Specific Inhibition of Interferon Signal Transduction Pathways by Adenoviral Infection*

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Adenoviral evolution has generated strategies to resist host cell antiviral systems, but molecular mechanisms for evasion of interferon (IFN) effects by adenoviruses during late-phase infection are poorly defined. In this study, we examined adenovirus type 5 (AdV) effects on IFN-γ-dependent gene expression and Janus family kinase-signal transducer and activator of transcription signaling components in human tracheobronchial epithelial cells. We found that AdV infection specifically inhibited IFN-γ-dependent gene expression in airway epithelial cells without evidence of epithelial cell injury or generation of a soluble extracellular inhibitor. Furthermore, infection with AdV for 18–24 h blocked phosphorylation/activation of the Stat 1 transcription factor that regulates IFN-γ-dependent genes. Although AdV also inhibited IFN-α-dependent phosphorylation of Stat1 and Stat2, interleukin-4-dependent phosphorylation of the related transcription factor Stat6 was not affected, indicating that the virus selectively affected specific signaling pathways. Our results indicate that AdV inhibition of the IFN-γ signal transduction cascade occurs through loss of ligand-induced receptor complex assembly and consequent component phosphorylation and suggest that lack of complex assembly is due to decreased expression of the IFN-γR2 chain of the IFN-γ receptor. IFN-γR2 is required at an early step in Janus family kinase-signal transducer and activator of transcription pathway activation and is expressed at low levels in airway epithelial cells, supporting the concept that adenoviral down-regulation of the level of this IFN-γ receptor component allows for persistent modulation of IFN-γ-dependent gene expression.

A central feature of the host response to viral infection is activation of cellular genes by interferons and other soluble mediators. Interferon (IFN)γ is an important mediator in the cellular response to viral infection based on its capacity to activate antiviral genes and induce a host cell antiviral state (1, 2). IFN-γ is produced by T cells and natural killer cells and mediates host cell effects through a high affinity receptor linked to a specific Janus family kinase-signal transducer and activator of transcription (JAK-STAT) signaling cascade (3, 4). Activation of this cascade is initiated by engagement and multimerization of the IFN-γ-receptor by IFN-γ, phosphorylation of IFN-γR1-associated Jak1- and IFN-γR2-associated Jak2-tyrosine kinases, and then phosphorylation of the IFN-γR1 chain (5–7). Phosphorylation of the IFN-γR1 chain of the IFN-γ receptor results in recruitment, phosphorylation, and subsequent release of Stat1 from the receptor (8). Activated and dimerized Stat1 translocates to the nucleus and binds specific DNA recognition sequences to activate transcription of IFN-γ-inducible genes (3). Through this pathway, Stat1 triggers an entire set of immune response genes oriented toward antiviral defense by either direct activation of gene transcription or induction of other transcription factors, such as interferon regulatory factor (IRF)-1, that amplify the antiviral response (9). Genes that are activated during IFN-γ-dependent immunity include intercellular adhesion molecule (ICAM)-1 for leukocyte recruitment and activation, major histocompatibility complex (MHC) class I and transporter associated with antigen processing-1 for viral antigen recognition, inducible nitric oxide synthase and IFN-α/β for antiviral toxicity, and other genes required for establishment of an antiviral state in host cells and attack of virus-infected cells by cytotoxic T cells (10–17).

Adenoviruses cause human respiratory infection including pharyngitis, croup, bronchitis, and pneumonia (18, 19). The success of adenoviruses in establishing productive infections of human airway epithelium likely depended upon the evolution of potent viral mechanisms for evasion of the innate and acquired immune response (20–22). IFN-γ regulation of antiviral genes through activated Stat1 is particularly important for immune system recognition and cytotoxic T cell attack of adenovirus-infected cells (15, 23–25), and adenoviral evolution has generated inhibitory strategies at more than one location in the IFN-γ-dependent immune response (17). For example, a 19-kDa glycoprotein encoded in the adenoviral early region 3 reduces the cytotoxic T cell response to virus-infected host cells by inhibiting MHC class I transport to the cell surface (26). We and others have shown that during early infection the adenoviral early region 1A (E1A) gene expresses a protein that inhibits gene expression through direct interaction with several cellular transcription factors including Stat1 and its cAMP-response-element-binding protein-binding protein (CBP) and p300 coactivator proteins (27, 28). However, we have also found that cellular levels of E1A decrease during the course of adenoviral infection (28). Interestingly, Stat1 activation is down-regulated during this later period of infection, thereby inhibiting IFN-γ-dependent gene activation at a step earlier in the activation cascade than inhibition by E1A (28). The existence of

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1 The abbreviations used are: IFN, interferon; JAK, Janus family kinase; STAT, signal transducer and activator of transcription; IRF, interferon regulatory factor; ICAM, intercellular adhesion molecule; MHC, major histocompatibility complex; CBP, cAMP-response-element-binding protein-binding protein; AdV, adenovirus; hTBE, human tracheobronchial epithelial; IL, interleukin; m.o.i., multiplicity of infection; Ab, antibody(ies); mAb, monoclonal Ab; LDH, lactate dehydrogenase.
multiple mechanisms for adenoviral inhibition of IFN-γ-dependent gene expression likely reflects the central nature of this pathway in host cell antiviral defense (2, 17, 29, 30).

In this report, we demonstrate that wild type adenovirus type 5 (AdV) infection for 18–24 h specifically inhibits IFN-γ-dependent gene expression in airway epithelial cells through direct effects on the IFN-γ-receptor complex. Experiments were performed with primary-culture human airway epithelial cells rather than transformed cell lines in order to exclude confounding effects of site of origin or oncogenes. We show that AdV blocks ligand-induced receptor complex assembly and receptor component phosphorylation and is associated with a specific decrease in expression of the IFN-γR2 chain of the IFN-γ-receptor, analogous to modulation of sensitivity to IFN-γ-induced proliferation, apoptosis, and MHC class I expression in T lymphocytes (31–33). This effect occurs later in the course of infection and may be required because adenoviral early gene expression and inhibitory effects are decreasing at this time (28). Our results support the concept that control of the level of IFN-γR2 allows for sustained modulation of immune response genes by AdV resulting in evasion of the airway defense response and establishment of a productive infection.

**EXPERIMENTAL PROCEDURES**

**Airway Epithelial Cell Isolation, Culture, and Stimulation—**Human tracheobronchial epithelial (hTBE) cells were obtained under a protocol approved by the Washington University human studies committee. Epithelial cells were isolated from tracheal and bronchial mucosal strips by enzymatic dissociation and cultured in Laboratory of Carcinogenesis (LHC)-8e medium on flasks or plates coated with collagen/albumin for study up to passage 10 as described previously (11, 28, 34).

Cells were treated with recombinant human IFN-γ (a gift from Genentech, San Francisco, CA) at a concentration of 100 units/ml and for durations that result in maximal effects on IFN-γ-dependent mRNA expression (4–20 h), and ICAM-1 surface protein expression (18 h) (11, 28, 34). Some cells were treated with recombinant human IFN-α (PBL Biomedical Labs, New Brunswick, NJ) at 100 units/ml for 1 h to assess Stat1 and Stat2 phosphorylation or with recombinant human interleukin (IL)-4 (a gift from Monsanto, St. Louis, MO) at 100 units/ml for 1 h to assess Stat6 phosphorylation.

**Viral Preparation and Infection—**Wild type AdV and an E1A-deficient mutant (AdV-d312) were gifts from S. Brody (Washington University). IFN-γ, ICAM-1, IRF-1, and GAPDH mRNA levels were determined by an enzyme-linked immunoassay as described previously (11, 11, 28, 38). ICAM-1 mRNA levels were determined using real-time RT-PCR (7900HT, ABI Prism) and GAPDH mRNA expression was used to control for variability in cell number. ICAM-1, IRF-1, and GAPDH mRNA levels were determined by real-time PCR using 1024-channel resolution over a 4-decade log range setting. IFN-γR2 mRNA levels were quantitated and normalized to GAPDH mRNA levels using densitometry (Gel Doc 1000, Bio-Rad, Hercules, CA) of radiographs. Probes for RNA blot analysis included (1) a 1.8-kb XbaI fragment from pCD1.8 containing the human ICAM-1 cDNA and pCDMSII (a gift from M. Dustin, Washington University and D. Staunton, Harvard University) (39); (2) the 2.0-kb EcoRI fragment from pUC19 containing human IFR-1 cDNA in pUC19 (a gift from Y. Henderson and A. Deisseroth, University of Texas, Houston) (9); (3) the 0.55-kb XbaI-HindIII fragment from pHcGAP containing human GAPDH cDNA in pBluescript SK+ (American Type Culture Collection) and the 3.2-kb EcoRI–HindIII fragment from pBSKshGR containing the human IFN-γR2 cDNA in pBluescript KS+ (a gift from R. Schreiber) (7, 40).

**Immunoblot Analysis—**Epithelial cell protein levels were assessed by immunoblot analysis using specific Abs against human JAK-STAT pathway components as described previously (6, 28, 35, 36, 41). Whole cell protein extracts were prepared by lysis of hTBE cell monolayers in 50 mM Tris, pH 8.0, 150 mM NaCl, 10 mM NaF, 2 mM sodium pyrophosphate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 0.75 mM dithiothreitol, 20 μM leupeptin, 1 μM aprotinin, and 0.5% Nonidet P-40. Protein concentrations were determined using a bicinonic acid protein reaction method (Pierce), and equal amounts of cell protein were subjected to SDS-PAGE in 7–10% polyacrylamide. Reconstituted proteins were electrochemically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), exposed to 5% nonfat milk or 3% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 to block nonspecific antigens, and then incubated with Ab against a specific cellular protein. Primary Ab binding was detected using goat anti-mouse or sheep anti-rabbit IgG conjugated to horseradish peroxidase (Roche Molecular Biochemicals) and an enhanced chemiluminescence detection system (Amersham Biosciences). Reprobing of membranes with a different primary Ab was done after washing in 62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol at 65 °C for 30 min to remove the previous Ab.

**Epithelial Cell Cytotoxicity Assays—**Release of the cytoplasmic enzyme lactate dehydrogenase (LDH) by hTBE cell monolayers was determined by a spectrophotometric assay of culture media based on the initial rate of oxidation of β-nicotinamide adenine dinucleotide in the presence of sodium pyruvate (42). Intracellular ATP content of hTBE cell monolayers was determined using a bioluminescence assay of cell extracts based on the reaction between ATP, Photinus pyralis firefly luciferase, and luciferin (Sigma) (43).

**Immunoprecipitation—**Tyrosine phosphorylation of proteins was detected by immunoprecipitation of JAK-STAT pathway components from hTBE cell extracts prepared for immunoblot analysis except that IFN-γR1 was isolated by lysis of hTBE cell monolayers in 25 mM Tris, pH 8.0, 150 mM NaCl, 10 mM NaF, 5 mM iodoacetamide, 1 mM phenylmethylsulfonfluoride, 1 mM sodium orthovanadate, 20 μM leupeptin, 1 μM aprotinin, and 1.5% Nonidet P-40 (6, 28, 35, 41). Protein concentrations were determined using the bicinonic acid protein reaction method, and equal amounts of cell protein extracts were treated with A or G-conjugated-Sepharose beads and were detected by immunoblot analysis using Ab against phosphotyrosine or the precipitated protein. Interactions between IFN-γ receptor complex components were examined by immunoprecipitation followed by immunoblot analysis of JAK-STAT pathway proteins from extracts prepared by lysis of hTBE cell monolayers in immunoblot lysis buffer adjusted to contain 250 mM NaCl and 1% digitonin without Nonidet P-40 in order to maintain protein–protein interactions (7, 44, 45).

**Flow Cytometry—**IFN-γR1 levels on the surface of hTBE cells were determined by fluorescence-activated flow cytometry as described previously (11) with minor modifications. Cell monolayers were harvested in phosphate-buffered saline with 1 mM EDTA, and 6 × 10^6 cells were suspended in 100 μl of phosphate-buffered saline containing 10% heat-inactivated fetal calf serum, 1.5 mM CaCl_2, 10 μg/ml of aprotinin, 10 μg/ml of antipain, 10 μg/ml of leupeptin, 10 μg/ml of BSA, 50 mM HEPES, and 0.5% bovine serum albumin before addition of 250 μl of 1:1 dilution of PE-conjugated mouse anti-human IFN-γR1 or isotype-matched control mAb. Primary Ab binding was detected using goat anti-mouse IgG F(ab′)_2-conjugated to fluorescein isothiocyanate (Sigma) followed by analysis on a FACScanLumen™ flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15-mW argon-ion laser. Data was acquired and analyzed using CELLQuest™, version 3.1.2 software (Becton Dickinson) using 1024-channel resolution over a 4-decade log range setting. IFN-γ-
γR2 levels on the surface of hTBE cells were determined similarly using 10 μg/ml of hamster mAb 2HUB-159 against human IFN-γR2 or species-matched control mAb. To increase sensitivity, primary Ab binding was detected using goat anti-Armenian hamster IgG conjugated to biotin (Jackson ImmunoResearch, West Grove, PA) followed by streptavidin conjugated to phycoerythrin (BD Pharmingen, San Diego, CA), and nonviable cells were detected and excluded from analysis using 7-aminoactinomycin D (Sigma).

Statistical Analysis—Enzyme-linked immunosassays and cytotoxicity assays were performed 2–4 times to assure reproducible results and were analyzed for statistical significance using a one-way analysis of variance for a factorial experimental design. The multicomparsion significance level for the one-way analysis of variance was 0.05. If significance was achieved by one-way analysis, post-analysis of variance comparison of means was performed using Scheffe F-tests (46).

RESULTS

Late Adenovirus Infection Inhibits IFN-γ-dependent Gene Expression—We have previously found that ICAM-1 levels on airway epithelial cells are particularly sensitive to induction by IFN-γ, and expression of E1A by AdV inhibits ICAM-1 and other IFN-γ-dependent gene expression by direct interaction of E1A with Stat1 and its CBP and p300 coactivator proteins (11, 28). We also found that infection with AdV for 24 h resulted in loss of IFN-γ-driven gene transcription and Stat1 activation, providing another potential mechanism for global inhibition of IFN-γ-dependent gene expression (28). To determine whether inhibition of IFN-γ-dependent gene expression was maintained after 12–24 h of infection, when expression of E1A and other early adenoviral genes was decreasing (28), we examined ICAM-1 expression on epithelial cells later in the course of adenoviral infection. Initial experiments indicated that AdV infection for 24 h had little effect on basal levels of ICAM-1 on hTBE cells, but inhibited IFN-γ-induced ICAM-1 surface protein expression (Fig. 1A). Infection with replication-deficient AdV-d312 did not have this capacity. AdV infection for 24 h also inhibited IFN-γ-induced ICAM-1 mRNA expression, which correlated with inhibition of ICAM-1 surface protein levels (Fig. 1B). IFN-γ-induced mRNA expression of the IRF-1 transcription factor in airway epithelial cells was also inhibited by AdV infection for 24 h (Fig. 1C). Accordingly, these results extend our previous work by demonstrating that AdV infection results in persistent, global inhibition of IFN-γ-dependent gene expression despite decreased cellular levels of E1A later in infection (28). Accordingly, subsequent experiments were directed at examining mechanisms for modifying the IFN-γ-driven JAK-STAT signaling pathway for activation of Stat1 that manifest after 12–24 h of AdV infection.

Adenovirus Infection Inhibits IFN-γ-induced Stat1 Phosphorylation—IFN-γ-induced transcription of immune response genes in airway epithelial cells is mediated through tyrosine phosphorylation of Stat1 (47). IFN-γ-induced Stat1 phosphorylation was markedly decreased in hTBE cells after 18 h of AdV infection, and this effect persisted to at least 24 h (Fig. 2A). However, because viral infection of epithelial cells may cause cell injury or death, AdV cytotoxic effects on hTBE cells were also examined to exclude this mechanism for altered cell signaling. Epithelial cells maintained the ability to exclude vital dyes such as trypan blue after 24 h of AdV infection, suggesting that altered Stat1 activation was not due to epithelial cell injury. To support this conclusion, epithelial cell LDH release and ATP levels were measured after AdV infection for 24 h. No significant release of cytoplasmic LDH from epithelial cells after AdV infection was observed, either with or without IFN-γ treatment (Fig. 2B). Furthermore, a significant decrease in ATP levels in epithelial cells was detected after infection with wild type AdV (Fig. 2C), but this decrease was similar to

2 T. D. Joseph and D. C. Look, unpublished observations.

![Fig. 1. Late adenovirus infection inhibits IFN-γ-dependent gene expression.](http://www.jbc.org/content/184/3/47138/F1)

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Adenovirus Infection Does Not Inhibit Stat6 Phosphorylation—Cytokine signaling through JAK-STAT pathways has become a paradigm for transduction of extracellular signals into cellular responses, particularly signals that rapidly activate host defense systems (3, 4). AdV may simply shut down any specific pathways in order to only disrupt systems that directly impair its ability to replicate. To examine the specificity of AdV effects on gene expression in epithelial cells, the activation of transcription factors controlled by other JAK-STAT pathways were assessed after AdV infection. Interestingly, AdV infection blocked phosphorylation of Stat1 (Fig. 3A) and Stat2 (Fig. 3B) in hTBE cells in response to the antiviral cytokine IFN-α. The
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Figure 2. Adenovirus infection inhibits IFN-γ-induced Stat1 phosphorylation. A, Stat1 phosphorylation was assessed by immunoblot analysis of extracts from hTBE cell monolayers that were left uninfected or were infected with AdV at an m.o.i. of 10 for the indicated times, with IFN-γ treatment during the final 1 h of infection. The positions of phosphorylated and unphosphorylated Stat1α, Stat1β, and β-actin are indicated by arrows. B, extracellular levels of LDH were determined by spectrophotometric assay of culture media from hTBE cell monolayers that were left uninfected or were infected with AdV at m.o.i. 10 for 24 h, with IFN-γ treatment during the final 1 h of infection. Values are expressed as mean percent of maximally permeabilized control cells (accomplished by treatment of hTBE cell monolayers with 0.01% saponin) ± S.D. (n = 4) and a significant difference from uninfected levels was not obtained. C, intracellular levels of ATP were assessed by bioluminescent assay of extracts from hTBE cell monolayers that were left uninfected without or with maximal permeabilization with 0.01% saponin or were infected with AdV or AdV-d312 at an m.o.i. of 10 for 24 h, with IFN-γ treatment for the final 1 h of infection. Values are expressed as mean percent of uninfected and unpermeabilized cells ± S.D. (n = 4), and a significant decrease from uninfected and unpermeabilized levels is indicated by an asterisk.

Figure 3. Adenovirus infection does not inhibit Stat6 phosphorylation. A, Stat1 phosphorylation was assessed by immunoprecipitation of Stat1 in extracts from hTBE cell monolayers that were left uninfected or were infected with AdV or AdV-d312 at an m.o.i. of 10 for 20 h, with IFN-α or IFN-γ treatment during the final 1 h of infection. For each condition, anti-Stat1 Ab and protein A-Sepharose were mixed with cell extract to generate an immune complex that was then subjected to immunoblot analysis against Ab to phospho-tyrosine and Stat6. B, Stat2 phosphorylation was assessed by immunoprecipitation of Stat2 in extracts from hTBE cell monolayers that were left uninfected or were infected with AdV or AdV-d312 at an m.o.i. of 10 for 20 h, with IFN-α treatment during the final 1 h of infection. Immunoprecipitation of Stat2 followed by immunoblot analysis against Ab to phospho-tyrosine and Stat2 were performed similar to A. C, Stat6 phosphorylation was assessed by immunoprecipitation of Stat6 in extracts from hTBE cell monolayers that were left uninfected or were infected with AdV or AdV-d312 at an m.o.i. of 10 for 20 h, with IL-4 treatment during the final 1 h of infection. Immunoprecipitation of Stat6 followed by immunoblot analysis against Ab to phospho-tyrosine and Stat6 were performed similar to A.

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Adenovirus Infection Does Not Generate a Soluble Extracellular IFN-γ Inhibitor—AdV appeared to inhibit the IFN-γ signal transduction cascade at or prior to Stat1 phosphorylation by the IFN-γ receptor. Using flow cytometry, a slight increase was found in the cell surface expression of the ligand-binding IFN-γR1 chain of the IFN-γ receptor on AdV-infected hTBE cells compared with cells that were uninfected or infected with AdV-d312 (Fig. 4A). Accordingly, altered IFN-γR1 expression did not account for loss of IFN-γ-dependent gene expression in airway epithelial cells infected with AdV. We also questioned whether a soluble factor released by cells infected with AdV could inhibit IFN-γ-induced Stat1 activation as has been described after infection of host cells by some nonviral pathogens (49, 50). To assess this possibility, media from hTBE cells infected with AdV for 24 h was transferred to uninfected epithelial cells, and IFN-γ-dependent Stat1 activation in the cells exposed to the transferred media was determined. In these experiments, no evidence of release of a soluble extracellular inhibitor of IFN-γ signaling was found (Fig. 4B). Taken together, these results suggest that AdV affected IFN-γ-dependent gene expression through modulation of the IFN-γ-dependent JAK-STAT pathway inside infected epithelial cells.

Adenovirus Infection Inhibits IFN-γ Receptor Complex Phosphorylation—The finding that AdV blocked Stat1 activation in response to IFN-γ led us to examine proximal intracellular events in the IFN-γ-dependent JAK-STAT pathway leading to Stat1 activation. IFN-γ-dependent Stat1 activation occurs after ligand-dependent assembly of a multimeric IFN-γ receptor complex followed by phosphorylation-initiated activation of selected components of the assembled complex. Down-regulated expression of components of the IFN-γ receptor complex has been demonstrated during host cell infection with certain pathogens including viruses (50, 51), providing precedent for the possibility that AdV could alter expression levels of components involved in IFN-γ signaling. However, we found that AdV infection did not substantially alter expression of IFN-γR1, Jak1, Jak2, or Stat1 in hTBE cells (Fig. 5A). Interestingly, AdV infection of airway epithelial cells efficiently blocked phosphorylation of each of these components, including the Jak kinases (Fig. 5B). Although IFN-γ activates each of these signaling components through tyrosine phosphorylation, Jak kinase activation is the initial phosphorylation event in the IFN-γ re-
fected with AdV at an m.o.i. of 10 for 20 h, with IFN-
were isolated from monolayers that were left uninfected or were in-
tracts from two sets of hTBE cell monolayers. In the first set, extracts
phosphorylation was assessed by immunoprecipitation of Stat1 in ex-
harvested after completion of the incubation period and either left
or centrifugation at 175,000
g (Centrifuged) for 18 h. Media aliquots underwent viral plaque assay
a 10-kDa pore size membrane (Filtered) or centrifugation at 175,000
were measured using flow cytometry with hTBE cell monolayers that
uninfected hTBE cell monolayers (to verify removal of viral particles) and were placed on a second set of
cell monolayers were mixed with
anti-Stat1 Ab and protein A-Sepharose to generate an immune complex
that was then subjected to immunoblot analysis against Ab to phospho-
tyrosine or Stat1.

Adenovirus Infection Inhibits IFN-γ-R2 Expression and IFN-γ Receptor Complex Assembly—To complete assessment of
known IFN-γ receptor components after AdV infection, we ex-
amined IFN-γ-R2 expression and function in airway epithelial
cells. However, IFN-γ-R2 protein and mRNA were not detecta-
ble using flow cytometry and total RNA blot analysis methods
previously established in our laboratory, suggesting that very
low levels of IFN-γ-R2 are expressed on uninfected hTBE cells.2
Therefore, to increase sensitivity for detection of IFN-γ-R2 pro-
tein, flow cytometry with an antibody detection system using multiple biotin-streptavidin interactions was used. Cell surface
IFN-γ-R2 was detected on uninfected epithelial cells using this
technique, and expression was down-regulated on cells infected
with AdV (Fig. 6A). Using polyadenylated RNA to increase
sensitivity in RNA blot analysis, IFN-γ-R2 mRNA was also detected in uninfected airway epithelial cells, and expression
was down-regulated by 89% in cells infected with AdV (Fig. 6B). To examine IFN-γ-R2 function in epithelial cells after AdV
infection, we used a coprecipitation assay that maintains in-
teractions between IFN-γ receptor components to detect Jak2
recruitment to IFN-γR1, an effect mediated by IFN-γR2 after
IFN-γ treatment (7, 44, 52). These experiments revealed that
IFN-γ-dependent association of Jak2 with IFN-γ-R1 was lost in
hTBE cells infected with AdV despite maintenance of constitu-
tive interaction between Jak1 and IFN-γ-R1 (Fig. 6C). Thus,
AdV infection inhibits IFN-γ signal transduction in human
airway epithelial cells by inhibition of ligand-induced recruit-
ment of Jak2 to the IFN-γ receptor complex with consequent
blockade of IFN-γ receptor complex assembly, phosphorylation,
and activation. This effect appears to be mediated by down-
regulation of the IFN-γ-R2 chain that is required for IFN-γ
signal transduction by the IFN-γ receptor complex (40, 53).

DISCUSSION

Our results indicate that AdV inhibit IFN-γ-dependent air-
way defense through a mechanism not previously described for
pathogens. Adenoviral effects on IFN-γ receptor complex as-
sembly and IFN-γ-R2 expression combined with other effects on
epithelial cell immune response gene expression may allow the
virus to evade the host response and establish a productive
infection. The importance of IFN-γ-R2 in the immune response
is illustrated by the observation that patients with mutations
that impair IFN-γ-R2 function have increased susceptibility to
infection by intracellular organisms (54, 55). In addition, the
level of IFN-γ-R2 serves as a normal physiologic modulator that
regulates proliferation, apoptosis, and IFN-γ-responsiveness in
T lymphocytes (31–33). The fact that IFN-γ-R2 is expressed at
low levels in airway epithelial cells and is required at an early
point in IFN-γ signaling (4, 7) suggests that inhibition of IFN-
γ-R2 expression allows for efficient and persistent modulation of
epithelial cell immune response genes by AdV.

Inhibition of IFN-γ-induced gene expression in host cells has
coevolved in several pathogens, and this effect occurs through
either direct effects on specific gene products (particularly
MHC class I or II molecules) or through more global effects on
IFN-γ signaling (20–22). Microbial inhibition of IFN-γ signal
transduction is often accomplished by decreasing the level of
specific IFN-γ receptor complex components. Inhibition of
IFN-γ signaling has been demonstrated through down-regula-
tion of Jak1 levels in human cytomegalovirus-infected endothe-

cellular immune response genes by AdV.
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Fig. 5. Adenovirus infection inhibits IFN-γ receptor complex phosphorylation. A, levels of IFN-γ receptor complex components that are phosphorylated after IFN-γ treatment were determined by immunoblot analysis of extracts from hTBE cell monolayers that were left uninfected or were infected with Adv or Adv-d312 at an m.o.i. of 10 for 20 h, with IFN-γ treatment during the final 1 h of infection. The positions of IFN-γR1, Jak1, Jak2, Stat1, and β-actin are indicated by arrows. B, phosphorylation of IFN-γ receptor complex components that are phosphorylated after IFN-γ treatment were assessed by immunoprecipitation of IFN-γR1, Jak1, Jak2, or Stat1 in extracts from hTBE cell monolayers that were left uninfected or were infected with Adv or Adv-d312 at an m.o.i. of 10 for 20 h, with IFN-γ treatment during the final 1 h of infection. For each condition, specific primary Ab and protein A-Sepharose was mixed with cell extract to generate an immune complex that was then subjected to immunoblot analysis against Ab to IFN-γR1, Jak1, Jak2, or Stat1 in extracts from hTBE cells monolayers that were left uninfected but treated with IFN-γ for 1 h (Control IP).

Fig. 6. Adenovirus infection inhibits IFN-γR2 expression and IFN-γ receptor complex assembly. A, cell surface protein levels of IFN-γR2 were measured using flow cytometry with hTBE cell monolayers that were left uninfected or were infected with Adv or Adv-d312 at an m.o.i. of 10 for 20 h. The fluorescence intensity of cells staining with control primary Ab and Ab against IFN-γR2 are indicated by arrows. B, levels of IFN-γR2 mRNA were determined by RNA blot analysis of polyadenylated RNA from hTBE cell monolayers that were left uninfected or were infected with Adv or Adv-d312 at an m.o.i. of 10 for 20 h, with IFN-γ treatment during the final 4 h of infection. The position of the 1.8-kb IFN-γR2, 3.3-kb ICAM-1, and 1.3-kb GAPDH mRNAs are indicated by arrows. C, interaction of IFN-γ receptor complex components was assessed by coimmunoprecipitation assay in which IFN-γR1 was immunoprecipitated out of extracts from hTBE cell monolayers that were left uninfected or were infected with Adv or Adv-d312 at an m.o.i. of 10 for 20 h, with IFN-γ treatment during the final 1 h of infection. For each condition, IFN-γR1 Ab and protein A-Sepharose were mixed with cell extract to generate an immune complex that was then subjected to immunoblot analysis against Ab to Jak1, Jak2, or IFN-γR1.

protein tyrosine phosphatase SHP-1 that mediates Jak2 dephosphorylation in a L. donovani-infected murine macrophage cell line (59, 60). Altered IFN-γ responses tend to be associated with infection by intracellular pathogens (54, 55), supporting the concept that inhibition of immune mechanisms for detection of infected host cells is useful for survival of these microbes. The reasons why AdV uses selective modulation of IFN-γ receptor complex assembly and IFN-γR2 levels to subvert IFN-γ-dependent immunity and the advantages of this inhibitory strategy compared with that used by other pathogens are currently unclear.

The present results demonstrate that AdV mechanisms for inhibition of IFN-γ signaling have a distinct temporal relationship. Adenoviral infection can be conceptually divided into two overlapping phases based on the major functions of viral genes that are expressed in a ordered, temporal pattern during the replication cycle of the virus, and adenoviral mechanisms that affect host cell antiviral responses often depend on specific viral gene expression (20, 61). In particular, during the early phase (the first 8–18 h of infection ending with viral DNA synthesis) when specific adenoviral genes (E1–4) sequester host cellular synthetic machinery for virus production and counteract host cell defenses, adenovirus interferes with events downstream to Stat1 activation. This effect correlates with peak expression of E1A, the first gene product transcribed from the viral genome. During the late-phase (12–36 h after the initiation of infection) when adenoviral genes (L1–5) direct the assembly of new virus and shutdown of host cell macromolecular synthesis, IFN-γ-dependent Stat1 phosphorylation is down-regulated. This effect on the IFN-γ-activated JAK-STAT pathway later in the viral replication cycle could assist diversion of host cellular energy toward production of viral progeny or may be required because adenoviral early gene expression and immune inhibitory effects are decreasing (28). The ability of adenoviruses to inhibit IFN-γ-inducible genes in the airway epithelium by multiple mechanisms with a distinct temporal relationship is presumably designed to assure a dampened immune response during all phases of the viral replication cycle.

The actions of AdV defined by our study of primary-culture cells clarify and extend previous work from other laboratories using established cell lines. Transformed cell lines with stable adenoviral E1A expression exhibit reduced IFN-γ-driven gene expression that correlates with decreased formation of DNA-binding complexes (62–64). Our work with intact virus demonstrates that adenoviral effects on Stat1 activation and DNA binding are not observed during the early phase of wild type
Adenoviral inhibition of interferon signaling

Adenoviral infection when E1A levels are high, but are found only during late-phase infection (28, and the present study). Stable E1A expression in certain cell lines appears to affect IFN-γ-dependent gene expression by decreasing cellular Stat1 levels (64), but we observed no effect on Stat1 expression during early- or late-phase adenoviral infection in the natural host cell. Taken together, the results indicate that transformed cell lines with stable expression of E1A may reflect conditions with prolonged or chronic viral infection or persistent viral remnants but may not be accurate models of initial adenoviral infection, at least in airway epithelium.

Airway epithelial cells respond to IFN-γ by rapidly expressing high levels of several genes that assist in immune recognition and thereby activate an MHC class I-restricted, CD8+ T cell-dependent cytotoxic response directed at cells expressing viral proteins (11, 28, 65, 66). IFN-γ also controls other airway defense mechanisms including induction of genes directed at intracellular antiviral toxicity and establishment of a host cell antiviral state (1, 2, 48). Because IFN-γ-dependent immunity and inflammation is a critical component of antiviral defense in standing viral mechanisms for altering IFN-dependent immunity (32, 33), we asked if AdV can activate Stat1 or prolong Stat1 activation in virus infected cells.

Conversely, as adenoviral vectors have lost the ability to affect IFN signal transduction, AdV also has the capacity to modulate the IFN-α-activated JAK-STAT pathway through inhibition of both Stat1 and Stat2 activation in airway epithelial cells. Understanding these viral mechanisms for altering IFN-dependent immune responses may allow for therapeutic strategies that block this viral capacity during infection with wild type viruses. In addition, by generating IFN-γ-activated JAK-STAT pathway through inhibition of both Stat1 and Stat2 activation in epithelial cells, understanding these viral mechanisms may direct reengineering of AdV vectors to reestablish this capacity (17).

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