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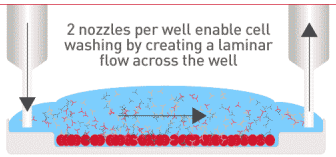

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Selective IκB Kinase Expression in Airway Epithelium Generates Neutrophilic Lung Inflammation[1]

Ruxana T. Sadikot,*¶ Wei Han,* M. Brett Everhart,† Ornella Zoia,* R. Stokes Peebles,* E. Duco Jansen,‡ Fiona E. Yull,§ John W. Christman,*¶ and Timothy S. Blackwell[2]*¶†

To determine whether NF-κB activation is sufficient to generate lung inflammation in vivo, we selectively expressed a constitutively active form of IκB kinase 1 (cIKK1) or IκB kinase 2 (cIKK2) in airway epithelium. After intratracheal administration of adenoviral vectors expressing cIKK1 or cIKK2 to transgenic reporter mice that express *Photinus* luciferase under the control of an NF-κB-dependent promoter, we detected significantly increased luciferase activity over time (up to 96 h). Compared with control mice treated with adenoviral vectors expressing β-galactosidase, lung bioluminescence and tissue luciferase activity were increased in NF-κB reporter mice treated with adenovirus (Ad)-cIKK1 or Ad-cIKK2. NF-κB activation in lungs of Ad-cIKK1- and Ad-cIKK2-treated mice was confirmed by immunoblots for RelA and EMSA from lung nuclear protein extracts. Mice treated with Ad-cIKK1 or Ad-cIKK2 showed induction of mRNA expression of several chemokines and cytokines in lung tissue. In lung lavage fluid, mice treated with Ad-cIKK1 or Ad-cIKK2 showed elevated concentrations of NF-κB-dependent chemokines macrophage-inflammatory protein 2 and KC and increased numbers of neutrophils. Coadministration of adenoviral vectors expressing a transdominant inhibitor of NF-κB with Ad-cIKK1 or Ad-cIKK2 resulted in abrogated NF-κB activation and other parameters of lung inflammation, demonstrating that the observed inflammatory effects of Ad-cIKK1 and Ad-cIKK2 were dependent on NF-κB activation by these kinases. These data show that selective expression of IκB kinases in airway epithelium results in NF-κB activation, inflammatory mediator production, and neutrophilic lung inflammation. Therapies targeted to NF-κB in lung epithelium may be beneficial in treating inflammatory lung diseases. ***The Journal of Immunology*****, 2003, 170: 1091–1098.**

The transcription factor NF-κB, along with AP-1, STAT, and C/EBP family transcription regulatory factors, coordinates the activation of many genes involved in host defense and inflammatory responses in the lungs. NF-κB activation is involved in the pathogenesis of a variety of inflammatory lung diseases including asthma, cystic fibrosis, pneumonia, and the acute respiratory distress syndrome, and may have a role in idiopathic pulmonary fibrosis and environmental lung diseases (1, 2). Although activation of NF-κB is necessary for maximal transcription of many adhesion molecules, enzymes, cytokines, and chemokines important in the initiation of inflammation, the independent effects of selective activation of this transcription factor complex in the lungs are undefined.

Five members of the mammalian NF-κB/Rel family have been identified. These include NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), RelB, and cRel. These proteins form homodimers or heterodimers and share the conserved Rel homology domain, which is involved in dimerization and DNA binding to specific cognate sequences. The preponderant form of NF-κB in most cells consists of a heterodimer containing p50 and RelA (p65), which contains a transactivation domain (1, 3). In quiescent cells, NF-κB complexes are sequestered in the cytoplasm by inhibitory proteins (IκB-α, IκB-β, IκB-ε) (4). Stimulation of the NF-κB pathway is mediated by diverse signal transduction cascades, resulting in phosphorylation of IκBs. Phosphorylated IκB-α (or IκB-β) is ubiquitinated and degraded by the 26S proteasome, allowing nuclear translocation of the NF-κB complexes (5, 6).

The specific protein kinases responsible for IκB phosphorylation have been identified in a high molecular mass complex called the signalsome. This protein complex contains two kinases (IκB kinase (IKK)[3]1 and IKK2) that have the ability to phosphorylate IκB and structural/regulatory subunits, including NF-κB essential modifier (or IKKγ), Cdc37, and Hsp90 (7–14). IKK1 and IKK2 have a similar primary structure and form homo- or heterodimers. IKK2 appears to be the principal kinase involved in IκB phosphorylation resulting from cell activation by the proinflammatory cytokines (TNF-α and IL-1β) and bacterial LPS (15–17). The function of IKK1 in generation of inflammation is less well defined; however, dimerization and phosphorylation of specific serine residues of IKK2 by IKK1 are required for kinase activity of the complex (18, 19). IKK1 appears to have an important role in development and may have additional functions independent of NF-κB (20).

In the present study, we hypothesized that expression of constitutively active forms of IKK1 or IKK2 in airway epithelium would be sufficient to induce an inflammatory response in the lungs. We and others have shown that airway epithelial cells can be stimulated to activate NF-κB and produce cytokines and chemokines that are important for directing innate immune responses in the

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[3] Abbreviations used in this paper: IKK, IκB kinase; Ad, adenovirus; Ad-luc, Ad-luciferase; β-gal, β-galactosidase; IT, intratracheal; cIKK, constitutively active IKK; ICCD, intensified charge-coupled device; MIP, macrophage-inflammatory protein; RPA, ribonuclease protection assay; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; LTR, long terminal repeat; RLU, relative light units; HLL, HIV-LTR/luciferase.

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lungs (21–23). Mediator production by airway epithelial cells is thought to contribute to a variety of lung inflammatory states.

Using direct intratracheal (IT) delivery of replication-deficient adenoviral vectors that express constitutively active IKK1 (cIKK1), IKK2 (cIKK2), or control vectors, we demonstrate that expression of cIKK1 or cIKK2, but not β-galactosidase (β-gal), in lung epithelium causes sufficient activation of NF-κB to direct cytokine expression and generate an intense neutrophilic lung inflammation. This response is abrogated by coadministration of adenoviral vectors expressing a dominant inhibitor of NF-κB, which indicates that the inflammatory state resulting from lung epithelial expression of cIKK1 and cIKK2 is specifically related to activation of the NF-κB pathway. Our findings suggest an important role for epithelial cell NF-κB activation in regulating the generation of neutrophilic lung inflammation.

Materials and Methods

Adenoviral vectors

For construction of replication-deficient adenoviral vectors that express activators of NF-κB, we obtained cIKK1 and cIKK2 from Dr. F. Mercurio (Signal Pharmaceutical, San Diego, CA). IKK1 and IKK2 were made constitutively active by Ser-Glu mutations in critical serine residues that are phosphorylated in the active kinase (Ser^{176}, Ser^{180} in IKK1 and Ser^{177}, Ser^{181} in IKK2) (12). The replication-deficient recombinant adenovirus (Ad) type 5 was used. An expression cassette containing a CMV promoter driving the expression of cIKK1 or cIKK2 was inserted into this vector. Adenoviral vectors expressing a dominant inhibitor of NF-κB (IκB-αDN), which represents a S36–40A mutant of the avian IκB-α that cannot be phosphorylated or degraded, and β-gal have been previously reported. Adenoviral vectors expressing luciferase were a gift from Dr. A. Powers (Vanderbilt University, Nashville, TN). Adenoviral vectors were propagated, purified, and stored at −70°C.

Animal model

Transgenic mice expressing *Photinus* luciferase cDNA under control of the proximal 5′ HIV-long terminal repeat (LTR) mice on a C57B6/DBA background weighing 20–30 g were used (24). Mice were treated with IT administration of adenoviral vectors after sedation with ketamine/xylazine. Mouse tracheas were directly exposed by surgical resection, pierced with a 26-gauge needle, and injected with 100 μl of the Ad preparation diluted in sterile PBS. The neck wound was closed with sterile sutures under aseptic conditions.

Mice were asphyxiated with CO_2 and lungs were removed. One lung was ground in 1 ml of reporter lysis buffer (Promega, Madison, WI) and stored at −20°C for luciferase assays and the other lung was frozen at −70°C. In some experiments, tracheas were cannulated and lungs were lavaged in situ with sterile pyrogen-free physiological saline that was instilled in four 1-ml aliquots and gently withdrawn with a 1-ml tuberculin syringe.

In vivo measurement of luciferase gene expression by bioluminescence

Mice were anesthetized and shaved over the chest and abdomen before imaging. Luciferin (1 mg/mouse in 200 μl of isotonic saline) was administered by i.p. injection, and mice were imaged with an intensified charge-coupled device (ICCD) camera (model C2400-32; Hamamatsu, Bridgewater, NJ) as previously reported (25, 26). This system consists of an image intensifier coupled to an 8-bit charge-coupled device camera, allowing for 256 intensity levels for each pixel. For the duration of photon counting, mice were placed inside a light tight box that also houses the camera. Light emission from the mouse was detected as photon counts using the ICCD camera and customized image processing hardware and software (Hamamatsu). The imaging duration (3 min) was selected to avoid saturating the camera during image acquisition. Quantitative analysis was accomplished by 1) defining a standard area (region of interest) in the 8-bit intensity image corresponding to the region of the chest overlying the mid lung zone and 2) determination of total integrated photon intensity over the region of interest. Photon counts were obtained before and after treatment with Ad so that each mouse could be used as its own control. For presentation, a 4-bit (16 intensity levels) digital false-color photon emission image was generated for each captured image according to the same false-color scale. To visualize the dimmer parts of the image, the brighter pixels in the images are displayed as white (thus appearing saturated); however, detected light emission for each image was well below the saturation limit for the camera.

Measurement of luciferase activity in lung tissue

Luciferase activity was measured in postmortem tissue samples by adding 100 μl of freshly reconstituted luciferase assay buffer (Promega) to 20 μl of lung tissue homogenate. Luciferase activity was measured in a Monolight 3010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA) and expressed as relative light units (RLU) normalized for protein content, which was measured according to the Bradford assay (27).

Lung lavage total and differential cell counts

Lung lavage fluid was centrifuged at 400 × *g* for 10 min to separate cells from supernatant. Supernatant was saved separately and frozen at −70°C. The cell pellet was suspended in serum-free RPMI 1640 culture medium, and total cell counts were determined on a grid hemocytometer. Differential cell counts were determined by staining cytocentrifuge slides with a modified Wright stain (Diff-Quick; Baxter, McGraw Park, IL) and counting 400–600 cells in complete cross-sections.

Macrophage-inflammatory protein (MIP) 2 and KC ELISA

MIP-2 and KC levels were measured using a sandwich ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Ribonuclease protection assay (RPA)

RPA employing both murine cytokine and chemokine templates were done with the RiboQuant multiprobe RPA system (BD PharMingen, San Diego, CA) according to the manufacturer's directions. Briefly, the probes were radiolabeled by adding [α-^{32}P]UTP to a reaction mixture that included template, transcription buffer, and T7 polymerase. After incubation, DNase was added and RNA probes were purified by phenol-chloroform extraction followed by ethanol precipitation. The probes were resuspended in hybridization buffer and diluted to 4×10^5 counts/min per μl. Twenty micrograms of total RNA from the lung was dried in a vacuum evaporator centrifuge and resuspended in 8 μl of hybridization buffer. Two micrograms of the labeled probe was added to each sample, heated to 90°C, and incubated overnight at 56°C. RNase was added, followed by proteinase K, and the resultant digest was phenol-chloroform extracted and precipitated with ethanol. Loading dye was added to the dried pellets, the samples were heated briefly to 90°C, and a 5% polyacrylamide gel was run, dried, and subjected to autoradiography.

Western blot

Twenty-five micrograms of protein from tissue homogenates or lung lavage cells was separated on a 10% acrylamide gel, transblotted, and immunodetected. cIKK1 was detected with Abs to a hemagglutinin tag present on the protein (Babco, Richmond, CA). cIKK2 was detected with Abs to a FLAG tag present on the protein (anti-$FLAGM_2$ mAb; Sigma-Aldrich, St. Louis, MO). IκB-αDN was detected with specific antiserum that does not cross-react with native murine IκB-α or β(24). Abs to RelA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

β-gal detection

Frozen sections of lung tissue were cut, fixed in 50% glutaraldehyde in PBS for 20 min, and stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) solution (5 mM potassium ferrocyanide, 5 mM potassium ferrocyanide, 2 mM $MgCL_2$, and 1 mg/ml 20% X-gal) for 12–18 h.

EMSA

After preparation of nuclear extracts, EMSA for NF-κB binding activity were done as previously described (28). A double-stranded oligonucleotide probe containing consensus NF-κB motif (Stratagene, La Jolla, CA) was used in these studies.

Lung histology

Lungs were removed en bloc after tracheal ligation, preserved in 10% formalin, and subsequently embedded in paraffin. H&E or Wright-Giemsa stain was then performed on 5-μm sections.

Statistical analysis

For comparison among groups, a one-way ANOVA was used with the Tukey-Kramer multiple comparisons test ($p < 0.05$ were considered to be significant).

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Results

IT administration of replication-deficient adenoviral vectors targets transgene expression to airway epithelium

We performed initial experiments with adenoviral vectors expressing luciferase (Ad-luc) and β-gal (Ad-β-gal) reporters to determine the timing and localization of transgene expression after IT injection. Mice were treated with Ad-luc at a dose of 3×10^9 PFU diluted in 100 μl of PBS (Fig. 1). Control mice were treated with 100 μl of sterile PBS by IT injection. To detect luciferase activity in vivo, wild-type C57B/6 mice were treated with 1 mg of luciferin by i.p. injection and imaged with an ICCD camera at 24, 72, and 96 h after administration of adenoviral vectors. Untreated mice and control mice treated with PBS showed no detectable bioluminescence, but mice treated with Ad-luc demonstrated luciferase activity (as detected by photon emission) from the chest by 24 h and this bioluminescence persisted through 96 h, which was the last time point evaluated (Fig. 1*A*). Of note, bioluminescence was not detected in any organ outside the chest following IT injection of adenoviral vectors. At 96 h, lungs were removed after i.p. luciferin injection and placed in a culture dish with RPMI 1640 medium. Fig. 1*B* shows ex vivo bioluminescence of lungs from three mice treated with Ad-luc. Bioluminescence was not detected from liver, spleen, or kidneys of these mice ex vivo and no bioluminescence was detected from any organ of mice treated with PBS alone (data not shown). These studies clearly demonstrate transgene expression primarily in the lungs that persists for at least 96 h after IT administration of adenoviral vectors.

To determine cellular localization of transgene expression in the lungs, we performed separate experiments in which mice were treated with Ad-β-gal at 3×10^9 PFU. Ninety-six hours after administration of Ad-β-gal, mice were harvested and X-gal staining was performed on frozen lung sections (Fig. 1*C*). Mice treated with Ad-β-gal showed staining almost exclusively in large airway epithelium. Staining was not detected on similarly prepared lung sections from PBS-treated controls (data not shown). These findings show that adenoviral vectors can be used to deliver transgenes selectively to airway epithelium.

Because of reports that high doses of adenoviral vectors can cause lung inflammation and that adenoviral vectors alone can activate NF-κB in cultured lung epithelial cell lines (23, 29–31), we performed experiments to define a titer of adenoviral vectors that results in transgene expression in the lungs in vivo without substantial nonspecific activation of NF-κB or other signs of inflammation. To accomplish this, we used a line of transgenic NF-κB reporter mice that possesses a portion of the proximal 5′ human HIV-LTR driving the expression of luciferase ((referred to as HLL mice (HIV-LTR/luciferase)) (24). The proximal HIV-LTR is a well-characterized NF-κB responsive promoter containing a TATA box, an enhancer region between −82 and −103 with two NF-κB motifs, and three Sp1 boxes from −46 to 78. In cell culture, NF-κB activation is absolutely required for transcriptional activity of the HIV-LTR. We have shown that luciferase activity in these transgenic mice reflects NF-κB activation over time (24). We have used bioluminescence imaging after luciferin injection to measure luciferase activity in these animals and have shown that this methodology correlates well with luciferase activity measured in a variety of tissues (25, 26). Using HLL mice, we performed dose-ranging experiments using 10^7–10^{11} PFU of Ad-β -gal delivered by IT injection. HLL mice were imaged after i.p. luciferin (1 mg) at baseline and at 24, 72, and 96 h after adenoviral administration. At doses of 10^{10} PFU and higher, increased bioluminescence from the lungs was detected (data not shown). Subsequent studies were done with 3×10^9 or lower PFU, doses that did not result in increased bioluminescent detection of luciferase activity in HLL mice at the time points evaluated.

At the doses of adenoviral vectors that did not result in nonspecific activation of NF-κB or vector-induced lung inflammation, we were

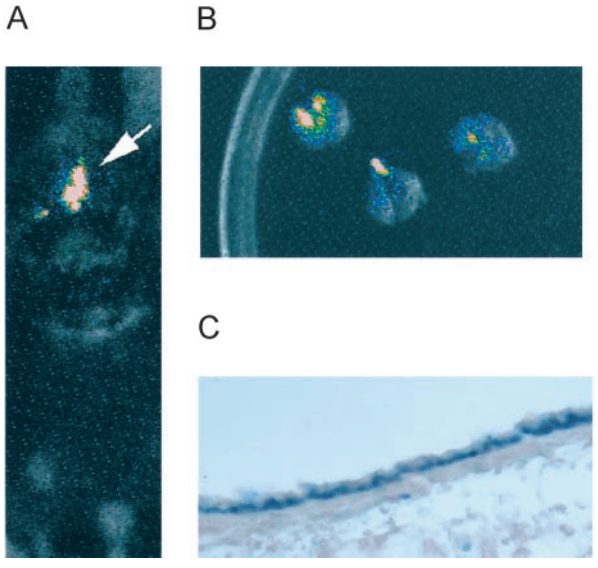

FIGURE 1. *A*, Bioluminescent imaging of a mouse 96 h after IT administration of adenoviral vectors expressing luciferase (Ad-luc) at 3×10^9 PFU/mouse. The mouse was imaged with an ICCD camera after i.p. injection of luciferin (1 mg). The arrow points to detected photon emission over the chest. *B*, Ex vivo bioluminescent imaging of lungs from three mice treated with Ad-luc. Luciferin was injected 96 h after Ad-luc treatment. Lungs were then harvested, placed in culture medium, and imaged. Pseudocolor represents intensity of the detected photon emission. *C*, β-gal staining in lung tissue 96 h after IT administration of Ad-β-gal at 3×10^9 PFU/mouse. Staining is seen predominantly in the bronchial epithelium.

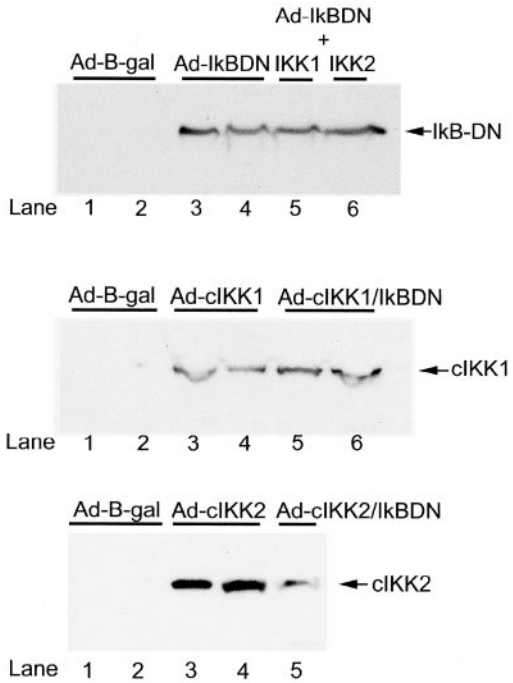

FIGURE 2. Western blots for detection of IκB-αDN (*top*), cIKK1 (*middle*), and cIKK2 (*bottom*) in lung tissue following IT administration of various adenoviral vectors expressing these gene products. The appropriate transgene product was seen when the specific vector was administered alone or in combination with another vector, and none of these products was seen after treatment with Ad-β-gal. Each lane represents the results from a separate mouse.

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able to identify specific transgene products expressed in lung tissue by Western blot (Fig. 2). Mice were treated with adenoviral vectors expressing 1) cIKK1 or cIKK2 (Ad-cIKK1 and Ad-cIKK2, respectively) at 3×10^8 PFU, 2) a dominant inhibitor of NF-κB, which is a S36/40A mutant of avian IκB-α, designated as IκB-αDN (Ad-IκB-αDN) at 3×10^9 PFU, or 3) Ad-β-gal at 3×10^9 PFU. Mice treated with Ad-cIKK1, Ad-cIKK2, or Ad-IκB-αDN demonstrated expression of cIKK1, cIKK2, or IκB-αDN in a corresponding fashion that was detectable in lungs harvested 96 h after IT injection of the specific adenoviral construct. These proteins were not detectable in homogenates from control mice treated with Ad-β-gal (Fig. 2). To determine whether transgene expression was limited to the epithelium, we performed separate experiments in which we evaluated transgene expression in lung lavage cells 96 h after IT injection of adenoviral vectors (Fig. 3). As shown, Ad-cIKK1 and Ad-cIKK2 expression was not detectable in lung lavage cells. Therefore, in these experiments, we were able to identify doses of adenoviral vectors that resulted in epithelial transgene expression without significant nontransgene-related activation of NF-κB.

Expression of cIKK in airway epithelium induces NF-κB activation in vivo in a transgene-specific manner

We investigated whether IT administration of Ad-cIKK1 could induce NF-κB activation and luciferase production in the lungs of HLL reporter mice. In these studies, mice were imaged by bioluminescence immediately before treatment (baseline) and at 24, 72, and 96 h after IT injection of adenoviral vectors. Luciferin was administered by i.p. injection (1 mg in 200 μl of PBS) 30 min before bioluminescence imaging and photonic counts were measured over a standardized area of the chest overlying lung tissue. Mice treated with Ad-cIKK1 showed a significant time-dependent increase in photonic counts, with peak activation at 72 h (5153 ± 127 photonic counts for Ad-cIKK1 compared with 1698 ± 667 photonic counts for Ad-β-gal controls, $p < 0.05$, $n = 5$; Fig. 4). Mice were sacrificed at 96 h and the final photonic counts were compared with luciferase activity measured in lung homogenates. Luciferase activity in lung tissue homogenates of mice treated with Ad-cIKK1 was much higher than in lung homogenates from mice treated with Ad-β-gal (238 ± 44 RLU/μg protein for cIKK1 group vs 134 ± 33 RLU/μg protein for β-gal group ($p < 0.05$)) and there was a close correlation between bioluminescent emission from the lungs at 96 h and luciferase activity in lung homogenates ($R^2 = 0.88$, $p < 0.001$; data not shown). In untreated mice, luciferase activity in lung homogenates was 110 ± 20 RLU/μg protein. These data indicate that treatment with Ad-cIKK1 activates NF-κB-dependent luciferase activity in lung tissue of HLL mice.

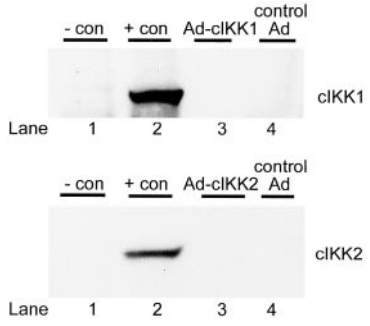

FIGURE 3. Western blots for detection of cIKK1 (*top*) and cIKK2 (*bottom*) in lung lavage cells following IT administration of Ad-cIKK1, Ad-cIKK2, or Ad-luc (control Ad). A positive control (+con) and negative control (−con) for immunodetection of cIKK1 or cIKK2 are included. Transgene expression was not identified in lavage cells.

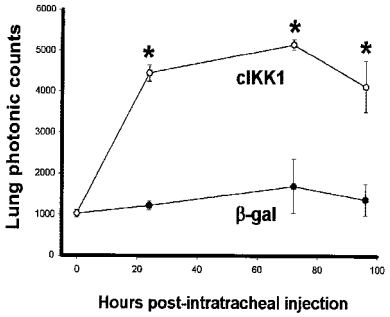

FIGURE 4. Time course for luciferase activity in HLL-transgenic reporter mice after treatment with IT injection of Ad-cIKK1 or Ad-β-gal as determined by bioluminescent detection. Mice were imaged at 24, 72, and 96 h after treatment with adenoviral vectors. Photon counts were measured over a standardized area of the lung 30 min after i.p. injection of luciferin (1 mg). Mice treated with Ad-β-gal showed no significant increase in photon emission at any time point, whereas mice treated with Ad-cIKK1 showed increased luciferase activity at each time point measured ($n = 5$ mice/group; *, $p < 0.05$ for comparison between groups at each time point).

To definitively show that luciferase production in this model is dependent on activation of NF-κB by cIKK1, we performed experiments using adenoviral vectors that express a transdominant inhibitor of NF-κB (IκB-αDN). Since this IκB-α mutant cannot be phosphorylated and degraded, NF-κB is sequestered in the cytoplasm and cannot be activated by IKK. We treated HLL mice with Ad-IκB-αDN or Ad-cIKK1 alone or the combination of Ad-cIKK1 plus Ad-IκB-αDN. In these experiments, Ad-cIKK1 was administered at a dose of 3×10^8 PFU and Ad-IκB-αDN was given at 3×10^9 PFU. Bioluminescent imaging of HLL mice was done at 24, 72, and 96 h after IT administration of adenoviral vectors. At 96 h, lungs were harvested for luciferase activity measurements and measurement of NF-κB activation. Bioluminescent imaging of mice at 72 h after treatment is shown in Fig. 5*A*. Baseline imaging of a mouse is shown as well as images of mice treated with Ad-IκB-αDN, Ad-cIKK1, and Ad-cIKK1 plus Ad-IκB-αDN. In these pseudocolor images in which white represents the greatest intensity of photon emission, minimal light emission is detected over the lungs (arrows) at baseline and after treatment with Ad-IκB-αDN. After Ad-cIKK1, light emission from the thorax is significantly increased; however, coadministration of Ad-IκB-αDN with Ad-cIKK1 completely abolishes the increase in light emission induced by cIKK1 expression. Fig. 5*B* summarizes detected photon emission from the lungs in these experiments at 72 h after IT injection. Mice treated with Ad-IκB-αDN did not show a significant increase in photon emission over baseline, whereas mice treated with Ad-cIKK1 alone had markedly increased photonic counts from the lungs (1800 ± 387 for Ad-IκB-αDN group vs 6100 ± 900 for Ad-cIKK1 group, $p < 0.001$). This increase in photon emission with Ad-cIKK1 treatment was completely blocked by the coadministration of a 10-fold excess of Ad-IκB-αDN (2000 ± 219). Similar differences among groups were found at 24 and 96 h after adenoviral vector treatment. Measurements of bioluminescence from the thorax correlated closely with postmortem measurements of luciferase activity in lung tissue from mice harvested 96 h after adenoviral vector administration (Fig. 5*C*). Treatment with Ad-cIKK1 resulted in increased lung luciferase activity (normalized for total protein content) compared with-treatment with Ad-IκB-αDN alone, and this induction of luciferase activity by cIKK1 was completely blocked by coadministration of Ad-cIKK1 plus Ad-IκB-αDN.

To evaluate nuclear translocation of NF-κB in the lungs in these experiments, we measured immunoreactive RelA in lung nuclear

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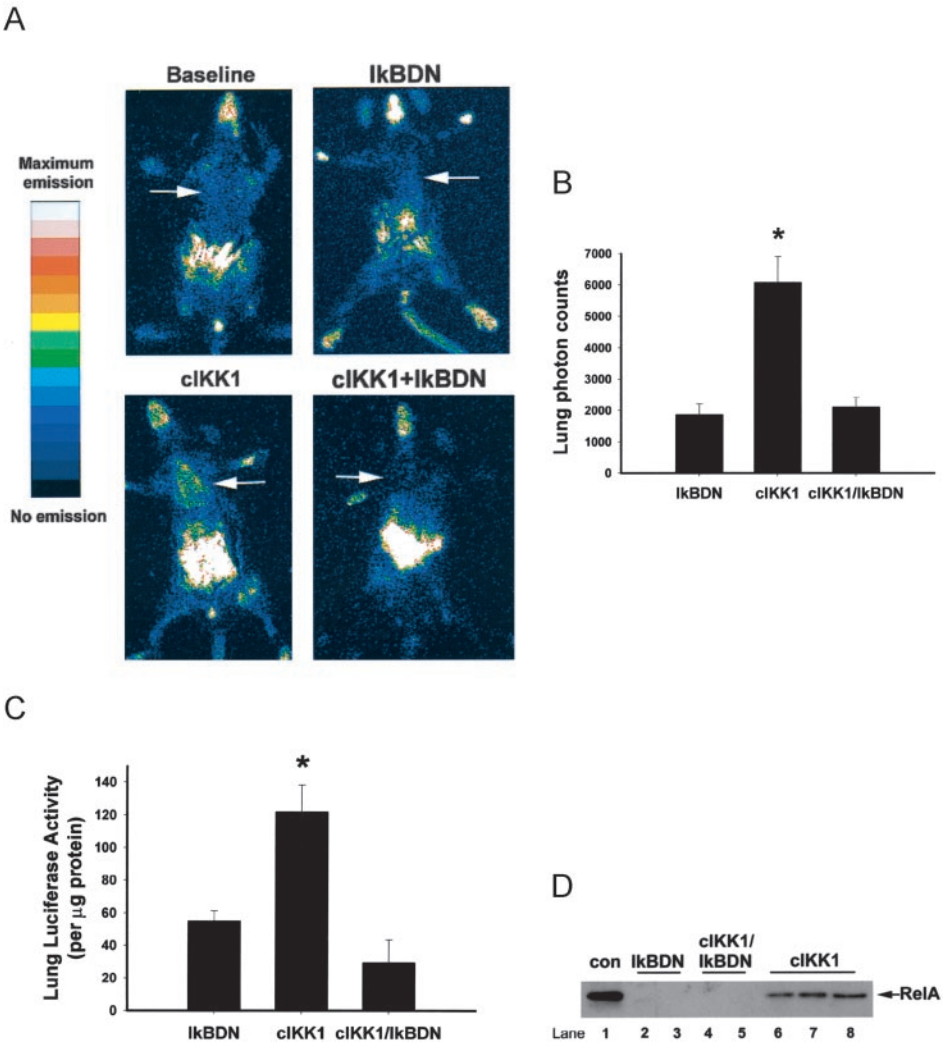

FIGURE 5. *A,* Representative bioluminescent images from HLL mice before treatment (baseline) and 72 h after IT treatment with adenoviral vectors expressing IκB-αDN (3×10^9 PFU), cIKK1 (3×10^8 PFU), or cIKK1 plus IκB-αDN. Photon emission from the area of the thorax overlying the lungs is identified by arrows. HLL mice had basal levels of luciferase activity in the abdomen and brain that were unaffected by adenoviral vector treatment in these experiments. *B,* Lung bioluminescent detection of luciferase activity 72 h after treatment with adenoviral vectors ($n = 4$ mice/group). *C,* Lung luciferase assays from lung homogenates (normalized for total protein) obtained from the same mice 96 h after treatment. *D,* Western blots from lung nuclear extracts of mice treated with adenoviral vectors for 96 h. RelA is identified only in mice treated with cIKK1 alone. A positive control for detection of RelA is shown in *lane 1* (con). Each lane is the result from a separate mouse (*, $p < 0.05$ for cIKK1 group compared with IκB-αDN or cIKK1 plus IκB-αDN group).

protein extracts by Western blot. Fig. 5*D* demonstrates the presence of RelA protein in nuclear extracts from three mice that were treated with Ad-cIKK1 and the absence of detectable RelA in extracts from four mice treated with either Ad-IκB-αDN alone or the combination of Ad-cIKK1 plus Ad-IκB-αDN. These data indicate that IκB-αDN prevented nuclear localization of RelA in response to treatment with the Ad-cIKK1. Also, the presence of nuclear RelA correlated with lung expression of luciferase in these experiments.

In addition to adenoviral vectors expressing cIKK1, we constructed adenoviral vectors expressing cIKK2. We administered Ad-cIKK2 or Ad-cIKK1 alone (3×10^9 PFU) or in combination (1.5×10^9 PFU of each) and evaluated lung bioluminescence at 24, 72, and 96 h. In this experiment, photon emission was highest in the group that received Ad-cIKK2 and intermediate in the group that received combined cIKK1 plus cIKK2. Fig. 6*A* shows detected photon emission from the lungs at 72 h after adenoviral vector administration. Mean photonic counts from the lungs of mice treated with Ad-cIKK2 were 18,340 $\pm$ 3,257 compared with 15,750 $\pm$ 1,780 photonic counts in the combined Ad-cIKK1 and Ad-cIKK2 group and 7,678 $\pm$ 789 in the Ad-cIKK1 group. Luciferase activity measurements in lung homogenates at 96 h were also highest in the group that received Ad-cIKK2 (Fig. 6*B*). Although both Ad-cIKK1 and Ad-cIKK2 induced luciferase activity in the lungs of HLL reporter mice, cIKK2 induced higher levels of luciferase in the lungs, and there was no synergistic effect of adding both vectors together.

We performed additional experiments to show that coadministration of Ad-IκB-αDN with Ad-cIKK2 blocks NF-κB activity and NF-κB-dependent luciferase activity in the lungs of HLL reporter mice. NF-κB activation was evaluated by EMSA from lung nuclear protein extracts 96 h after adenoviral vector treatment (Fig. 7). Bands on EMSA were identified by cold and nonspecific competition and Ab supershifts as containing RelA/p50 heterodimers and p50 homodimers as indicated. As shown, lung nuclear protein extracts from mice treated with Ad-cIKK1 (Fig. 7, *lanes 3* and *4*) or Ad-cIKK2 (*lanes 7* and *8*) demonstrated intense binding to a consensus NF-κB oligonucleotide, whereas nuclear protein extracts from mice treated with Ad-IκB-αDN in combination with Ad-cIKK1 or Ad-cIKK2 had minimal binding of the RelA/p50 heterodimer band (*lanes 5* and *6* and *9* and *10*, respectively). Induction of lung luciferase activity by Ad-cIKK2 in these experiments was also blocked by coadministration of Ad-IκB-αDN (data not shown). These data clearly indicate that NF-κB activation in lungs results from treatment with adenoviral vectors that express cIKK1 or cIKK2 and that this activation can be specifically inhibited by expression of IκB-αDN.

Activation of NF-κB in the lungs results in increased cytokine expression and neutrophilic lung inflammation

To evaluate the effects of NF-κB activation in lung epithelium, we used multiprobe RPA from total lung RNA to determine cytokine

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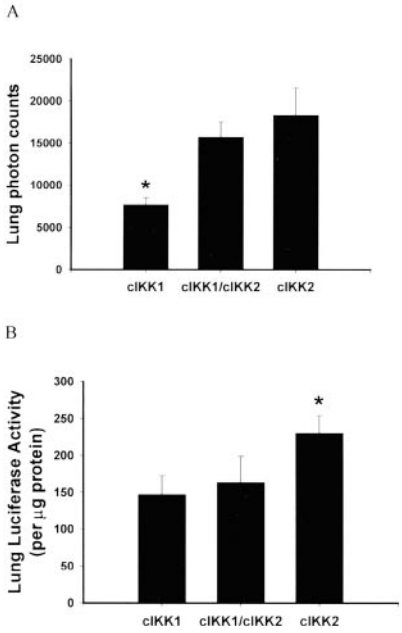

FIGURE 6. *A*, Lung bioluminescent detection of luciferase activity 72 h after administration of Ad-cIKK1 (3×10^9 PFU), Ad-cIKK2 (3×10^9 PFU), or a combination of these vectors (cIKK1/cIKK2; 1.5×10^9 PFU each). Photon emission was highest in mice expressing cIKK2. *B*, Luciferase assays done from lung homogenates obtained 96 h after treatment ($n = 5$ or 6 mice/group; *, $p < 0.05$ for cIKK2 group compared with cIKK1 group).

and chemokine gene expression 96 h after treatment with Ad-IκB-αDN alone, Ad-cIKK1, Ad-cIKK2, or a combination of Ad-cIKK1 (or Ad-cIKK2) plus Ad-IκB-αDN. In HLL mice that were treated with Ad-cIKK1 or Ad-cIKK2 alone, mRNA expression of a variety NF-κB-dependent chemokines was induced, including RANTES, eotaxin, MIP-1α, MIP-1β, MIP-2, IFNγ-inducible protein 10, and monocyte chemoattractant protein 1 (Fig. 8*A*). Increased expression of all of these chemokines was blocked by combined treatment with Ad-cIKK1 (or cIKK2) and Ad-IκB-αDN. Expression of mRNA for cytokines TNF-α and IL-6 was also found in the lungs of mice treated with Ad-cIKK1 or Ad-cIKK2, whereas in mice that received Ad-IκB-αDN in addition to Ad-cIKK1 or Ad-cIKK2, mRNA for these cytokines was only minimally detectable (Fig. 8*B*).

Levels of the CXC chemokines MIP-2 and KC were measured in lung lavage of HLL mice 96 h after treatment with adenoviral vectors because these NF-κB-dependent chemokines are thought to be important for neutrophil recruitment (1). In untreated mice,

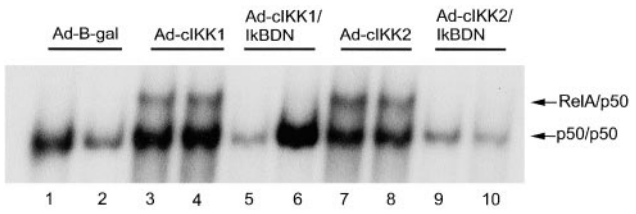

FIGURE 7. EMSA using lung nuclear protein extracts from mice treated with Ad-β-gal, Ad-cIKK1, Ad-cIKK2, or the combination of Ad-cIKK1 plus Ad-IκB-αDN or cIKK2 plus Ad-IκB-αDN. Two NF-κB bands composed of RelA/p50 heterodimers and p50 homodimers are identified. NF-κB binding activity is greatest in mice treated with Ad-cIKK1 (*lanes 3* and *4*) or Ad-cIKK2 (*lanes 7* and *8*) alone. Appearance of the RelA/p50 heterodimer is blocked by coexpression of IκB-αDN (*lanes 5* and *6* or *lanes 9* and *10*, respectively). RelA/p50 binding was not detected following treatment with the control vector Ad-β-gal. Each lane is the result from a separate mouse.

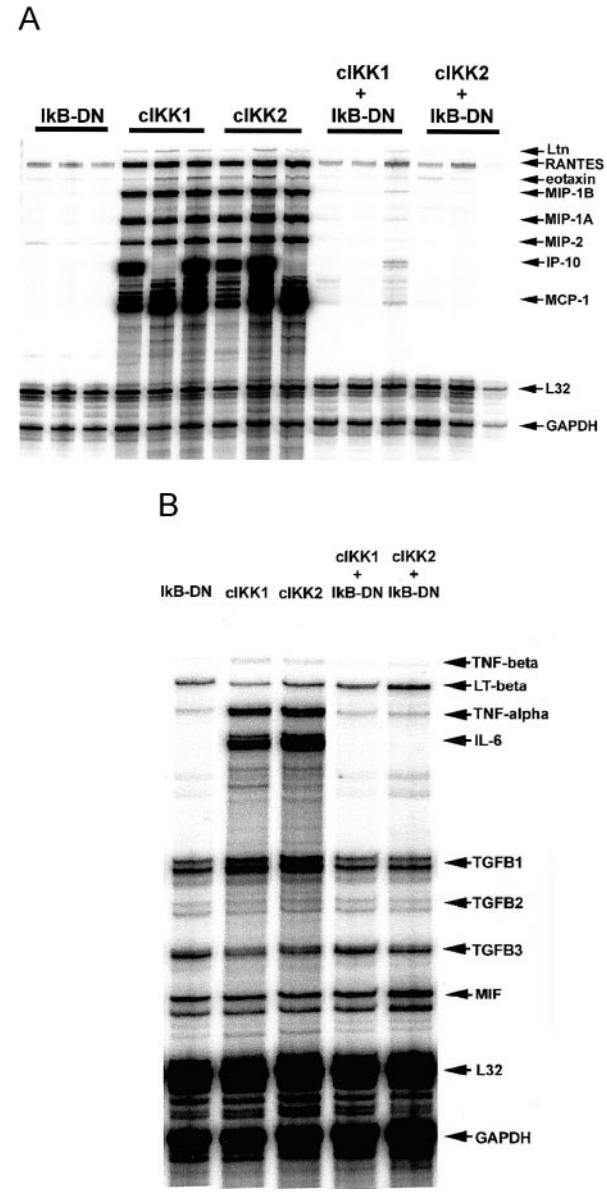

FIGURE 8. RPA for chemokines (*A*) and cytokines (*B*) using total RNA from lung tissue of mice treated with Ad-IκB-αDN (IκB-DN), Ad-cIKK1, Ad-cIKK2, Ad-cIKK1 plus Ad-IκB-αDN, or Ad-cIKK2 plus Ad-IκB-αDN. Each lane represents results from a single animal. The position mRNA of detected chemokines and cytokines and constitutively expressed mRNAs L32 and GAPDH are indicated.

no MIP-2 or KC was detectable in lung lavage fluid and in mice treated with Ad-β-gal small amounts of both chemokines were present (Fig. 9*A*). However, mice treated with Ad-cIKK1 or Ad-cIKK2 had significant elevation of lung lavage levels of these chemokines compared with mice treated with Ad-β-gal. Chemokine levels of mice coadministered Ad-cIKK1 plus Ad-IκB-αDN or Ad-cIKK2 plus Ad-IκB-αDN were similar to those seen after treatment with Ad-β-gal.

We investigated whether NF-κB activation in lung epithelium results in recruitment of neutrophils to lungs or otherwise alters lung histology. Fig. 9*B* shows cell counts from lung lavage obtained 96 h after adenoviral vector administration. In these experiments, mice treated with Ad-cIKK1 or Ad-cIKK2 had abundant numbers of neutrophils that could be recovered in lung lavage compared with mice that were treated with Ad-β-gal. Simultaneous

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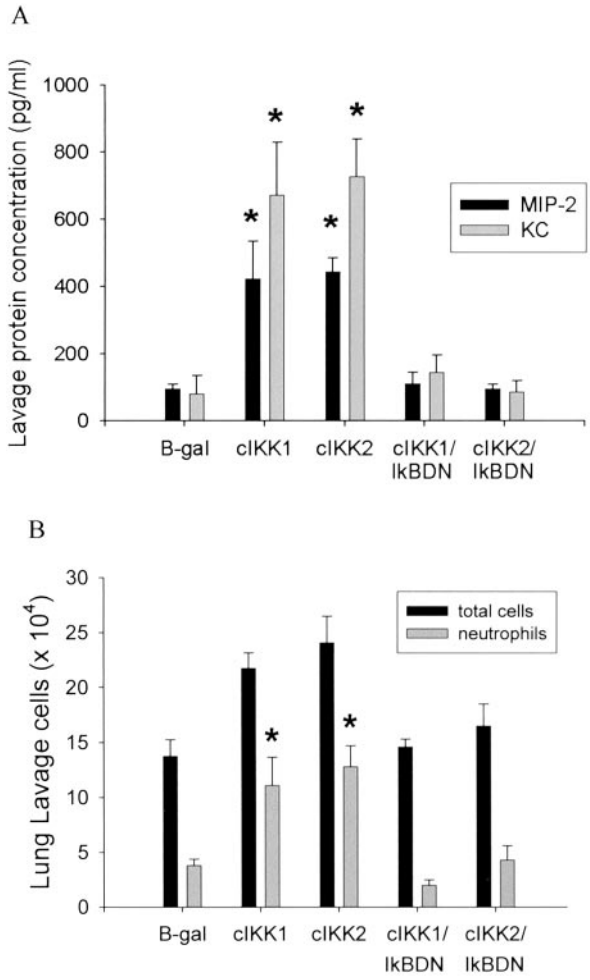

FIGURE 9. *A*, NF-κB-dependent chemokines MIP-2 and KC in lung lavage from mice 96 h after treatment with adenoviral vectors. Mice treated with Ad-cIKK1 or Ad-cIKK2 showed significantly higher concentrations of both chemokines than mice treated with Ad-β-gal or the combination of Ad-cIKK1 plus Ad-IκB-αDN or Ad-cIKK2 plus Ad-IκB-αDN. *B*, Total cell counts and neutrophils in lung lavage. Mice treated with Ad-cIKK1 or Ad-cIKK2 showed a significant neutrophilic influx that was attenuated in mice treated with the combination of Ad-cIKK1 plus Ad-IκB-αDN or Ad-cIKK2 plus Ad-IκB-αDN ($n = 4$ mice/group; ∗, $p < 0.05$ for cIKK1 and cIKK2 groups compared with all other groups).

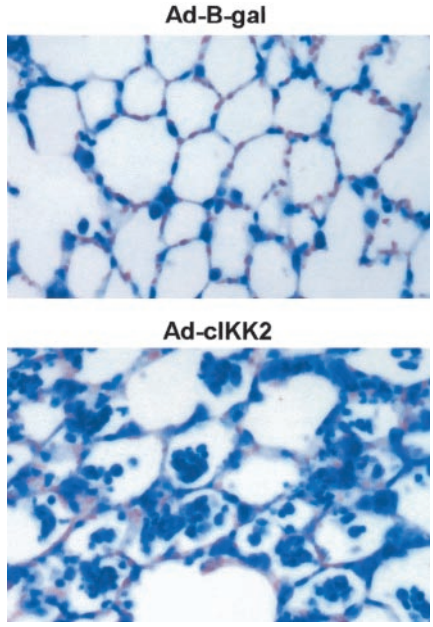

FIGURE 10. Representative histology of lungs 96 h after IT administration of Ad-β-gal or Ad-cIKK2. Wright-Giemsa-stained lung sections from mice treated with Ad-β-gal appear normal, whereas mice treated with Ad-cIKK2 developed alveolitis (original magnification, ×200).

administration of Ad-IκB-αDN with Ad-cIKK1 or Ad-cIKK2 abolished the increase in neutrophil counts induced by expression of cIKK1 or cIKK2 alone. In untreated mice, lung lavage neutrophil counts were very low ($<1 \times 10^4$ cells) and similar to the group treated with Ad-IκB-αDN alone (data not shown). Significant numbers of lymphocytes or eosinophils were not identified in the lung lavage in any of the treatment groups.

We examined lung histology of mice 96 h after treatment with adenoviral vectors to assess the extent of inflammatory changes. Lung tissue from mice that were treated with either Ad-cIKK1 or Ad-cIKK2 showed pronounced inflammatory changes that included intra-alveolar accumulation of inflammatory cells, predominantly neutrophils. Alveolar septal thickening was also observed in lungs of these mice. Fig. 10 shows representative lung histology from a mouse treated with Ad-cIKK2 (similar lung inflammation was found after treatment with Ad-cIKK1). No histological abnormalities were identified in mice treated with Ad-β-gal (Fig. 10) or Ad-IκB-αDN. As with other parameters of lung inflammation, coadministration of Ad-IκB-αDN with Ad-cIKK1 or cIKK2 prevented the histological changes seen in mice treated with Ad-cIKK1 or Ad-cIKK2 alone (data not shown).

Discussion

In this study, we used transgenic NF-κB reporter mice and adenoviral vectors to mediate activation and inhibition of NF-κB specifically in lung epithelium. This approach employs novel methodology to study this signal transduction pathway in live animals. Expression of constitutively active forms of IKK1or IKK2 in airway epithelium activates NF-κB with resultant cytokine and chemokine production and neutrophilic lung inflammation. This inflammatory response is blocked by a dominant inhibitor of NF-κB, showing that generation of inflammation by Ad-cIKK1 and Ad-cIKK2 is specifically dependent on NF-κB. Together, these data show that activation of NF-κB in airway epithelium by cIKK1 or cIKK2 is sufficient to produce neutrophilic lung inflammation.

Recombinant adenoviral vectors are efficient vehicles for delivery of genes and are useful for targeting airway epithelium. Ad offer the advantage of high titer, stability, and the ability to achieve gene transfer independent of the cell replication status. Several recent studies have used adenoviral vectors to manipulate NF-κB activation to study the impact of this pathway on inflammation. For example, expression of wild-type IKK2 was shown to induce synovial inflammation after intra-articular gene transfer in rats, and this inflammation was inhibited by expression of a dominant negative form of IκB-α (32). Another group found that expression of adenoviral-delivered RelA in the pancreas resulted in pancreatitis (33) and coadministration of Ad-IκB-α diminished the inflammatory response. In addition, several groups, including ours, have used adenoviral vectors expressing a dominant inhibitor of the NF-κB pathway to block NF-κB in target cells and tissues to assess the impact on NF-κB-mediated inflammation (24, 34, 35). These studies show that adenoviral vector-mediated gene delivery can be used to investigate the NF-κB pathway; however, there are issues that can limit the utility of this approach. At high doses, replication-deficient adenoviral vectors have been shown to cause

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a significant inflammatory response (29–31). This inflammatory response has been described to consist of an early phase (within 24 h) characterized by the accumulation of neutrophils and macrophages, followed by a later phase that develops after 5 days, consisting primarily of lymphocytes (29–31). In our studies, we have shown that a dose of replication-deficient Ad could be selected to achieve high-level transgene expression in airway epithelium with minimal viral vector-induced inflammation. In contrast, the inflammatory response induced by expression of Ad-cIKK1 or Ad-cIKK2 was significantly greater than control Ad and was specifically inhibited by expressing a transdominant NF-κB inhibitor. By carefully controlling these experiments, we have been able to show that adenoviral vector-mediated expression of cIKK1 or cIKK2 results in lung inflammation through activation of NF-κB by these kinases.

The differential roles of the two IKKs are not completely understood; however, IKK2 is thought to be the principal kinase involved in induction of NF-κB by inflammatory stimuli such as TNF-α and IL-1β (15–17, 36). Our study shows that a constitutively active mutant form of either IKK is capable of activating NF-κB in vivo and inducing a NF-κB-dependent inflammatory response. In our studies, treatment with Ad-cIKK2 induced higher levels of NF-κB-dependent luciferase in the lungs of HLL reporter mice than treatment with Ad-cIKK1. Although it is possible that expression of cIKK2 resulted in more efficient activation of NF-κB than cIKK1, we cannot exclude the possibility that differences in NF-κB activation in these studies resulted from disparity in the level of expression of cIKK1 and cIKK2. Regardless, the major finding in these studies is that both kinases are competent to cause NF-κB activation and inflammation in vivo.

The specific functions of the NF-κB activation pathway in various lung cell types are not well defined. Although airway epithelial cells in culture have been shown to activate NF-κB and produce chemokines in response to various stimuli, the role of these cells in regulation of acute neutrophilic inflammation has not been specifically evaluated. In this study, we show that bronchial epithelial cells are fully capable of inducing an inflammatory response mediated through NF-κB activation and it is possible that targeting airway epithelial cells for anti-inflammatory therapy would be beneficial for treatment of inflammatory lung diseases.

In summary, we have shown that airway delivery of adenoviral vectors can be used to study signal transduction pathways in lung epithelial cells. Using this strategy, we have demonstrated that selective activation of the NF-κB transcription factor complex in lung epithelium by expression of cIKKs is sufficient to induce cytokine/chemokine gene expression and to generate neutrophilic lung inflammation. We believe that therapies directed toward inhibition of NF-κB in specific lung cell types could be beneficial for treatment of a variety of inflammatory lung diseases.

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