the apoptosis of AMs [33–35]. Under light anesthesia, mice were injected with Cl_2MBP liposomes or control PBS liposomes 2 days prior to RSV infection. Optimal conditions of AM depletion were determined by differential counting of BAL cells and by staining of lung-tissue sections with fluorescein isothiocyanate–labeled F4/80 monoclonal antibody (MAb) (Serotec).

Statistical analysis. Statistical analysis was carried out using the SigmaStat 3.0 program (Jandel). The data were analyzed by Wilcoxon test and analysis of variance for multiple comparisons.

Results

Activation of NF-κB in the lung by RSV. The activation of NF-κB in the lung was determined by EMSA of nuclear extracts from lung tissue of pRSV-infected 4–6-week-old BALB/c mice (24 h). Control animals were inoculated inl with 1 μg/50 μL lipopolysaccharide (LPS) (*Escherichia coli* strain O111:B4; Sigma), a known activator of NF-κB, or with PBS. A low level of NF-κB DNA–binding activity was detected in lung nuclear protein extracts of PBS-inoculated animals (figure 1*A, lane 2*). Note that the intensity of the NF-κB–binding complex may slightly vary among experiments, individual animals, and length of the autoradiography exposure. Nuclear activity of NF-κB dramatically increased in the lungs of pRSV-infected mice (figure 1*A, lane 3*), as well as in those of LPS-inoculated animals (figure 1*A, lane 4*). Sequence specificity of the nuclear protein DNA–binding complex was confirmed by competition with unlabeled oligonucleotides in the EMSA. Competition assays with either unlabeled oligonucleotide RANTES wt or RANTES Mut, an oligonucleotide that contained a mutation of the NF-κB contact points, were added together with the radiolabeled RANTES wt probe. As shown in figure 1*A,* the RANTES wt probe was able to completely compete for the binding of the RSV-inducible complexes, whereas the Mut probe failed to compete (compare lanes 5, 6, and 7). To examine the activation of NF-κB in the lung at later time points during the course of infection, pRSV-infected mice were killed at days 5, 7, 11, and 21, and the lung nuclear protein extracts were tested by EMSA. As shown in figure 1*B,* NF-κB–binding activity was clearly detectable at days 5 and 7, whereas it was no longer detectable at days 11 and 21 after infection (data not shown).

To determine whether NF-κB activation was specifically due to RSV and to exclude the confounding effect of cell-derived factors potentially present in the purified viral preparations, we performed the following control experiments. Groups of BALB/c mice were inoculated inl side by side with 50 μL of pRSV, cRSV (i.e., lysate/supernatant of RSV-infected Hep-2 cells not sucrose-gradient purified), lysate/supernatant of uninfected HEp-2 cells, PBS, or 70% sucrose (used for preparation of pRSV). As shown in figure 2*A* and 2*B,* both pRSV and cRSV remarkably induced NF-κB DNA–binding activity in the lung. On the other hand, PBS, uninfected HEp-2 cell lysates, and sucrose had no significant effect on NF-κB activation (figure 2*A*). These results suggest that the effect on lung NF-κB activation that we observed with both RSV preparations (pRSV and cRSV) was specifically due to the virus. Thus, because the use of the different control preparations gave similar results,

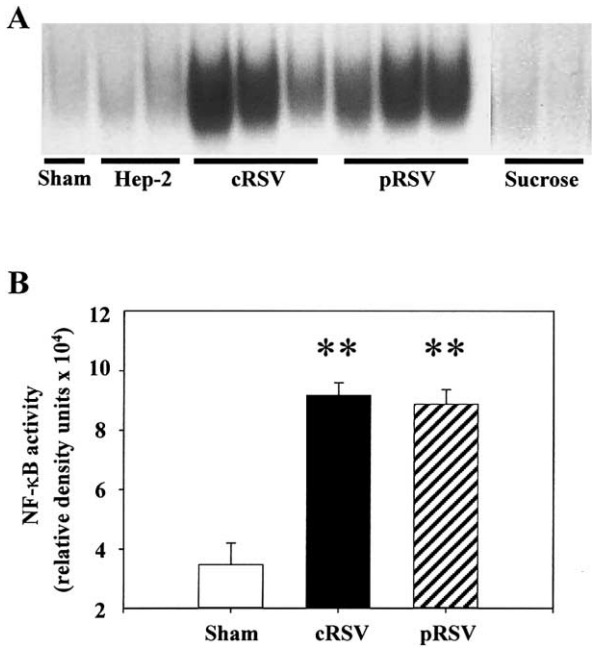

Figure 2. Role of viral preparation in respiratory syncytial virus (RSV)–induced NF-κB activation. *A,* Electrophoretic mobility shift assay (EMSA) showing representative NF-κB complexes of lung nuclear protein extracts from mice inoculated intranasally with PBS (Sham), uninfected HEp-2 cell lysate, crude RSV (cRSV), purified RSV (pRSV), or 70% sucrose (all at 24 h). *B,* Densitometry analysis of NF-κB–binding complexes (mean ± SE; $n = 4$–13 animals/group). $^{**}P < .001$, vs. sham-infected animals.

subsequent studies were performed with PBS-inoculated control animals (referred to hereafter as "sham-infected").

Identification of nuclear NF-κB subunits in RSV-infected lung tissue. We and others have shown that increased NF-κB DNA–binding activity in RSV-infected lung epithelial cells in vitro [22–24, 26] is associated with the simultaneous increase in nuclear abundance (i.e, nuclear translocation) of the potent NF-κB transcriptional subunit RelA, as well as the subunits p50 and c-Rel [22, 25]. Thus, the profile of NF-κB subunits in RSV-infected lung was examined by determining whether the inducible complexes in the EMSA cross-reacted with antibodies specific for various NF-κB subunits (i.e., supershift assay). In this assay, the presence of small amounts of individual NF-κB family members can be detected by the presence of a supershifted band, representing an antibody-protein-DNA complex, or by the reduction or disappearance of the protein-DNA complex. For the purposes of the present study, lung nuclear protein extracts were isolated from mice that had been inoculated with RSV for 0.5, 6, and 24 h. As shown in figure 3, an NF-κB–binding complex was detectable in lung nuclear protein extracts as early as 0.5 h and later at 6 and 24 h after RSV inoculation (figure 3, *lane PI*). The addition of anti-p50 (NF-κB1) and anti-cRel antibodies consistently supershifted and caused a reduction or disappearance of the RSV-inducible complex from lung extracts obtained at different time points after infection (figure 3; supershifted bands are indicated by arrowheads). On the other hand, anti-p52, -RelB, and -RelA antibodies did not produce a supershifted band. We noticed, however, that the anti-RelA antibody slightly reduced the formation of the RSV-induced complex in lung nuclear protein extracts from mice infected for 6 h, suggesting its presence in the DNA-protein complex. Nuclear translocation (i.e., activation) of the RelA subunit was confirmed by more-extensive immunofluorescence studies in frozen sections of the lung, using specific antibodies that recognize p65/RelA proteins (authors' unpublished data). These data confirm that RSV infection causes changes in the subcellular distribution of the NF-κB subunits cRel, p50, and RelA in RSV-infected lungs.

Involvement of AMs in NF-κB activation by RSV. The brisk activation of NF-κB by pRSV seen in our experiments as early as 30 min–1.5 h after inoculation suggests that these early events in the lung may occur independently of viral replication. To address this question, mice were injected with either pRSV (10^7 pfu) or UV-RSV (prepared from the same pRSV pool, identical volume of inoculum [50 μL], and total protein concentration). Lack of viral replication in the UV-RSV preparation was determined by a plaque assay. As shown in figure 4, inoculation with both pRSV and UV-RSV resulted in rapid activation of NF-κB in lung tissue at 1.5 h (figure 4*A, lanes 3–7,* and 4*B*). Macrophages have been shown elsewhere in vitro to express a number of inducible NF-κB–dependent genes when exposed to both replicating and inactivated RSV [6, 36]. Thus, to determine whether macrophages were the source of activated lung NF-κB during this early response, AMs were depleted by injecting BALB/c mice with Cl_2MBP liposomes prior to RSV inoculation. This treatment resulted in a >70% reduction in the number of AMs (RSV, 84% ± 3%; RSV/Cl_2MBP liposomes, 25% ± 3%) up to 48 h posttreatment, as determined by differential counts and staining with a cell-specific MAb and in agreement with other reports [37]. Depletion of AMs by Cl_2MBP liposomes greatly reduced or abolished lung NF-κB binding in response to intact RSV, as well as UV-RSV, at 1.5 h after virus inoculation (figure 4*A, lanes 8–12,* and 4*B*).

At 24 h, we observed a decrease in the intensity of NF-κB binding complexes and in the number of responding animals after UV-RSV inoculation, compared with intact, replicating pRSV (figure 5*A, lanes 3–7,* and 5*B*). Although the difference in NF-κB binding activity did not reach statistical significance, it was consistent across separate experiments. Of interest, AM depletion had a negligible effect on NF-κB activation seen 24 h postinoculation with either intact RSV or UV-RSV (figure 5*A, lanes 8–12,* and 5*B*). These results suggest that (1) AMs are a major source of lung NF-κB response to RSV at the earliest time points and that this response does not require viral replication and (2), in addition to the macrophagic population, other tissue-resident cells (such as epithelial cells) or inflammatory/immune cells that are recruited to the lung [14] are likely

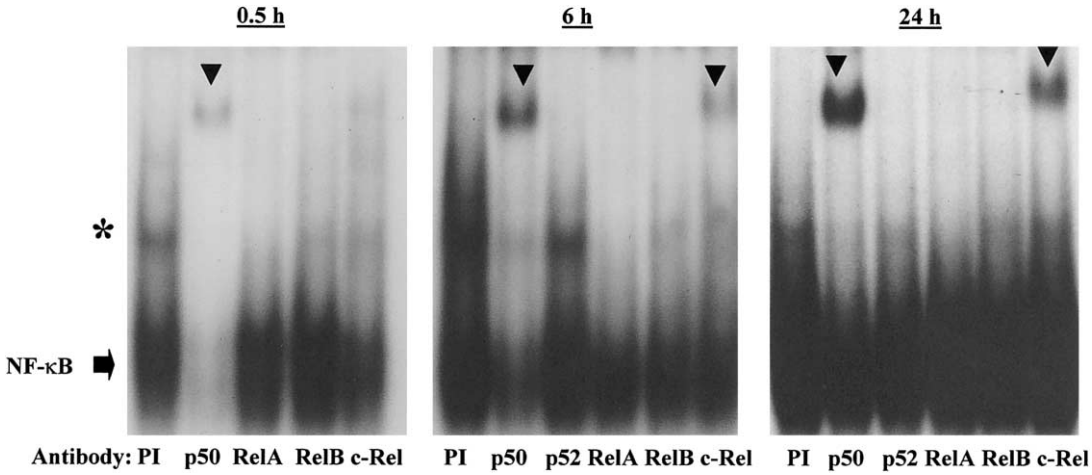

Figure 3. Identification of NF-κB subunits in lung tissue of respiratory syncytial virus (RSV)–infected mice by electrophoretic mobility shift assay (EMSA) supershift. Mice were infected with RSV. At 0.5, 6, and 24 h after infection, extracted lung nuclear proteins were used for the EMSA in the presence of the following antibodies: preimmune serum (PI), rabbit IgG anti-p50 (NF-κB1), anti-p52 (NF-κB2), anti-p65 (RelA), anti-RelB, and anti–c-Rel. By supershift, the NF-κB subunits p50 and c-Rel were identified in lung nuclear protein extracts *(arrowheads)*. At the 6-h time point, a reduction of the protein-DNA complex in the presence of anti-RelA can be seen (compare lanes PI and RelA). *, Nonspecific band. Results are representative of 9 mice/time point.

to become a preponderant component of the lung NF-κB response starting at 24 h after RSV inoculation.

Role of TLR4 in RSV-induced NF-κB activation. Two studies have recently shown that TLR4 may be involved in the innate response to RSV [38, 39]. RSV F glycoprotein stimulates cytokine production by monocytes via TLR4, and indirect evidence in vitro has suggested that TLR4 may be involved in RSV F–mediated NF-κB activation [38]. Therefore, we examined the role of TLR4 in RSV-induced NF-κB activation in the lung. C3H/HeJ mice have a spontaneous point mutation in the intracellular domain of TLR4 [40] and a profound defect in IL-6 production by macrophages in response to RSV F protein [38]. C3H/HeJ and control C3H/HeSnJ mice (with intact TLR4 signal pathways) were inoculated with pRSV (1×10^7 pfu) or UV-RSV or were sham-inoculated, and lung nuclear protein extracts were tested by EMSA at 1.5 and 24 h. In C3H/HeJ mice, neither intact RSV nor UV-RSV was able to induce a sizable NF-κB DNA–binding activity response at 1.5 h, whereas, in C3H/HeSnJ mice, both viral preparations induced a robust NF-κB response (figure 6, *upper panels*). At 24 h, however, only intact pRSV appeared to be able to activate lung NF-κB in C3H/HeJ mice, whereas both pRSV and UV-RSV induced NF-κB binding activity in control C3H/HeSnJ mice (figure 6, *lower panels*). Similarly, macrophage-enriched splenocytes isolated from C3H/HeJ mice did not show, in most of the experimental conditions, NF-κB binding in response to in vitro stimulation with sham, pRSV, or UV-pRSV preparations. On the other hand, cells obtained from control C3H/HeSnJ mice were able to respond to pRSV or UV-pRSV stimulation by activating NF-κB (H.A.H., personal observation).

Discussion

Lower respiratory tract infections caused by RSV are characterized by profound inflammation of the airway mucosa, which contributes to disease manifestations, including air flow limitation, lung atelectasis/emphysema, and hypoxemia [41]. Significant advances in our understanding of the mechanisms that mediate RSV-induced inflammation have derived from detailed gene promoter analysis, which has mainly been conducted in lung- or bronchial-derived epithelial cell lines. These studies have clearly proved that NF-κB is the "master switch" that controls the expression of numerous RSV-inducible genes [22, 24, 27, 28]. Recently, using high-density oligonucleotide microarray analysis of a tightly regulated expression system controlling expression of a nondegradable isoform of the IκBα inhibitor, we have been able to identify ~380 genes that are controlled by NF-κB in RSV-infected cells [42]. Despite this solid in vitro evidence, the in vivo signaling and transcriptional pathways that mediate airway mucosa inflammation in RSV or other respiratory viral infections are still largely unknown.

In the present study, using an experimental mouse model, we present the first direct evidence that RSV activates NF-κB in the lungs (figure 1*A*). RSV-inducible NF-κB–binding activity occurs rapidly after viral inoculation, persists in the lung tissue for at least 7 days after infection, and is no longer detectable 11 days after inoculation (figure 1*B*), when the virus is cleared from the lung [14, 31, 43]. The lack of NF-κB–binding activity in the lung after inoculation with control preparations, such as the extracts of the uninfected cells that are routinely used to grow RSV, argues in favor of the specificity of viral components in the activation

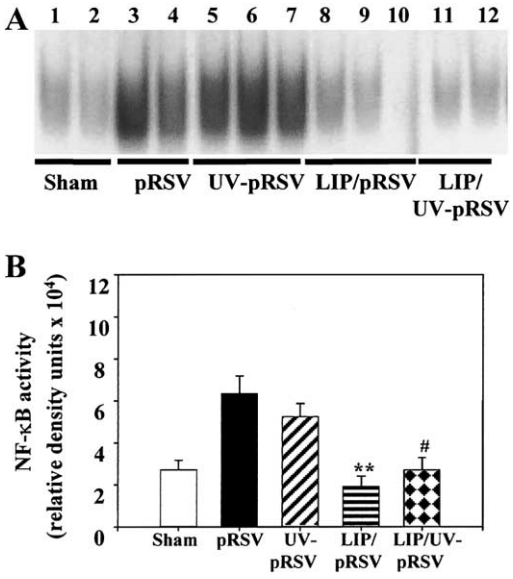

Figure 4. Role of replicating virus and alveolar macrophages (AMs) in lung NF-κB activation (1.5 h). Mice were inoculated with PBS (Sham), purified respiratory syncytial virus (pRSV), or UV-inactivated pRSV (UV-pRSV) or were depleted of AMs by liposome treatment prior to inoculation with pRSV (LIP/pRSV) or with UV-RSV (LIP/UV-pRSV). *A,* At 1.5 h after inoculation, lung nuclear proteins were used for electrophoretic mobility shift assay, as described in figure 1. The figure is a representative experiment of 3 independent experiments with sham- (n = 12 mice), RSV- (n = 12 mice), UV-RSV– (n = 10 mice), and liposome-treated mice (LIP/pRSV [n = 7 mice] and LIP/UV-pRSV [n = 7 mice]). *B,* The bar graph shows the quantification (mean ± SE) of the NF-κB–binding complexes. $^{**}P < .001$, vs. pRSV-infected mice; $^{\#}P < .05$, vs. UV-pRSV–inoculated mice.

of this pathway (figure 2). Previous studies by us and others have demonstrated increased NF-κB DNA–binding activity in cultured lung epithelial cells after RSV infection [22–24, 26]. We have also shown by subcellular fractionation, Western immunoblot analysis, and immunofluorescence that one mechanism for the RSV-enhanced activation of NF-κB binding in epithelial cells is through cytoplasmic nuclear translocation of the subunits p50, c-Rel, and RelA [22], all of which were detectable in the lung DNA–nuclear protein complexes (figure 3) [25]. Although the cellular source of activated NF-κB cannot be precisely identified by EMSA, immunofluorescence studies performed 24 h after inoculation suggested that epithelial cells represent a significant component of NF-κB activation by RSV (authors' unpublished data). Nonetheless, in other models of acute lung injury and inflammation, AMs have been shown to be required for NF-κB activation in the lung [44, 45]. Indeed, 2 observations in our study suggest that AMs are critically involved in the earliest phase of lung response to RSV (figures 4 and 5). First, the rapid appearance of NF-κB–binding activity after inl inoculation of RSV did not require intact replicating virus. In this regard, activation of transcription factors or inducible expression of cytokine genes in epithelial cells requires viral replication [2], whereas, in other cell types, including macrophages, binding of nonreplicating RSV or virus-surface proteins has been shown to induce NF-κB activation and production of cytokines or chemokines [6, 36, 38]. Second, the depletion of AMs by Cl_2MBP liposome treatment abolished both replicating RSV- and UV-RSV–induced activation of NF-κB at 1.5 h after inoculation. However, liposome treatment did not significantly affect NF-κB activity at 24 h, despite the fact that the lung was still depleted of AMs. This finding suggests that, at later time points of RSV infection, other cells that are recruited to the lung become a sizable source of the lung NF-κB response to RSV, in addition to the AMs. In this regard, we have shown that NF-κB activation is still detectable 5–7 days after inoculation, a time point at which lymphocytes are a major component of the inflammatory infiltrate in the lung [31]. Future studies will be focused on a more precise characterization of these cellular sources of NF-κB activity in RSV-infected lung tissue.

TLRs are type I transmembrane proteins, which have been identified as central regulators of innate immune response against a variety of microbes [46]. TLR4 is the first discovered human homologue of the *Drosphila* Toll, is the predominant receptor for

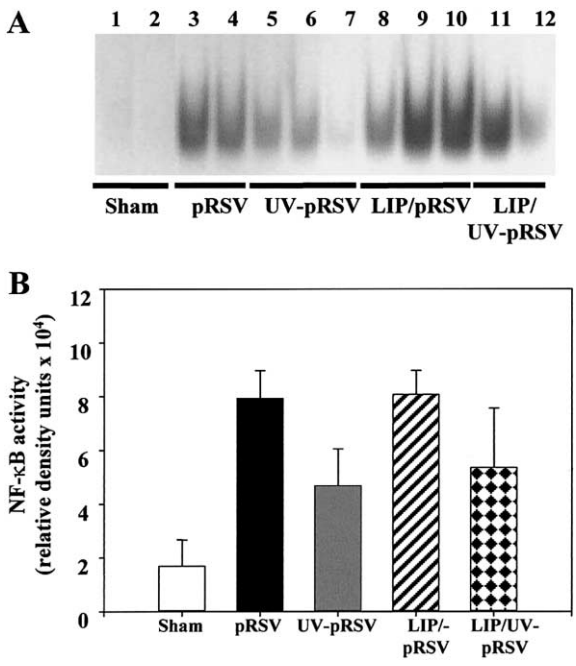

Figure 5. Role of replicating virus and alveolar macrophages (AMs) in lung NF-κB activation (24 h). Mice were inoculated as described in figure 4. *A,* At 24 h, lung nuclear proteins were used for electrophoretic mobility shift assay. The figure shows a representative experiment of 3 independent experiments with sham- (n = 12 mice), purified respiratory syncytial virus– (pRSV; n = 12 mice), UV-inactivated RSV– (UV-pRSV; n = 10 mice), and liposome-treated mice (LIP/pRSV [n = 7 mice] and LIP/UV-pRSV [n = 7 mice]). *B,* The bar graph represents the quantification (mean ± SE) of the NF-κB binding complexes (differences not statistically significant).

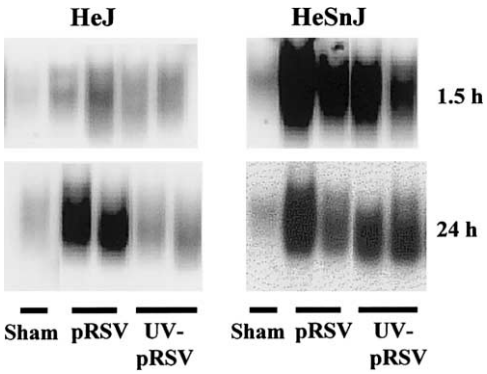

Figure 6. Involvement of Toll-like receptor 4 in NF-κB activation by respiratory syncytial virus (RSV). C3H/HeJ (HeJ) and C3H/HeSnJ (HeSnJ) mice were inoculated with PBS (Sham), purified RSV (pRSV), or UV-inactivated pRSV (UV-pRSV) for 1.5 *(top panels)* or 24 *(lower panels)* h. Mice were killed, and lung nuclear proteins were tested by electrophoretic mobility shift assay for NF-κB–binding activity. The figure is a representative experiment with 6 mice/group.

gram-negative bacterial LPS [40, 47], and is preferentially expressed on resting monocytes/macrophages, polymorphonuclear leukocytes, and dendritic cells [48]. Ligation of the TLR4 promotes dimerization of the receptor and recruitment of the adapter protein MyD88 with autophosphorylation of the serine/threonine protein kinase IRAK, leading to the activation of the IKK–NF-κB signal pathway [49]. TLR4 recently has been shown to be involved in the release of IL-6 by human and mouse monocytes/macrophages stimulated in vitro by affinity-purified preparations of RSV A2 F glycoprotein [38]. Here, we have extended these observations to an in vivo experimental model, providing the first direct evidence that TLR4 is a critical regulator of RSV-induced NF-κB activation. In C3H/Je mice, a strain that carries a functional missense point mutation within the *Tlr4* gene region encoding the cytoplasmic tail of this receptor [40], the amount of RSV-inducible NF-κB–binding activity detected in lung extracts or isolated macrophages was consistently reduced (in some animals, even undetectable), compared with TLR4-competent C3H/HeSnJ mice (figure 6). Of interest, although at the earliest time points after inoculation (1.5 h) both intact pRSV and inactivated UV-RSV failed to induce NF-κB responses in C3H/Je mice, at the 24 h time point, the same pRSV preparation, but not the UV-RSV one, was able to induce NF-κB activation similar to that observed in C3H/HeSnJ. Thus, in agreement with the AM depletion experiments, this finding suggests the possible existence of distinct but sequentially integrated, RSV-inducible NF-κB responses in the lung that occur in the initial phases after viral inoculation. The first response occurs at the earliest time points after inoculation, is mostly AM- and TLR4-dependent, and does not require RSV replication. The second response, at later points, involves, in addition to AMs, cells that are tissue resident (such as airway epithelial cells) and/or inflammatory and immune cells that are recruited to the lung, are TLR4-independent, and require some degree of viral replication (full replication and/or abortive). The relevance of these initial TLR4- and AM-mediated events in the progression of the inflammatory and immune responses in the lung, as well as the implications for the immunopathogenesis of naturally acquired RSV infection, remain to be determined. Collectively with our findings, the recent observation that RSV-infected TLR4-deficient mice exhibit impaired NK and $CD14^+$ cell trafficking and deficient NK function and viral clearance, compared with mice expressing TLR4 [39], suggests that NF-κB may be a central activator of not only inflammatory responses but also innate mucosal immunity to RSV [50]. Studies addressing this hypothesis are currently in progress.

References

1. Hall CB. Respiratory syncytial virus and parainfluenza virus. N Engl J Med **2001**;344:1917–28.
2. Garofalo RP, Welliver RC, Ogra PL. Clinical aspects of bronchial reactivity and cell-virus interaction. In: Ogra PL, Lamm ME, Bienenstock J, Mestecky J, Strober W, McGhee JR, eds. Mucosal immunology. 2nd ed. San Diego: Academic Press, **1999**:1223–37.
3. Graham BS, Johnson TR, Peebles RS. Immune-mediated disease pathogenesis in respiratory syncytial virus infection. Immunopharmacology **2000**; 48:237–47.
4. Zhang Y, Luxon B, Casola A, Garofalo RP, Jamaluddin M, Brasier AR. Expression of RSV-induced chemokine gene networks in lower airway epithelial cells revealed by cDNA microarrays. J Virol **2001**;75:9044–58.
5. Patel JA, Kunimoto M, Sim TC, et al. Interleukin-1 alpha mediates the enhanced expression of intercellular adhesion molecule–1 in pulmonary epithelial cells infected with respiratory syncytial virus. Am J Respir Cell Mol Biol **1995**;13:602–9.
6. Becker S, Quay J, Soukup J. Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus–infected human alveolar macrophages. J Immunol **1991**;147:4307–12.
7. Panuska JR, Merolla R, Rebert NA, et al. Respiratory syncytial virus induces interleukin-10 by human alveolar macrophages: suppression of early cytokine production and implications for incomplete immunity. J Clin Invest **1995**;96:2445–53.
8. Mastronarde JG, Monick MM, Hunninghake GW. Oxidant tone regulates IL-8 production in epithelium infected with respiratory syncytial virus. Am J Respir Cell Mol Biol **1995**;13:237–44.
9. Saito T, Deskin RW, Casola A, et al. Respiratory syncytial virus induces selective production of the chemokine RANTES by upper airway epithelial cells. J Infect Dis **1997**;175:497–504.
10. Olszewska-Pazdrak B, Casola A, Saito T, et al. Cell-specific expression of RANTES, MCP-1, and MIP-1 alpha by lower airway epithelial cells and eosinophils infected with respiratory syncytial virus. J Virol **1998**;72:4756–64.
11. Garofalo R, Mei F, Espejo R, et al. Respiratory syncytial virus infection of human respiratory epithelial cells up-regulates class I MHC expression through the induction of IFN-β and IL-1α. J Immunol **1997**;157:2506–13.
12. Matsuzaki Z, Okamoto Y, Sarashina N, Ito E, Togawa K, Saito I. Induction of intercellular adhesion molecule–1 in human nasal epithelial cells during respiratory syncytial virus infection. Immunology **1996**;88:565–8.
13. Jamaluddin M, Wang S, Garofalo RP, et al. IFN-beta mediates coordinate expression of antigen-processing genes in RSV-infected pulmonary epithelial cells. Am J Physiol Lung Cell Mol Physiol **2001**;280:L248–57.
14. Haeberle HA, Kuziel WA, Dieterich HJ, Casola A, Gatalica Z, Garofalo RP. Inducible expression of inflammatory chemokines in respiratory syncytial virus–infected mice: role of MIP-1α in lung pathology. J Virol **2000**;75:878–90.
15. Tripp RA, Jones L, Anderson LJ. Respiratory syncytial virus G and/or SH

glycoproteins modify CC and CXC chemokine mRNA expression in the BALB/c mouse. J Virol **2000**;74:6227–9.
16. Hussell T, Pennycook A, Openshaw PJ. Inhibition of tumor necrosis factor reduces the severity of virus-specific lung immunopathology. Eur J Immunol **2001**;31:2566–73.
17. Garofalo RP, Patti J, Hintz KA, Hill V, Ogra PL, Welliver RC. Macrophage inflammatory protein–1α, and not T-helper type 2 cytokines, is associated with severe forms of bronchiolitis. J Infect Dis **2001**;184:393–9.
18. Harrison AM, Bonville CA, Rosenberg HF, Domachowske JB. Respiratory syncytial virus–induced chemokine expression in the lower airways: eosinophil recruitment and degranulation. Am J Respir Crit Care Med **1999**; 159:1918–24.
19. Barnes PJ, Karin M. Nuclear factor–κB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med **1997**;336:1066–71.
20. Beg AA, Baldwin ASJ. The I kappa B proteins: multifunctional regulators of Rel/NF-kappa B transcription factors. Genes Dev **1993**;7:2064–70.
21. Karin M, Delhase M. The I kappa B kinase (IKK) and NF-kappa B: key elements of proinflammatory signalling. Semin Immunol **2000**;12:85–98.
22. Garofalo RP, Sabry M, Jamaluddin M, et al. Transcriptional activation of the interleukin-8 gene by respiratory syncytial virus infection in alveolar epithelial cells: nuclear translocation of the RelA transcription factor as a mechanism producing airway mucosal inflammation. J Virol **1996**;70:8773–81.
23. Mastronarde JG, He B, Monick MM, Mukaida N, Matsushima K, Hunninghake GW. Induction of interleukin (IL)–8 gene expression by respiratory syncytial virus involves activation of nuclear factor (NF)–κB and NF–IL-6. J Infect Dis **1996**;174:262–7.
24. Bitko V, Velazquez A, Yank L, Yang Y-C, Barik S. Transcriptional induction of multiple cytokines by human respiratory syncytial virus requires activation of NF-κB and is inhibited by sodium salicylate and aspirin. Virology **1997**;232:369–78.
25. Jamaluddin M, Casola A, Garofalo RP, et al. The major component of IkBα proteolysis occurs independently of the proteasome pathway in respiratory syncytial virus–infected pulmonary epithelial cells. J Virol **1998**;72:4849–57.
26. Fiedler MA, Wernke-Dollries K, Stark JM. Inhibition of viral replication reverses respiratory syncytial virus–induced NF-κB activation and interleukin-8 gene expression in A549 cells. J Virol **1996**;70:9079–82.
27. Thomas H, Friedland JS, Sharland M, Becker S. Respiratory syncytial virus–induced RANTES production from human bronchial epithelial cells is dependent on nuclear factor–κB nuclear binding and is inhibited by adenovirus-mediated expression of inhibitor of κBα. J Immunol **1998**;161: 1007–16.
28. Casola A, Garofalo RP, Haeberle H, et al. Multiple *cis* regulatory elements control RANTES promoter activity in alveolar epithelial cells infected with respiratory syncytial virus. J Virol **2001**;75:6428–39.
29. Kisch AL, Johnson KM. A plaque assay for respiratory syncytial virus. Proc Soc Exp Biol Med **1963**;112:583.
30. Jamaluddin M, Garofalo RP, Ogra PL, Brasier AR. Inducible translational regulation of the NF-IL6 transcription factor by respiratory syncytial virus infection in pulmonary epithelial cells. J Virol **1996**;70:1554–63.
31. Graham BS, Perkins MD, Wright PF, Karzon DT. Primary respiratory syncytial virus infection in mice. J Med Virol **1988**;26:153–62.
32. Bohrer H, Qiu F, Zimmermann T, et al. Role of NF-κB in the mortality of sepsis. J Clin Invest **1997**;100:972–85.
33. Thepen T, McMenamin C, Oliver J, Kraal G, Holt PG. Regulation of immune response to inhaled antigen by alveolar macrophages: differential effects of in vivo alveolar macrophage elimination on the induction of tolerance vs. immunity. Eur J Immunol **1991**;21:2845–50.
34. Berg JT, Lee ST, Thepen T, Lee CY, Tsan MF. Depletion of alveolar macrophages by liposome-encapsulated dichloromethylene diphosphonate. J Appl Physiol **1993**;74:2812–19.
35. van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods **1994**;174:83–93.
36. Stadnyk AW, Gillan TL, Anderson R. Respiratory syncytial virus triggers synthesis of IL-6 in BALB/c mouse alveolar macrophages in the absence of virus replication. Cell Immunol **1997**;176:122–6.
37. Leemans JC, Juffermans NP, Florquin S, et al. Depletion of alveolar macrophages exerts protective effects in pulmonary tuberculosis in mice. J Immunol **2001**;166:4604–11.
38. Kurt-Jones EA, Popova L, Kwinn L, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol **2000**;1:398–401.
39. Haynes LM, Moore DD, Kurt-Jones EA, Finberg RW, Anderson LJ, Tripp RA. Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. J Virol **2001**;75:10730–7.
40. Poltorak A, He X, Smirnova I, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. Science **1998**;282:2085–8.
41. Ruuskanen O, Ogra PL. Respiratory syncytial virus. Curr Probl Pediatr **1993**;23:50–79.
42. Tian B, Zhang Y, Luxon B, et al. Identification of NF-κB–dependent gene networks in respiratory syncytial virus–infected cells. J Virol **2002**;76: 6800–14.
43. Taylor G, Stott EJ, Hughes M, Collins AP. Respiratory syncytial virus infection in mice. Infect Immun **1984**;43:649–55.
44. Lentsch AB, Shanley TP, Sarma V, Ward PA. In vivo suppression of NF-κB and preservation of IkBα by interleukin-10 and interleukin-13. J Clin Invest **1997**;100:2443–8.
45. Lentsch AB, Czermak BJ, Bless NM, Ward PA. NF-kappaB activation during IgG immune complex–induced lung injury: requirements for TNF-alpha and IL-1beta but not complement. Am J Pathol **1998**;152:1327–36.
46. Aderem A, Ulevitch RJ. Toll-like receptors in the induction of the innate immune response. Nature **2000**;406:782–7.
47. Hoshino K, Takeuchi O, Kawai T, et al. Cutting edge: toll-like receptor 4 (TLR4)–deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol **1999**;162:3749–52.
48. Muzio M, Bosisio D, Polentarutti N, et al. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J Immunol **2000**;164:5998–6004.
49. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol **2001**;2:675–80.
50. Garofalo RP, Haeberle H. Epithelial regulation of innate immunity to respiratory syncytial virus. Am J Respir Cell Mol Biol **2000**;23:581–5.