

Impaired Innate Host Defense Causes Susceptibility to Respiratory Virus Infections in Cystic Fibrosis

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Summary

Viral infection is the primary cause of respiratory morbidity in cystic fibrosis (CF) infants. Here, we identify that host factors allow increased virus replication and cytokine production, providing a mechanism for understanding the severity of virus disease in CF. Increased virus is due to lack of nitric oxide synthase 2 (NOS2) and 2', 5' oligoadenylate synthetase (OAS) 1 induction in response to virus or IFN γ . This can be attributed to impairment of activation of signal transducer and activator of transcription (STAT)1, a fundamental component to antiviral defense. NO donor or NOS2 overexpression provides protection from virus infection in CF, suggesting that NO is sufficient for antiviral host defense in the human airway and is one strategy for antiviral therapy in CF children.

Introduction

Cystic fibrosis (CF) is the most common lethal genetic disorder among Caucasians, affecting an estimated 30,000 persons in the US (Cystic Fibrosis Foundation, 2000). The gene responsible for CF (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989) produces the cystic fibrosis transmembrane conductance regulator (CFTR), a polypeptide of 1480 amino acids with molecular mass of 168 kDa, and function of a cAMP-dependent Cl⁻ channel (Anderson et al., 1991; Sheppard and Welsh, 1999). CF is characterized by chronic lung infections with bacteria, mostly *Pseudomonas aeruginosa*, intense neutrophil-dominated airway inflammation, and progressive lung disease, which is the major cause of morbidity and mortality. Bacterial colonization of CF lung is usually established in the first decade of life (Rosenfeld and Ramsey, 1992). Little is known about the factors associated with initial colonization in CF lung, but viral infections predispose CF lung to bacterial colonization. Although chronic bacterial infection occurs in older CF

children, 39% of CF children in the first year of life are hospitalized with respiratory compromise related to respiratory virus infection. Furthermore, individuals hospitalized with respiratory symptoms during infancy are six times more likely to acquire *Pseudomonas aeruginosa* during early childhood (Armstrong et al., 1998). Studies show a relationship between viral respiratory tract infection with respiratory syncytial virus, parainfluenza virus, and influenza virus and pulmonary exacerbation and disease progression in CF children (Hiatt et al., 1999; Hordvik et al., 1989; Petersen et al., 1981; Wang et al., 1984). Although CF patients have no higher incidence of viral infection, severity of viral infection is amplified.

The innate antiviral response of human cells involves distinct cellular programs (Iordanov et al., 2001). In the presence of dsRNA, a common viral intermediate, 2', 5' oligoadenylate synthetase (2', 5' OAS), and dsRNA-dependent protein kinase (PKR) promote inhibition of host cell protein synthesis by activating RNase L to degrade viral and cellular RNA and by phosphorylating the α subunit of translation initiation factor, eIF2, to block its recycling from an inactive form, respectively. This prevents viral replication, eventually leading to the self-elimination of the infected cell via apoptosis. This program is probably most efficient for viral infections that are initiated by a small number of infected cells at a local site of virus entry. A second program is the production of antiviral interferons (IFN) by mucosal cells and serves the purpose of preparing adjacent naive cells for resistance to viral invasion. This program requires survival of infected cells and expression of antiapoptotic genes through activation of nuclear factor- κ B (NF- κ B) transcription factor. NF- κ B and interferon regulatory factors (IRF) 3 and 7 are required for production of type 1 interferons (Grandvaux et al., 2002). Subsequently, IFN induces antiviral pathways including PKR, 2', 5' OAS/RNase L system, and Mx proteins (Samuel, 1991; Stark et al., 1998). Mx proteins are IFN-inducible, high-abundance GTPases which interfere with viral replication, impairing the growth of negative-strand RNA viruses at the level of viral transcription and other steps (Stark et al., 1998). dsRNA or IFN- γ are also potent activators of nitric oxide synthase 2 (NOS2)- and nitric oxide (NO)-dependent antiviral pathways. High-level NO synthesis results in a large variety of reactive products, which can inhibit viral replication by modifying a number of target molecules essential for replication (Biron, 1999). STAT1, a member of a family of proteins that transduce signals from cell surface receptors to the nucleus and activate transcription by binding directly to regulatory DNA elements, is essential for host antiviral defense. IFN- α and IFN- γ lead to phosphorylation of STAT1 and binding to unique elements in a number of IFN-stimulated genes (ISGs), activating transcription (Haque and Williams, 1998). Although many antiviral genes are induced or activated in direct response to viral dsRNA, fundamental components of antiviral defense are activation of PKR, 2', 5' OAS, and NOS2 via the IFN/STAT1 pathways. In support of this, STAT1-deficient mice, which display

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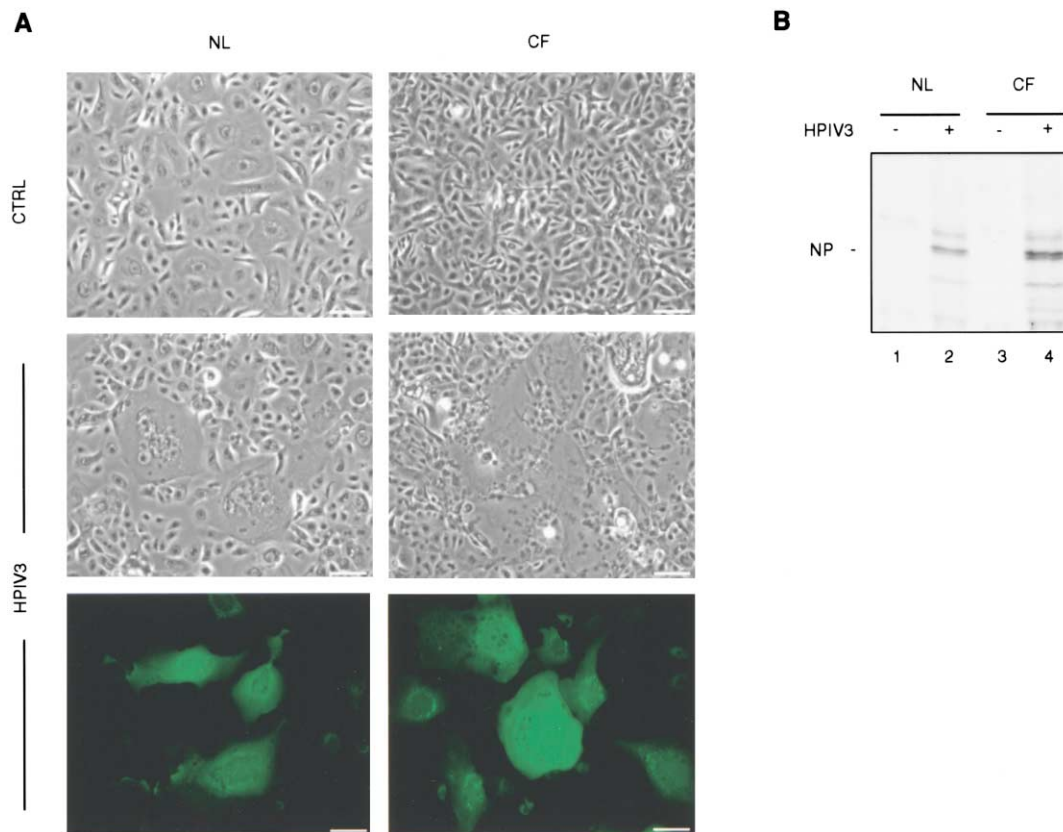


Figure 1. Increased HPIV3 Replication in CF Cells

(A) Phase contrast picture of NL and CF cells, uninfected (upper panels) or 24 hr after HPIV3 infection (middle panels) and immunofluorescence staining for HPIV3 NP 24 hr postinfection (lower panels) ($n = 3$). Bars, 100 μm .

(B) Equal amounts (20 μg) of ^{35}S -methionine-labeled new protein synthesized in NL and CF cells were immunoprecipitated by HPIV3 anti-RNP antibody and loaded in each lane ($n = 3$).

a complete lack of responsiveness to IFN, are highly sensitive to infection by virus (Durbin et al., 1996; Meraz et al., 1996).

In this context, we hypothesized that CF airway epithelial cells may be less effective in eliminating viral infection due to an impairment of the antiviral host defense mechanisms in CF lung. Here, we show that CF airway epithelial cells allow increased replication of parainfluenza virus and an increased production of pro-inflammatory cytokines. Investigation of the innate and interferon (IFN)-mediated antiviral pathways reveals that the antiviral pathway of nitric oxide synthesis is absent in CF. Furthermore, upregulation of 2', 5' OAS1 does not occur in CF cells in response to IFN or dsRNA. This can be attributed to impaired STAT1 activation, which may be a central mechanism responsible for the deficiencies in CF antiviral host defense.

Results

Increased Viral Replication in CF

CF and normal (NL) human airway epithelial cells (HAEC) were infected with human parainfluenza virus 3 (HPIV3) (0.1 moi) and syncytia (cell-cell fusion) formation evaluated (Figure 1A). Cell-cell fusion was increased in CF cells compared to NL 24 hr after infection (middle panel).

Immunofluorescent staining for HPIV3 N-protein (NP) revealed greater size and number of syncytia containing virus in CF cells (lower panel). To confirm that the NP present in the cell lysate was from viral replication and not from added virus, new protein synthesized was evaluated by ^{35}S -methionine incorporation followed by SDS polyacrylamide gel electrophoresis of cell lysates immunoprecipitated with anti-RNP antibody which recognizes HPIV3 NP. NP was detected at ~ 2 -fold higher level in CF than NL (Figure 1B).

IFN Pretreatment Protects CF Cells from Virus

First identified because of their ability to interfere with virus replication, IFNs are fundamental in host antiviral defense (Biron, 1999; Briscoe et al., 1996; Durbin et al., 1996; Grandvaux et al., 2002; Isaacs et al., 1957; Karaghiosoff et al., 2000; Karupiah et al., 1993; Samuel, 1991; Stark et al., 1998). To investigate IFN antiviral effects in CF, CF cells were pretreated with 1000 U/ml IFN- α , IFN- γ , or no cytokine for 24 hr, and then infected with HPIV3 (0.1 moi). Syncytia formation was prevalent in untreated CF cells (Figure 2A, upper-right panel), but pretreatment with IFN- α or IFN- γ prevented viral syncytia formation (Figure 2A, lower panels). Evaluation of HPIV3 N-mRNA expression revealed that more virus N-mRNA was formed in infected CF than in NL cells,

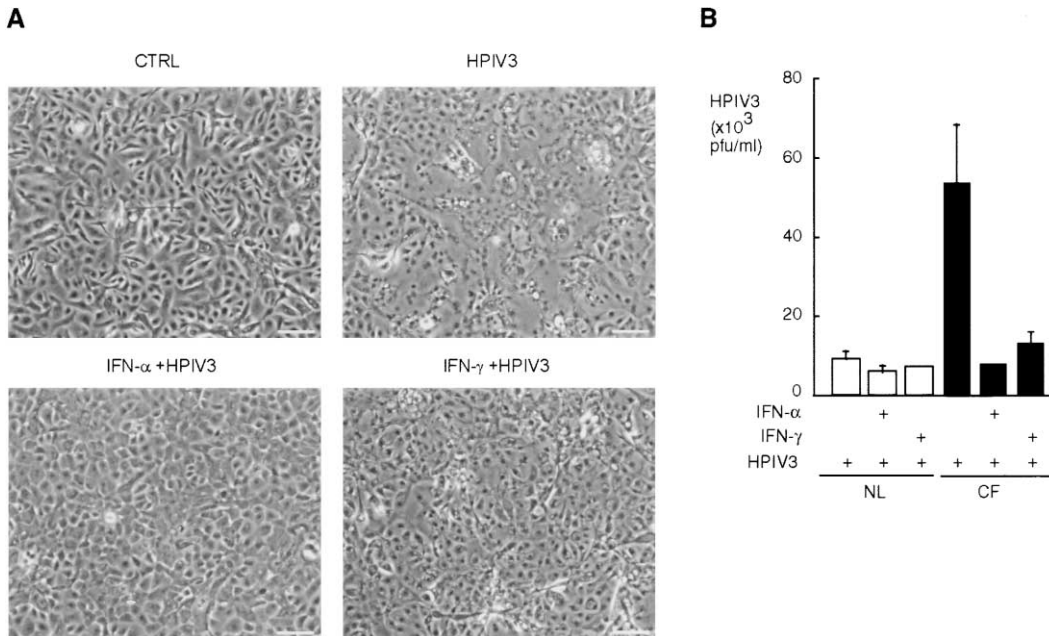


Figure 2. IFN Pretreatment Protects CF Cells from HPIV3 Infection

(A) Phase contrast pictures of CF cells, untreated (upper-left panel), infected with HPIV3 (upper-right panel), or pretreated with IFN- α (lower-left panel) or IFN- γ (lower-right panel) 24 hr before HPIV3 infection (n = 2). Bars, 100 μ m.

(B) Infectious viral particles in media overlying cells untreated or pretreated with IFN- α (1000 U/ml) or IFN- γ (1000 U/ml) measured by plaque assay [plaque forming units (pfu)/ml $\times 10^3$] after HPIV3 infection (0.1 moi). Infectious viral particles are higher titer in media overlying CF cells (n = 5) than NL (n = 3) [$p = 0.015$].

and IFN- α or IFN- γ pretreatment significantly reduced the N-mRNA in both NL and CF cells (data not shown). Media overlying cells were evaluated for infectious HPIV3 particles by plaque assay. CF produced ~ 6 -fold more infectious HPIV3 as compared to NL (CF: 53 ± 15 , range 30~70, n = 5; NL: 8 ± 2 , range 6~10, n = 3 [$\times 10^3$ pfu/ml]). IFN- α and IFN- γ pretreatment reduced virus in CF to NL levels (Figure 2B). Innate antiviral pathways in NL cells appeared effective in eliminating viral replication, but IFN pretreatment reduced viral load by ~ 1.5 -fold. IFN- α and IFN- γ pretreatment reduced virus in CF by 7- and 5-fold, respectively ($p < 0.05$, student's t test).

Increased viral replication may result in an increase in proinflammatory cytokine production and contribute to severity of virus infection in vivo (Matsukura et al., 1996; Zhu et al., 1996). Thus, cytokine production by cells was evaluated. Supernatant from CF cells 24 hr after HPIV3 infection had higher IL-6 and IL-8 compared to NL, although baseline levels were similar [(baseline: IL-6 pg/ml, CF 13 ± 1 , NL 12 ± 2 ; IL-8 pg/ml, CF 195 ± 87 , NL 164 ± 30 ; n = 3, $p > 0.05$ CF versus NL), (24 hr postinfection: IL-6 pg/ml, CF 2568 ± 1996 , NL 208 ± 62 ; IL-8 pg/ml, CF 11920 ± 8606 , NL 2822 ± 245 , n = 3, $p < 0.05$, 24 hr comparison, CF versus NL, Mann-Whitney test)].

Expression of Antiviral Proteins in CF

CF and NL cells infected with HPIV3 (0.1 moi) or treated with IFN- α for 24 hr expressed MxA. IFN- α induced higher MxA compared to HPIV3, while IFN- γ did not

induce MxA (Figure 3A). MxA was produced at later times after HPIV3 infection as compared to IFN- α stimulation (data not shown). IFN- α is synthesized by lung epithelial cells after viral infection (Gao et al., 1999), and virus-induced MxA expression is likely a consequence of IFN- α (Pavlovic et al., 1992; Ronni et al., 1997). Similar levels of IFN- α were produced by CF and NL in response to virus, reaching peak levels in media overlying cells by 6 hr postinfection (data not shown).

Western analyses for IRF-1, PKR, RNase L, and 2', 5' OAS1 were performed with cell lysates collected at 4, 16, and 24 hr after stimulation with virus mimic, dsRNA, or IFN- γ . PKR and IRF-1 were induced by IFN- γ and polyIC in both NL and CF. RNase L did not change before or after stimulation but was present in both cell types. Although NL cells increased 2', 5' OAS1 after stimulation, CF cells failed to upregulate expression of 2', 5' OAS1 (Figure 3B). Viral induction of NOS2 in CF and NL was assessed 24 hr after HPIV3 infection (0, 0.2, 0.4, 1.0 moi). NL showed a dose-dependent induction of NOS2 by HPIV3, but CF had no detectable NOS2. Expression of MxA confirmed the presence of viral infection (Figure 3C). Reverse transcription of RNA and polymerase chain reaction of cDNA (RT-PCR) analysis of NOS2 mRNA in CF and NL confirmed lack of NOS2 induction in CF in response to HPIV3 (data not shown).

Early in virus infection, host defenses including NOS2 may be induced by dsRNA through PKR signaling pathways, independent of IFN- γ , in NL cells (Uetani et al., 2000). However, by 24 hr after infection, large amounts of IFN- γ are produced which lead to activation of numerous downstream target genes. Specifically, IFN- γ is a

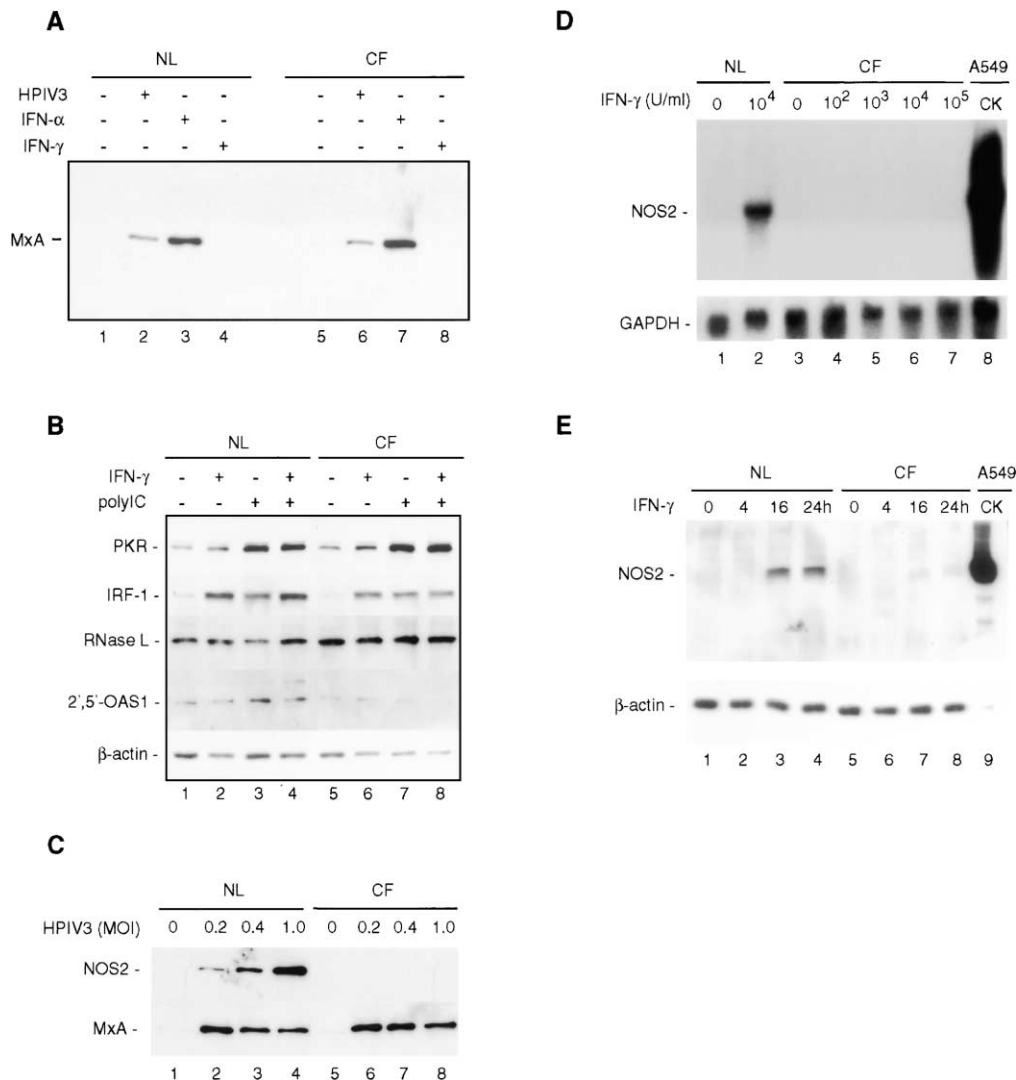


Figure 3. Impaired Antiviral Pathways in CF Cells

(A) Western analysis of MxA in CF and NL cells, untreated, infected with HPIV3 (0.1 moi), or stimulated by IFN- α (1000 U/ml) or IFN- γ (1000 U/ml) for 24 hr (n = 2).

(B) Western analysis of PKR, IRF-1, RNase L, and 2', 5'- OAS1 in CF and NL cells, untreated, or treated with IFN- γ (1000 U/ml), polyIC (100 ng/ml), or by mixture of IFN- γ and polyIC (n = 3).

(C) Western analysis for NOS2 and MxA in NL and CF cells, uninfected and infected with HPIV3 (n = 2).

(D) Northern analysis for NOS2 in total RNA (4 μ g/lane) from CF or NL cells 24 hr after IFN- γ stimulation. Total RNA (5 μ g/lane) from A549 cells 8 hr after stimulation with 10⁵ U/ml IFN- γ , 0.5 ng/ml IL-1 β , and 10 ng/ml TNF- α (cytokine mixture, CK) was used as positive control (n = 2).

(E) Western analysis of NOS2 protein in cell lysate (50 μ g total protein/lane) from NL or CF cells 24 hr after IFN- γ stimulation (n = 3).

potent inducer of NOS2 gene expression in normal human airway cells (Guo et al., 1997; Uetani et al., 2000). Here, Northern analysis of NOS2 expression revealed that NL cells expressed NOS2 mRNA upon IFN- γ exposure, while CF cells did not (Figure 3D). Western analysis of proteins extracted at different time points after IFN- γ stimulation showed that NL produced NOS2 protein as early as 16 hr, while CF had no detectable NOS2 (Figure 3E). We also tested induction of NOS2 by polyIC, and combinations of cytokines (IFN- γ , IL-1 β , TNF- α) in replicate experiments (n = 3). NOS2 was not induced in CF cells by any combination of stimuli (data not shown).

Similar IFN Response in CF and NL Cells

Based upon findings of defective induction of two antiviral pathways, we expanded evaluation of the IFN response in CF. We compared gene expression profiles in CF and NL at baseline (Figure 4A) and 8 hr after IFN (Figures 4B and 4C) by a custom-constructed ISG/AU/dsRNA cDNA microarray, which contains 2921 genes specific for viral and IFN responses. IFN responses were similar between CF and NL with only 0.9% and 0.5% difference in IFN- α - and IFN- γ -induced changes in gene expression (correlation of CF to NL response: IFN- α R² = 0.931; IFN- γ R² = 0.940). IFN- α induced 81 genes and

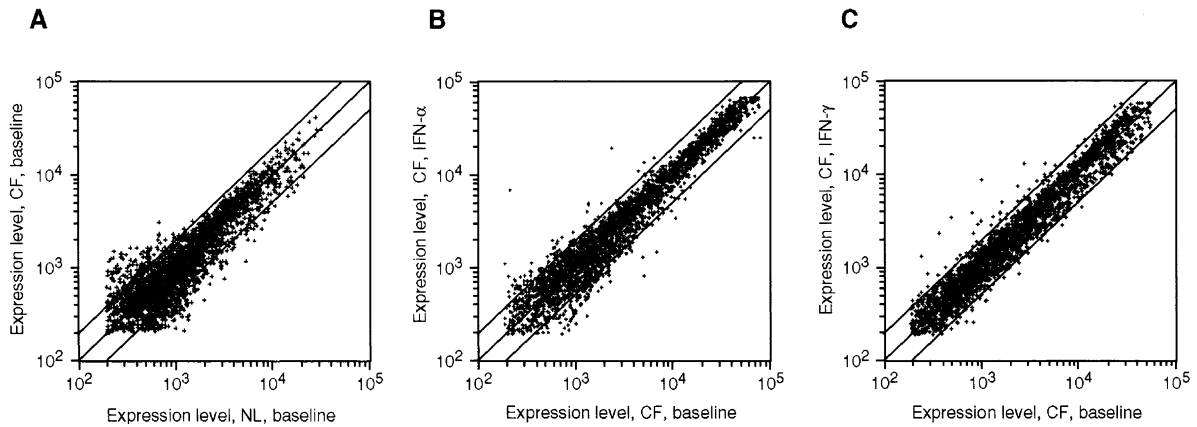


Figure 4. Gene Expression Profile of CF and NL Cells
(A) Baseline gene expression of CF cells compared to NL. (B) Gene expression 8 hr after IFN- α or (C) IFN- γ treatment in CF cells.

repressed 68 genes; IFN- γ induced 27 genes and repressed 33 genes. This similarity of CF response to NL accounts for the effectiveness of IFN pretreatment in inhibiting virus replication in CF cells. On the other hand, a baseline comparison between CF and NL evaluated by ISG/AU/dsRNA microarray identified 226 differentially expressed genes. In CF cells, 136 genes (4.6% of total genes) were 2-fold upregulated, and 90 genes (3% of total genes) were 2-fold downregulated as compared to NL. This baseline difference was confirmed by transcriptosome analysis on Affymetrix HG-U133A GeneChips. Table 1 highlights the genes that are different (≥ 1.2 -fold change) and relevant to IFN, antiviral effects, and/or NOS2 induction. Notable findings include decreased JAK1, a receptor-associated kinase essential for IFN signaling, and increased IRF2, a competitive

inhibitor of IRF-1. Both genes are key to antiviral defense and specifically to NOS2 induction (Briscoe et al., 1996; Kamijo et al., 1994; Nelson et al., 1993). The 2', 5' OAS1 was also lower in CF at baseline, confirming the Western blot analysis (Figure 3B).

Transcription Factors in CF

Further experiments were performed to investigate the mechanism of deficiency of antiviral host defense in CF, and specifically the reduced NOS2 expression in CF. To evaluate signal transduction proteins IRF-1 and NF- κ B, which are important to the host antiviral response including NOS2 induction, we treated CF and NL with IFN- γ (10^3 U/ml), tumor necrosis factor- α (TNF- α) (10 ng/ml), or synthetic dsRNA (polyIC) (100 ng/ml) as a mimic of virus infection, then the transcription factor activation

Table 1. Gene Expression in CF Cells Relative to NL at Baseline

UniGene	Gene Description	Ratio CF/NL	Genebank
Cytokine-Related Genes			
Hs.93913	IL-6: interleukin 6	3.1	NM_000600
Hs.624	IL-8: interleukin 8	2.22	NM_000584
Hs.1722	IL-1 α : interleukin 1 α	3.13	M15329
Hs.285115	IL-13 receptor, α 1	1.42	U81380
Hs.25954	IL-13 receptor, α 2	2.26	NM_000640
Hs.196384	PTHS2: prostaglandin-endoperoxide synthase 2	2.59	NM_000963
Hs.372783	SOD 2: superoxide dismutase 2, mitochondrial	2.79	X15132
Hs.211600	TNFAIP3: tumor necrosis factor α 3	1.51	A1738896
Interferon/Virus-Related Genes			
Hs 83795	IRF 2: interferon regulatory factor 2	1.33	NM_002199
Hs 115541	JAK2: Janus kinase 2	2.00	AF001362
Hs.86958	IR-2: interferon receptor 2	>2	L41944
Hs.179972	IFI α : interferon α -induced protein	1.30	NM_018011
Hs.50651	JAK 1: Janus kinase 1	<0.5 ^a	
Hs.82396	2', 5' OAS1: 2', 5'-oligoadenylate synthetase 1	0.43	NM_002534
Apoptosis			
Hs.381231	caspase 8	>2	NM_00128
Hs.9216	caspase 7	1.75	NM_001227
Others			
Hs.234642	APQ3: aquaporin 3	0.47	NM_004925
Hs.89603	MUC1: mucin 1	0.37	NM_002456

^a Ratio from cDNA microarray data. Gene expression level is below detection limit on Affymetrix genechip. All other ratios are from Affymetrix genechip.

analyzed in whole-cell extract (WCE) by electrophoretic mobility shift assay (EMSA). In contrast to previous reports of reduced IRF-1 expression in whole lungs of CF mice (Kelley and Elmer, 2000; Widdicombe, 2000), IRF-1 was strongly activated by IFN- γ in both CF and NL. Its activation by TNF- α or polyIC was weaker but similar in CF and NL (Figure 5A). Similarly, NF- κ B was activated by dsRNA or TNF- α in both CF and NL (Figure 5B). Quantitation of total NF- κ B (p65 and p50) showed no difference between CF and NL (NF- κ B relative units: nonstimulated, CF 990 ± 380 , NL 1090 ± 360 ; IFN- γ , CF 970 ± 490 , NL 1240 ± 350 ; polyIC, CF 2000 ± 310 , NL 2670 ± 280 ; TNF- α CF 3250 ± 140 , NL 4130 ± 790 ; $n = 3$, all $p > 0.05$).

Activation of STAT1 is essential for NOS2 expression and the antiviral response (Gao et al., 1997; Guo et al., 1997; Heitmeier et al., 1999). To evaluate STAT1, CF and NL were exposed to IFN- γ (10^3 U/ml) for 30 min, then WCE collected and analyzed by EMSA with 32 P-labeled GAS oligo duplex. CF had lower STAT1 activation compared to NL (Figure 5C). Impairment of STAT1 activation was consistent in CF, and $\sim 60\%$ of NL (Figure 5D). STAT1 is important for not only NOS2 expression, but also for STAT1 itself. To evaluate STAT1 production in CF, CF and NL cells exposed to IFN- γ for 24 hr were evaluated by Western blot using rabbit polyclonal anti-STAT1 Ab. 2fTGH and U3A, human fibroblast cell lines with and without expression of STAT1 (Muller et al., 1993), were used as positive and negative controls for STAT1 expression. Baseline STAT1 protein in CF was less than NL, and 24 hr after IFN- γ , NL expressed more STAT1 than CF (Figure 5E). STAT1 protein in CF was only 53% of that in NL (CF = 1.6 ± 0.7 , NL = 3.0 ± 1.3 , $n = 4$, $p < 0.05$). Furthermore, after IFN- γ stimulation, STAT1 in CF was significantly lower than NL (CF = 5.1 ± 0.6 , NL = 10.6 ± 2.0 , $p < 0.01$) (Figure 5F).

Overexpression of NOS2 or NO Donor Protects CF from Virus

Previous work suggests that loss of NOS2 expression in cells leads to increased susceptibility to viral infection (Flodstrom et al., 2001; Karupiah et al., 1998; Noda et al., 2001). Induction of NOS2 prior to infection is associated with inhibition of viral replication (Reiss and Komatsu, 1998; Sanders, 1999). Since CF cells are unable to express NOS2, NOS2 expression construct or NO donors were used to correct the NO deficiency. We introduced NOS2-transgene into CF cells by transfecting the cells with NOS2 expression plasmid (pCCF37). Control CF cells were transfected with reverse sequence NOS2 (R-NOS2) plasmid (pCCF38), or liposome reagent without plasmid, or left untreated. All cells were infected with HPIV3 (0.5 moi) 24 hr after transfection. Alternatively, two types of NO donors, S-nitroso-N-acetyl penicillamine (SNAP) or deta NONOate, were added to cells at the time of viral infection. NOS2 was expressed in CF transfected with pCCF37 but not in control CF cells (Figure 6A). Indicative of viral production, HPIV3 NP was present in untreated and control transfected cells but not in CF cells expressing the NOS2 transgene. Quantitated as nitrite and nitrate in the media, NO production in CF cells transfected with NOS2 transgene was similar to

levels produced by NL cells stimulated with IFN- γ [$\text{NO}_2^- + \text{NO}_3^-$ (μM): CF cells + NOS2 transgene, 8.0 ± 1.0 ; NL cells + IFN- γ , 9.5 ± 0.5]. NO donor compounds produced higher levels of NO in the media [$\text{NO}_2^- + \text{NO}_3^-$ (μM): SNAP, 50 ± 20 ; detaNO, 29 ± 1]. NO donors SNAP and deta NONOate, decreased viral load ~ 2.5 -fold. Strikingly, CF cells transfected with NOS2 transgene (pCCF37) had nearly undetectable infectious virus in the overlying media (Figure 6B). NOS2 overexpression may be more efficient than NO donors because NOS2 transgene provides continuous generation of intracellular NO.

Discussion

Here, CF airway epithelial cells are shown at the cellular level to be more susceptible to HPIV3 infection than NL. Increased virus is due to lack of specific antiviral host defense in CF, including NOS2 and 2', 5' OAS 1 which may be attributed to impairment of activation of STAT1. In support of the biological relevance of ~ 6 -fold increase of virus, murine studies have shown that loss of innate host defenses leads to a moderate increase in virus, but significantly more severe clinical outcomes (Flodstrom et al., 2001; Kosugi et al., 2002; Noda et al., 2001; Xiang et al., 2002; Zhou et al., 1999). For example, even though the increase of virus is modest in organs of NOS2-deficient (NOS2 $^{-/-}$) mice with cytomegalovirus (Noda et al., 2001) or coxsackievirus B4 (Flodstrom et al., 2001) as compared to wild-type, the NOS2 knockout mice have higher mortality and decreased virus clearance. Likewise, CMV replication is only moderately enhanced as evidenced by 5-fold increase in viral titers in mice pretreated with a specific inhibitor of NOS2, but this results in viral persistence and latency (Kosugi et al., 2002). Mice triple deficient in Mx, RNase L, and PKR have increased susceptibility to virus, although viral titers are not significantly elevated in tissues (Xiang et al., 2002; Zhou et al., 1999). More severe clinical outcomes with modest increase of virus may occur due to inherent viral properties and/or altered host cellular response (Garcia-Sastre, 2001, 1998; Seo et al., 2002). For example, virulence may be increased with moderately higher titers due to more efficient inhibition of host antiviral pathways. Conversely, greater activation of signaling pathways, such as NF- κ B, due to increased dsRNA produced during increased viral replication, may amplify proinflammatory cytokine production (Matsukura et al., 1996; Zhu et al., 1996). In this study, CF cells released more IL-6 and IL-8 than NL in response to virus. Higher levels of IL-6 and IL-8, which are involved in neutrophil accumulation and degranulation and contribute to greater airway inflammation and more severe respiratory symptoms with virus (Matsukura et al., 1996; Zhu et al., 1996). For example, severity of clinical symptoms with rhinovirus is primarily related to high levels of IL-6 in nasal secretions (Zhu et al., 1996). CF airways, even in infants, contain higher levels of proinflammatory cytokines, particularly IL-6 and IL-8, irrespective of bacterial colonization (Aldallal et al., 2002; Noah et al., 1997). Thus, it has been hypothesized that inflammation is intrinsic to the CF neonatal airway prior to infection. Here, baseline IL-6 and IL-8 secretion are similar in CF and

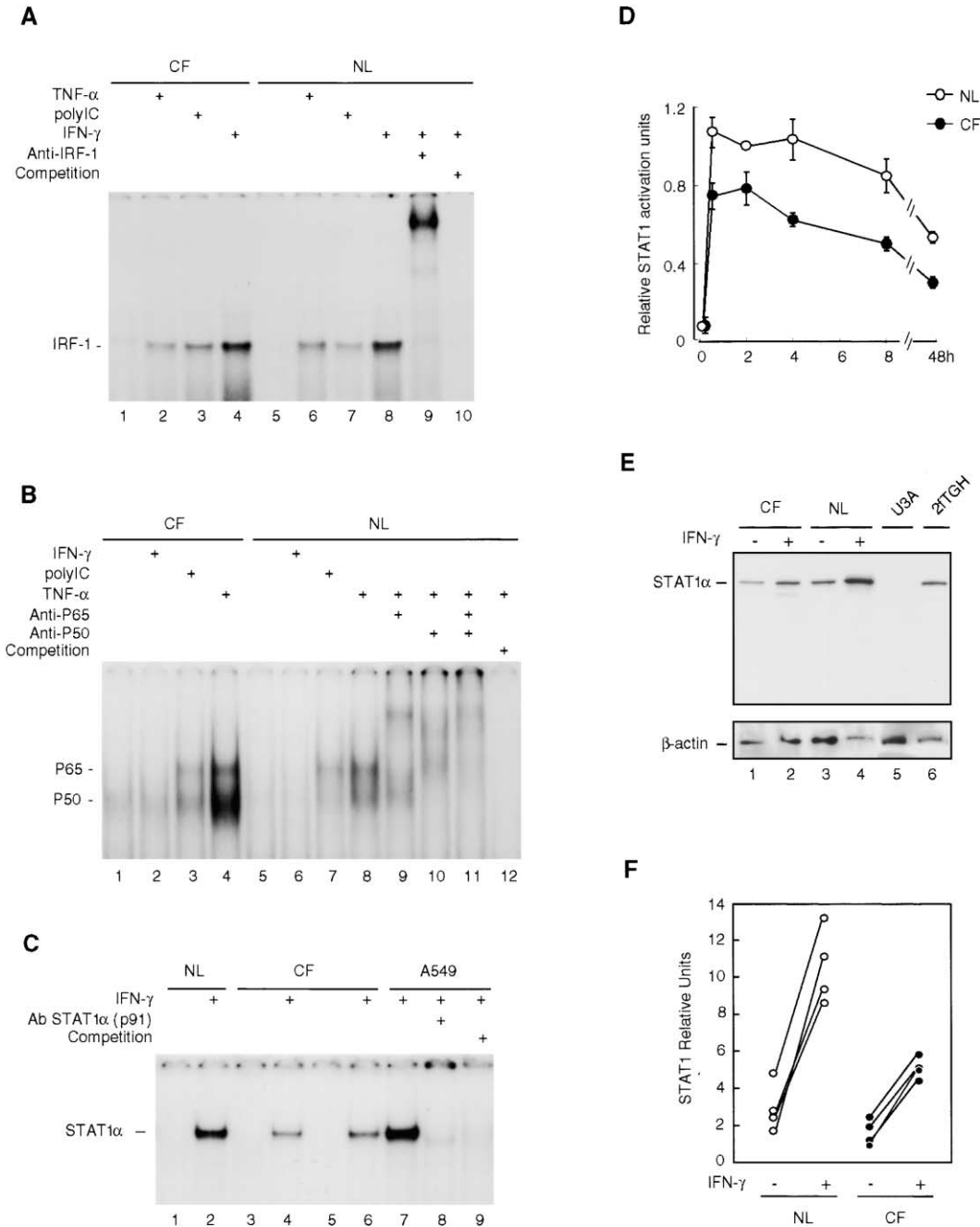


Figure 5. Activation and Expression of Transcription Factors in CF Cells

(A) WCE (4 μ g) from CF and NL cells, untreated or treated with TNF- α , polyIC, or by IFN- γ for 3 hr were evaluated for IRF-1 by EMSA (n = 4).
 (B) NF- κ B activation was evaluated by EMSA in cells stimulated with TNF- α , polyIC, or by IFN- γ for 1 hr (n = 3).
 (C) CF and NL cells were stimulated with IFN- γ for 30 min and WCE collected to evaluate for STAT1 activation by EMSA. IFN- γ -stimulated A549 was a positive control, and supershift with anti-STAT1 (p91) and competition with unlabeled GAS probe confirmed presence of STAT1 in the complex.
 (D) STAT1 activation at different times was quantitated in four independent EMSA experiments, which were averaged and expressed as relative units normalized to NL value at 2 hr.
 (E) Cell lysate (20 μ g total protein/lane) from CF or NL 24 hr after IFN- γ stimulation was evaluated for STAT1 (p91) expression by Western analysis. Lysates from 2fTGH and U3A were used as positive and negative controls.
 (F) Quantitation of Western analysis of STAT1 expression in cell lysate from four pairs of NL and CF cells, unstimulated or 24 hr after IFN- γ .

NL, but IL-6 and IL-8 mRNA are higher, which may account for the greater release of cytokines upon viral infection. Thus, virus may be one stimulus for the in-

creased cytokine production in CF airways. Taken together, the susceptibility of CF infants to virus may be explained by increased virus and cytokine production,

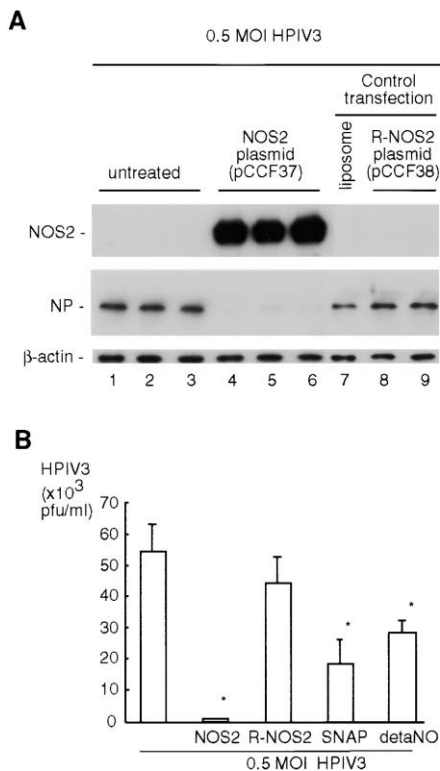


Figure 6. NOS2 Overexpression or NO Donors Protect CF Cells from HPIV3 Infection

(A) Western analysis for NOS2 and HPIV3 NP in CF cells infected by HPIV3, transfected with NOS2 expression construct (NOS2, pCCF37), exposed to reagent alone, or transfected with reverse sequence NOS2 expression construct (R-NOS2, pCCF38) 24 hr prior to infection (n = 2).

(B) Plaque assay using media overlying CF cells 24 hr after HPIV3 infection (0.5 moi). 24 hr prior to infection, CF cells were transfected with NOS2 expression construct (NOS2, pCCF37), reverse sequence NOS2 expression construct (R-NOS2, pCCF38), reagent alone (liposome), or left untreated. At the time of infection, some untreated CF cells were exposed to NO donors, SNAP, or deta NONOate (detaNO). Untreated cells have higher titer of infectious virus production than cells with NOS2-transgene or with NO donors [n = 3, *p < 0.02].

which results in greater airway inflammation and the severe respiratory symptoms of CF infants with virus infection.

Despite defects in antiviral defenses, pretreatment with IFNs protected CF from virus. The biologic consequences, including antiviral effects, of IFN are mediated by multiple independent genes. Induction of over 600 genes has been identified in response to IFNs (de Veer et al., 2001). Thus, it is difficult to assign IFN antiviral action to any specific gene. Redundancy of antiviral defense is supported by the fact that pretreatment with exogenous IFN leads to a protective antiviral state despite defects in various antiviral pathways. However, it is clear that if the early antiviral defenses are lacking, using a strategy of knockout of specific antiviral genes, virus infection can lead to devastating effects despite the presence of intact IFN pathways (Kosugi et al., 2002; Noda et al., 2001; Xiang et al., 2002; Zhou et al., 1999). For example, mice deficient in Mx, RNase L, and PKR, which are markedly susceptible to viral infections, are

nevertheless rescued by pretreatment with IFN (Zhou et al., 1999). Thus, virus-inducible, cell-autonomous innate defenses are important to inhibiting virus, and indeed may be crucial to host defense against viruses with strategies that interfere with IFN signaling, such as HPIV3.

STAT1 is required for IFN signal transduction in the cell and essential for the survival response to virus infection (Durbin et al., 1996; Meraz et al., 1996). Despite numerous downstream targets of STAT1 activation, loss of NOS2 has been identified as a primary factor in the susceptibility of STAT1 null animals to virus (Karupiah et al., 1993). Although not clearly understood, decreased STAT1 also produces a deficient antiviral state and loss of NOS2, while other IFN-mediated genes respond normally (Briscoe et al., 1996; Karaghiosoff et al., 2000). In two prior studies, nonfunctional JAK1 or Tyk2, receptor-associated kinases in the IFN signaling pathway, resulted in decreased STAT1 protein and activation, and a defective antiviral state, although the response to IFN- α or - γ was intact (Briscoe et al., 1996; Karaghiosoff et al., 2000). The Tyk2-deficient cells displayed a phenotype remarkably similar to the CF cells: increased virus replication in cells, impairment of STAT1 activation, with almost all IFN-dependent pathways intact except for NOS2. Altogether, these and the present study suggest that a threshold of STAT 1 may be required for the antiviral state, expression of NOS2, and perhaps other antiviral genes, such as 2', 5' OAS. Alternatively, a JAK1- or Tyk2-dependent signal may be required, in addition to STAT1, for expression of NOS2, and for the antiviral state (Briscoe et al., 1996).

Because IFN/STAT1 pathways are so effective in preventing viral infection, many viruses have developed mechanisms to evade the interferon system of the host. All members of the paramyxovirus family interfere with IFN signaling, although by different mechanisms (Andrejeva et al., 2002; Young et al., 2000). HPIV3 inhibits IFN signaling, through specific reduction of serine phosphorylation of STAT1 α (Young et al., 2000). Serine phosphorylation is intact in CF (data not shown), but CF cells with impaired IFN activation of STAT1 may be particularly vulnerable to serine phosphorylation block by HPIV3, resulting in more effective interference with IFN signaling. While interference with IFN signaling is a common strategy by which paramyxovirus circumvents antiviral defenses (Andrejeva et al., 2002; Young et al., 2000), viral proteins which block NOS pathways have not been reported. Our data support that HPIV3 may not have specific strategies to escape NO effects. NO inhibits virus replication and even latency of virus, including coxsackievirus, influenza A & B, murine cytomegalovirus, vaccinia, ectromelia, and herpes simplex-1 (Croen, 1993; Flodstrom et al., 2001; Karupiah et al., 1998; Karupiah and Harris, 1995; Rimmelzwaan et al., 1999; Saura et al., 1999; Zaragoza et al., 1997). Here, HPIV3 is also shown to be inhibited by NO. Two specific virus targets of NO, ribonucleotide reductase and viral protease, have been suggested on the basis of in vitro exposure of viral protein to NO donors in cell free systems (Croen, 1993; Lepoivre et al., 1991; Saura et al., 1999). These two known targets are absent in HPIV3. Although viral proteins may be targets of NO, NO also affects host proteins, which is relevant to HPIV3 since it requires host

proteins for transcription and replication (De et al., 1993). Known targets for NO modification include thiol groups and tyrosine, and NO may bind to heme iron in proteins (Grisham et al., 1999). In lung epithelial cells, over 40 cellular proteins are modified by tyrosine nitration, with consequences on activity and function (Aulak et al., 2001). Tyrosine nitration is decreased by NOS inhibitors and in NOS2 knockout cells; thus, NO modification of both host and viral proteins and subsequent effects on protein expression and activity are also likely reduced in CF cells which lack NOS2.

It is interesting to speculate about whether CFTR has a direct effect or is a modifier gene for expression of STAT1, 2', 5' OAS1, or NOS2. Inhibition of CFTR function results in reduced NOS2 mRNA in human tracheal epithelial cell lines, while overexpression of human CFTR in CF mice intestinal epithelium leads to NOS2 expression in the ileum (Steagall et al., 2000). These results suggest that NOS2 expression may be directly related to the presence of functional CFTR. In addition, the present findings suggest that STAT1 and NOS2 may be potential gene modifiers of the disease severity in CF lung disease. An important component of the innate host defense in the airway is the ability of respiratory epithelial cells to produce NO continuously in vivo (Sanders et al., 1998). The continuous production of NO in the airways is due in part to expression of NOS2 (Guo et al., 1995). CF infants at birth prior to the onset of respiratory symptoms/infection have exhaled NO 3-fold lower than in healthy controls, suggesting that the defect in NOS2 expression occurs prior to onset of recurrent infections (Elphick et al., 2001). Here, NOS2 is conclusively shown to be sufficient for antiviral defense in human airway epithelial cells. The success of overexpression of NOS2 in CF cells, or pretreatment with IFN, in protection from viral infection indicates that these approaches are promising in prevention of CF lung infection. Although less effective, provision of NO donors provided significant reduction of viral production and may be an alternative strategy for treatment of CF patients.

Experimental Procedures

Cell Culture, Virus, and Cytokines

HAEC were obtained through bronchoscopy brushing, from explanted lungs, or from segments of bronchus obtained from surgery and cultured by methods previously described (Guo et al., 2000; Uetani et al., 2000). An aliquot of cultured cells was immunostained to confirm epithelial phenotype. In addition, all cells were genotyped for 86 common *CFTR* mutations by Genzyme Genetics (Boston, Massachusetts). All eight samples from explant CF lungs were confirmed to be homozygous $\Delta F508/\Delta F508$. Eleven samples from control non-CF lungs were all wild-type *CFTR*.

A549 cells and CV-1 cells were maintained as previously described (Choudhary et al., 2001; Guo et al., 2000). HPIV3 (HA-1, NIH 47885) was a kind gift from Dr. De. Human IFN- γ was a gift from Genentech Inc. (South San Francisco, California). The IFN- α was purchased from Sigma-Aldrich (St. Louis, Missouri). Recombinant human IL-1 β and TNF- α were from Genzyme.

RNA Isolation and Northern Analysis

Total RNA was extracted by GTC-CsCl gradient method (Erzurum et al., 1993a). Northern analysis was carried out using ^{32}P -dCTP-labeled human NOS2 cDNA by methods previously described (Uetani et al., 2000).

Plaque Assay and Immunofluorescent Staining

Culture supernatants were collected, and the yield of infectious HPIV3 in cells that underwent specific treatments was measured by plaque assay on CV-1 cells as previously described (De et al., 1995). 24 hr postinfection, cells cultured on cover slides were stained for HPIV3 by the method previously described (Choudhary et al., 2001).

^{35}S -Methionine Labeling and Immunoprecipitation

CF and NL cells in 12-well plate were infected with HPIV3 at 0.1 moi. At 12 hr postinfection, the medium was replaced with methionine-free DMEM and incubation was continued in 37°C. At 14 hr postinfection, the cells were labeled with 50 μCi of ^{35}S -methionine in 1 ml methionine-free DMEM for 6 hr. Cells were washed with DPBS and cell lysates were prepared and 20 μg of protein was immunoprecipitated by antibody against HPIV3 N-protein as previously described (De et al., 2000) and analyzed in an SDS-10% polyacrylamide gel.

IL-6 and IL-8 ELISA

Production of human IL-6 and IL-8 in the supernatant from CF and NL cells 24 hr after HPIV3 infection was evaluated using Quantikine human IL-6 and IL-8 ELISA (R&D Systems, Minneapolis, Minnesota). All samples were diluted ten times using appropriate calibration buffer.

Custom cDNA Microarray and Affymetrix Gene Array

RNA extracted from CF and NL cells at baseline or after 8 hr IFN treatment were evaluated for gene expression profile using custom-constructed cDNA microarray as previously described (Frevet et al., 2003). The ISG/AU/dsRNA array used in this study contains 1013 ISGs, 1464 AU-rich genes, 18 genes potentially involved in AU directed mRNA decay, 54 ribosomal genes, 288 dsRNA-responsive genes, and 84 housekeeping genes (NOS2 is not on this array).

Affymetrix HG-U133A GeneChips were also used in this study to evaluate baseline gene expression in CF and NL cells as previously described (Lipshutz et al., 1999; Yang et al., 2000).

Western Analysis

Whole-cell lysates were prepared and Western analysis performed as previously described (Uetani et al., 2000). The primary antibodies used included rabbit polyclonal antibody against NOS2 (Merck, Rahway, New Jersey), rabbit polyclonal antibody against C terminus of IRF-1 (Santa Cruz Biotechnology, Santa Cruz, California), rabbit polyclonal antibody against PKR (Carpick et al., 1997), mouse monoclonal antibody against RNase L (Dong and Silverman, 1995), rabbit polyclonal antibodies against MxA and HPIV3 N-protein (Choudhary et al., 2001), and rabbit polyclonal antibody against 2', 5' OAS1 (Ghosh et al., 2001).

WCE and EMSA

WCE were prepared and EMSA performed by methods previously described (Guo et al., 1997; Uetani et al., 2000). To specifically identify NF- κB , IRF-1, and STAT1 α (p91) proteins in binding complexes, 2–4 μg of rabbit anti-p65, anti-p50, anti-IRF-1, or anti-STAT1 α (p91) polyclonal Ab (Santa Cruz Biotechnology) was added to the binding reaction mix and incubated for 30 min at room temperature before adding the ^{32}P -labeled oligonucleotide.

NOS2 Expression Construct and Transient Transfection

Human NOS2 expression construct was made by inserting full-length NOS2 cDNA into a pAVS6 vector (Erzurum et al., 1993b). A control construct was also made by inserting reverse sequence NOS2 cDNA into a pAVS6 vector. Transient transfection was performed on cells at 90% confluence using LipofectAMINE PLUS reagent (Invitrogen Corporation, Carlsbad, California).

Nitrite and Nitrate Quantitation

NO production was quantitated by measuring total nitrite and nitrate in the media, using ISO-NO MarkII isolated nitric oxide meter and nitric oxide sensor (ISO-NOP) (World Precision Instruments, Inc., Sarasota, Florida). Data were collected and analyzed by Duo18.

Statistical Analysis

The data are reported as means \pm standard deviation of the mean (SD). Two-tailed t test statistics or the Mann-Whitney test was used as appropriate at a significance level of 0.05.

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References

2000. Cystic Fibrosis Foundation. Patient Registry 2000 Annual Report (Bethesda, Maryland, Cystic Fibrosis Foundation), pp. 1.
- Aldallal, N., McNaughton, E.E., Manzel, L.J., Richards, A.M., Zabner, J., Ferkol, T.W., and Look, D.C. (2002). Inflammatory response in airway epithelial cells isolated from patients with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 166, 1248–1256.
- Anderson, M.P., Rich, D.P., Gregory, R.J., Smith, A.E., and Welsh, M.J. (1991). Generation of cAMP-activated chloride currents by expression of CFTR. *Science* 251, 679–682.
- Andrejeva, J., Young, D.F., Goodbourn, S., and Randall, R.E. (2002). Degradation of STAT1 and STAT2 by the V proteins of simian virus 5 and human parainfluenza virus type 2, respectively: consequences for virus replication in the presence of alpha/beta and gamma interferons. *J. Virol.* 76, 2159–2167.
- Armstrong, D., Grimwood, K., Carlin, J.B., Carzino, R., Hull, J., Olinisky, A., and Phelan, P.D. (1998). Severe viral respiratory infections in infants with cystic fibrosis. *Pediatr. Pulmonol.* 26, 371–379.
- Aulak, K.S., Miyagi, M., Yan, L., West, K.A., Massillon, D., Crabb, J.W., and Stuehr, D.J. (2001). Proteomic method identifies proteins nitrated in vivo during inflammatory challenge. *Proc. Natl. Acad. Sci. USA* 98, 12056–12061.
- Biron, C.A. (1999). Initial and innate responses to viral infections—pattern setting in immunity or disease. *Curr. Opin. Microbiol.* 2, 374–381.
- Briscoe, J., Rogers, N.C., Witthuhn, B.A., Watling, D., Harpur, A.G., Wilks, A.F., Stark, G.R., Ihle, J.N., and Kerr, I.M. (1996). Kinase-negative mutants of JAK1 can sustain interferon-gamma-inducible gene expression but not an antiviral state. *EMBO J.* 15, 799–809.
- Carpick, B.W., Graziano, V., Schneider, D., Maitra, R.K., Lee, X., and Williams, B.R. (1997). Characterization of the solution complex between the interferon-induced, double-stranded RNA-activated protein kinase and HIV-1 trans-activating region RNA. *J. Biol. Chem.* 272, 9510–9516.
- Choudhary, S., Gao, J., Leaman, D.W., and De, B.P. (2001). Interferon action against human parainfluenza virus type 3: involvement of a novel antiviral pathway in the inhibition of transcription. *J. Virol.* 75, 4823–4831.
- Croen, K.D. (1993). Evidence for antiviral effect of nitric oxide. Inhibition of herpes simplex virus type 1 replication. *J. Clin. Invest.* 91, 2446–2452.
- De, B.P., Burdsall, A.L., and Banerjee, A.K. (1993). Role of cellular actin in human parainfluenza virus type 3 genome transcription. *J. Biol. Chem.* 268, 5703–5710.
- De, B.P., Gupta, S., and Banerjee, A.K. (1995). Cellular protein kinase C isoform zeta regulates human parainfluenza virus type 3 replication. *Proc. Natl. Acad. Sci. USA* 92, 5204–5208.
- De, B.P., Hoffman, M.A., Choudhary, S., Huntley, C.C., and Banerjee, A.K. (2000). Role of NH(2)- and COOH-terminal domains of the P protein of human parainfluenza virus type 3 in transcription and replication. *J. Virol.* 74, 5886–5895.
- de Veer, M.J., Holko, M., Frevel, M., Walker, E., Der, S., Paranjape, J.M., Silverman, R.H., and Williams, B.R. (2001). Functional classification of interferon-stimulated genes identified using microarrays. *J. Leukoc. Biol.* 69, 912–920.
- Dong, B., and Silverman, R.H. (1995). 2–5A-dependent RNase molecules dimerize during activation by 2–5A. *J. Biol. Chem.* 270, 4133–4137.
- Durbin, J.E., Hackenmiller, R., Simon, M.C., and Levy, D.E. (1996). Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell* 84, 443–450.
- Elphick, H.E., Demoncheaux, E.A., Ritson, S., Higenbottam, T.W., and Everard, M.L. (2001). Exhaled nitric oxide is reduced in infants with cystic fibrosis. *Thorax* 56, 151–152.
- Erzurum, S.C., Danel, C., Gillissen, A., Chu, C.S., Trapnell, B.C., and Crystal, R.G. (1993a). In vivo antioxidant gene expression in human airway epithelium of normal individuals exposed to 100% O₂. *J. Appl. Physiol.* 75, 1256–1262.
- Erzurum, S.C., Lemarchand, P., Rosenfeld, M.A., Yoo, J.H., and Crystal, R.G. (1993b). Protection of human endothelial cells from oxidant injury by adenovirus-mediated transfer of the human catalase cDNA. *Nucleic Acids Res.* 21, 1607–1612.
- Flodstrom, M., Horwitz, M.S., Maday, A., Balakrishna, D., Rodriguez, E., and Sarvetnick, N. (2001). A critical role for inducible nitric oxide synthase in host survival following coxsackievirus B4 infection. *Virology* 281, 205–215.
- Frevel, M.A., Bakheet, T., Silva, A.M., Hissong, J.G., Khabar, K.S., and Williams, B.R. (2003). p38 Mitogen-activated protein kinase-dependent and -independent signaling of mRNA stability of AU-rich element-containing transcripts. *Mol. Cell. Biol.* 23, 425–436.
- Gao, J., Morrison, D.C., Parmely, T.J., Russell, S.W., and Murphy, W.J. (1997). An interferon-gamma-activated site (GAS) is necessary for full expression of the mouse iNOS gene in response to interferon-gamma and lipopolysaccharide. *J. Biol. Chem.* 272, 1226–1230.
- Gao, J., De, B.P., and Banerjee, A.K. (1999). Human parainfluenza virus type 3 up-regulates major histocompatibility complex class I and II expression on respiratory epithelial cells: involvement of a STAT1- and CIITA-independent pathway. *J. Virol.* 73, 1411–1418.
- Garcia-Sastre, A. (2001). Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. *Virology* 279, 375–384.
- Garcia-Sastre, A., Egorov, A., Matasov, D., Brandt, S., Levy, D.E., Durbin, J.E., Palese, P., and Muster, T. (1998). Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. *Virology* 252, 324–330.
- Ghosh, A., Sarkar, S.N., Rowe, T.M., and Sen, G.C. (2001). A specific isozyme of 2'-5' oligoadenylate synthetase is a dual function proapoptotic protein of the Bcl-2 family. *J. Biol. Chem.* 276, 25447–25455.
- Grandvaux, N., tenOever, B.R., Servant, M.J., and Hiscott, J. (2002). The interferon antiviral response: from viral invasion to evasion. *Curr. Opin. Infect. Dis.* 15, 259–267.
- Grisham, M.B., Jourdain, D., and Wink, D.A. (1999). Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites: implications in inflammation. *Am J. Physiol.* 276, G315–G321.
- Guo, F.H., De Raeve, H.R., Rice, T.W., Stuehr, D.J., Thunnissen, F.B., and Erzurum, S.C. (1995). Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium in vivo. *Proc. Natl. Acad. Sci. USA* 92, 7809–7813.
- Guo, F.H., Uetani, K., Haque, S.J., Williams, B.R., Dweik, R.A., Thunnissen, F.B., Calhoun, W., and Erzurum, S.C. (1997). Interferon gamma and interleukin 4 stimulate prolonged expression of inducible nitric oxide synthase in human airway epithelium through synthesis of soluble mediators. *J. Clin. Invest.* 100, 829–838.
- Guo, F.H., Comhair, S.A., Zheng, S., Dweik, R.A., Eissa, N.T., Thomassen, M.J., Calhoun, W., and Erzurum, S.C. (2000). Molecular mechanisms of increased nitric oxide (NO) in asthma: evidence for transcriptional and post-translational regulation of NO synthesis. *J. Immunol.* 164, 5970–5980.

- Haque, S.J., and Williams, B.R. (1998). Signal transduction in the interferon system. *Semin. Oncol.* 25, 14–22.
- Heitmeier, M.R., Scarim, A.L., and Corbett, J.A. (1999). Prolonged STAT1 activation is associated with interferon-gamma priming for interleukin-1-induced inducible nitric-oxide synthase expression by islets of Langerhans. *J. Biol. Chem.* 274, 29266–29273.
- Hiatt, P.W., Grace, S.C., Kozinetz, C.A., Raboudi, S.H., Treece, D.G., Taber, L.H., and Piedra, P.A. (1999). Effects of viral lower respiratory tract infection on lung function in infants with cystic fibrosis. *Pediatrics* 103, 619–626.
- Hordvik, N.L., Konig, P., Hamory, B., Cooperstock, M., Kreutz, C., Gayer, D., and Barbero, G. (1989). Effects of acute viral respiratory tract infections in patients with cystic fibrosis. *Pediatr. Pulmonol.* 7, 217–222.
- Iordanov, M.S., Wong, J., Bell, J.C., and Magun, B.E. (2001). Activation of NF-kappaB by double-stranded RNA (dsRNA) in the absence of protein kinase R and RNase L demonstrates the existence of two separate dsRNA-triggered antiviral programs. *Mol. Cell. Biol.* 21, 61–72.
- Isaacs, A., Lindenmann, J., and Valentine, R.C. (1957). Virus interference II. Some properties of interferon. *Proc. R. Soc. Lond. B* 147, 268–273.
- Kamijo, R., Harada, H., Matsuyama, T., Bosland, M., Gerecitano, J., Shapiro, D., Le, J., Koh, S.I., Kimura, T., Green, S.J., et al. (1994). Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* 263, 1612–1615.
- Karaghiousoff, M., Neubauer, H., Lassnig, C., Kovarik, P., Schindler, H., Pircher, H., McCoy, B., Bogdan, C., Decker, T., Brem, G., et al. (2000). Partial impairment of cytokine responses in Tyk2-deficient mice. *Immunity* 13, 549–560.
- Karupiah, G., Xie, Q.W., Buller, R.M., Nathan, C., Duarte, C., and MacMicking, J.D. (1993). Inhibition of viral replication by interferon-gamma-induced nitric oxide synthase. *Science* 261, 1445–1448.
- Karupiah, G., and Harris, N. (1995). Inhibition of viral replication by nitric oxide and its reversal by ferrous sulfate and tricarboxylic acid cycle metabolites. *J. Exp. Med.* 181, 2171–2179.
- Karupiah, G., Chen, J.H., Nathan, C.F., Mahalingam, S., and MacMicking, J.D. (1998). Identification of nitric oxide synthase 2 as an innate resistance locus against ectromelia virus infection. *J. Virol.* 72, 7703–7706.
- Kelley, T.J., and Elmer, H.L. (2000). In vivo alterations of IFN regulatory factor-1 and PIAS1 protein levels in cystic fibrosis epithelium. *J. Clin. Invest.* 106, 403–410.
- Kerem, B., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M., and Tsui, L.C. (1989). Identification of the cystic fibrosis gene: genetic analysis. *Science* 245, 1073–1080.
- Kosugi, I., Kawasaki, H., Arai, Y., and Tsutsui, Y. (2002). Innate immune responses to cytomegalovirus infection in the developing mouse brain and their evasion by virus-infected neurons. *Am. J. Pathol.* 161, 919–928.
- Lepoivre, M., Fieschi, F., Coves, J., Thelander, L., and Fontecave, M. (1991). Inactivation of ribonucleotide reductase by nitric oxide. *Biochem. Biophys. Res. Commun.* 179, 442–448.
- Lipshutz, R.J., Fodor, S.P., Gingeras, T.R., and Lockhart, D.J. (1999). High density synthetic oligonucleotide arrays. *Nat. Genet.* 21, 20–24.
- Matsukura, S., Kokubu, F., Noda, H., Tokunaga, H., and Adachi, M. (1996). Expression of IL-6, IL-8, and RANTES on human bronchial epithelial cells, NCI-H292, induced by influenza virus A. *J. Allergy Clin. Immunol.* 98, 1080–1087.
- Meraz, M.A., White, J.M., Sheehan, K.C., Bach, E.A., Rodig, S.J., Dighe, A.S., Kaplan, D.H., Riley, J.K., Greenlund, A.C., Campbell, D., et al. (1996). Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. *Cell* 84, 431–442.
- Muller, M., Laxton, C., Briscoe, J., Schindler, C., Improt, T., Darnell, J.E., Jr., Stark, G.R., and Kerr, I.M. (1993). Complementation of a mutant cell line: central role of the 91 kDa polypeptide of ISGF3 in the interferon-alpha and -gamma signal transduction pathways. *EMBO J.* 12, 4221–4228.
- Nelson, N., Marks, M.S., Driggers, P.H., and Ozato, K. (1993). Interferon consensus sequence-binding protein, a member of the interferon regulatory factor family, suppresses interferon-induced gene transcription. *Mol. Cell. Biol.* 13, 588–599.
- Noah, T.L., Black, H.R., Cheng, P.W., Wood, R.E., and Leigh, M.W. (1997). Nasal and bronchoalveolar lavage fluid cytokines in early cystic fibrosis. *J. Infect. Dis.* 175, 638–647.
- Noda, S., Tanaka, K., Sawamura, S., Sasaki, M., Matsumoto, T., Mikami, K., Aiba, Y., Hasegawa, H., Kawabe, N., and Koga, Y. (2001). Role of nitric oxide synthase type 2 in acute infection with murine cytomegalovirus. *J. Immunol.* 166, 3533–3541.
- Pavlovic, J., Haller, O., and Staeheli, P. (1992). Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle. *J. Virol.* 66, 2564–2569.
- Petersen, N.T., Hoiby, N., Mordhorst, C.H., Lind, K., Flensburg, E.W., and Bruun, B. (1981). Respiratory infections in cystic fibrosis patients caused by virus, chlamydia and mycoplasma—possible synergism with *Pseudomonas aeruginosa*. *Acta Paediatr. Scand.* 70, 623–628.
- Reiss, C.S., and Komatsu, T. (1998). Does nitric oxide play a critical role in viral infections? *J. Virol.* 72, 4547–4551.
- Rimmelzwaan, G.F., Baars, M.M., de Lijster, P., Fouchier, R.A., and Osterhaus, A.D. (1999). Inhibition of influenza virus replication by nitric oxide. *J. Virol.* 73, 8880–8883.
- Riordan, J.R., Rommens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.L., et al. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 245, 1066–1073.
- Rommens, J.M., Iannuzzi, M.C., Kerem, B., Drumm, M.L., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D., Hidaka, N., et al. (1989). Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* 245, 1059–1065.
- Ronni, T., Matikainen, S., Sareneva, T., Melen, K., Pirhonen, J., Keskinen, P., and Julkunen, I. (1997). Regulation of IFN-alpha/beta, MxA, 2',5'-oligoadenylate synthetase, and HLA gene expression in influenza A-infected human lung epithelial cells. *J. Immunol.* 158, 2363–2374.
- Rosenfeld, M., and Ramsey, B. (1992). Evolution of airway microbiology in the infant with cystic fibrosis: role of nonpseudomonal and pseudomonal pathogens. *Semin. Respir. Infect.* 7, 158–167.
- Samuel, C.E. (1991). Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities. *Virology* 183, 1–11.
- Sanders, S.P. (1999). Asthma, viruses, and nitric oxide. *Proc. Soc. Exp. Biol. Med.* 220, 123–132.
- Sanders, S.P., Siekierski, E.S., Porter, J.D., Richards, S.M., and Proud, D. (1998). Nitric oxide inhibits rhinovirus-induced cytokine production and viral replication in a human respiratory epithelial cell line. *J. Virol.* 72, 934–942.
- Saura, M., Zaragoza, C., McMillan, A., Quick, R.A., Hohenadl, C., Lowenstein, J.M., and Lowenstein, C.J. (1999). An antiviral mechanism of nitric oxide: inhibition of a viral protease. *Immunity* 10, 21–28.
- Seo, S.H., Hoffmann, E., and Webster, R.G. (2002). Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. *Nat. Med.* 8, 950–954.
- Sheppard, D.N., and Welsh, M.J. (1999). Structure and function of the CFTR chloride channel. *Physiol. Rev.* 79, S23–S45.
- Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., and Schreiber, R.D. (1998). How cells respond to interferons. *Annu. Rev. Biochem.* 67, 227–264.
- Steagall, W.K., Elmer, H.L., Brady, K.G., and Kelley, T.J. (2000). Cystic fibrosis transmembrane conductance regulator-dependent regulation of epithelial inducible nitric oxide synthase expression. *Am. J. Respir. Cell Mol. Biol.* 22, 45–50.
- Uetani, K., Der, S.D., Zamanian-Daryoush, M., de La Motte, C., Lieberman, B.Y., Williams, B.R., and Erzurum, S.C. (2000). Central role

- of double-stranded RNA-activated protein kinase in microbial induction of nitric oxide synthase. *J. Immunol.* **165**, 988–996.
- Wang, E.E., Prober, C.G., Manson, B., Corey, M., and Levison, H. (1984). Association of respiratory viral infections with pulmonary deterioration in patients with cystic fibrosis. *N. Engl. J. Med.* **311**, 1653–1658.
- Widdicombe, J.H. (2000). Yet another role for the cystic fibrosis transmembrane conductance regulator. *Am. J. Respir. Cell Mol. Biol.* **22**, 11–14.
- Xiang, Y., Condit, R.C., Vijaysri, S., Jacobs, B., Williams, B.R., and Silverman, R.H. (2002). Blockade of interferon induction and action by the E3L double-stranded RNA binding proteins of vaccinia virus. *J. Virol.* **76**, 5251–5259.
- Yang, J., Moravec, C.S., Sussman, M.A., DiPaola, N.R., Fu, D., Hawthorn, L., Mitchell, C.A., Young, J.B., Francis, G.S., McCarthy, P.M., and Bond, M. (2000). Decreased SLIM1 expression and increased gelsolin expression in failing human hearts measured by high-density oligonucleotide arrays. *Circulation* **102**, 3046–3052.
- Young, D.F., Didcock, L., Goodbourn, S., and Randall, R.E. (2000). Paramyxoviridae use distinct virus-specific mechanisms to circumvent the interferon response. *Virology* **269**, 383–390.
- Zaragoza, C., Ocampo, C.J., Saura, M., McMillan, A., and Lowenstein, C.J. (1997). Nitric oxide inhibition of coxsackievirus replication in vitro. *J. Clin. Invest.* **100**, 1760–1767.
- Zhou, A., Paranjape, J.M., Der, S.D., Williams, B.R., and Silverman, R.H. (1999). Interferon action in triply deficient mice reveals the existence of alternative antiviral pathways. *Virology* **258**, 435–440.
- Zhu, Z., Tang, W., Ray, A., Wu, Y., Einarsson, O., Landry, M.L., Gwaltney, J., Jr., and Elias, J.A. (1996). Rhinovirus stimulation of interleukin-6 in vivo and in vitro. Evidence for nuclear factor kappa B-dependent transcriptional activation. *J. Clin. Invest.* **97**, 421–430.