Kinetic Differences and Synergistic Antiviral Effects Between Type I and Type III Interferon Signaling Indicate Pathway Independence

Emily A. Voigt^{1,2} and John Yin^{1,2}

The spread of acute respiratory viral infections is controlled by type I and III interferon (IFN) signaling. While the mechanisms of type I IFN signaling have been studied in detail, features that distinguish type III IFN signaling remain poorly understood. Type III IFNs play an essential role in limiting infections of intestinal and respiratory epithelial surfaces; however, type III IFNs have been shown to activate similar genes to type I IFNs, raising the question of how these IFNs differ and their signals interact. We measured the kinetics of type I and III IFN activation, functional stability, and downstream antiviral responses on A549 human lung epithelial cells. Similar kinetics were found for transcriptional upregulation and secretion of type I and III IFNs in response to infection by an RNA virus, peaking at 12 h postinfection, and both protein types had similar stabilities with functional half-lives extending beyond 2 days. Both IFNs activated potent cellular antiviral responses; however, responses to type III IFNs were delayed by 2–6 h relative to type I IFN responses. Combined treatments with type I and III IFNs produced enhanced antiviral effects, and quantitative analysis of these data with a Bliss interaction model provides evidence for independence of type I and III IFN downstream signaling pathways. This novel synergistic interaction has therapeutic implications for treatment of respiratory virus infections.

Introduction

INTERFERONS (IFNs) ARE CRUCIAL CYTOKINES for innate immune protection of human lung epithelial cells against viral infections. IFNs secreted by lung epithelial cells in response to infection are a complex mixture of biologically active molecules, including type I and III IFNs (Calven and others 2013). Type I IFNs, which classically include IFN- α and IFN- β , act through a common receptor to induce a large set of interferon-stimulated genes (ISGs), including MxA, IFIT2, PKR, RNaseL, and OAS, with diverse antiviral functions that act together to establish a cellular antiviral state (AVS) that is prohibitive of virus replication and spread (Zhou and others 1999). These direct antiviral functions include varied activities such as inhibition of viral penetration (Whitaker-Dowling and others 1983), control of viral transcription (Haller and Kochs 2002; Haller and others 2007), viral genome sequestration (Xiao and others 2013), RNA cleavage (Dong and others 1994), translation inhibition (Munir and Berg 2013), inhibition of virion assembly (Xiao and others 2013), and induction of cellular apoptosis (Li and others 2008; Stawowczyk and others 2011).

In addition, type I IFNs play immunoregulatory roles and affect further innate and adaptive immune responses by inducing natural killer cell cytotoxicity, upregulating MHC I

expression, and promoting T-cell expansion and activation (Stager and Kaye 2004; Curtsinger and others 2005; Kolumam and others 2005; Curtsinger and Mescher 2010; Hervas-Stubbs and others 2010). The mechanisms and functions of the type I IFNs have been extensively studied and reviewed (Randall and Goodbourn 2008; Sadler and Williams 2008; Schoggins and others 2011; Liu and others 2012).

While type I IFNs have been widely studied, a recent work shows that the more recently discovered type III IFNs are crucial to many respiratory infection responses. The type III IFNs, which include IFN- λ_1 (IL-29), IFN- λ_2 (IL-28A), and IFN- λ_3 (IL-28B), are induced by all classes of viral genomes in a broad range of host cells, including human lung epithelial cells (Ank and others 2006). These molecules display antiviral activities similar to IFN- α/β , though they do not act through the type I IFN receptor but rather through their own type III IFN receptor complex (Sheppard and others 2003).

Both IFN- α and IFN- λ receptor stimulation induces promoter recruitment of a common set of transcription factors (Zhou and others 2007), and signaling through Janus kinase 1 (JAK1) and STAT factor phosphorylation, including STAT2, whose activation was previously considered specific to type I IFN signaling (Krause and Pestka 2005). This signaling then leads to induction of typical ISGs with final effects

¹Department of Chemical and Biological Engineering, University of Wisconsin–Madison, Madison, Wisconsin.

²Systems Biology Theme, Wisconsin Institute for Discovery, Madison, Wisconsin.

similar to those of type I IFNs that include inhibition of virus replication, induction of apoptosis, MHC I antigen expression, and inhibition of cell proliferation (Dumoutier and others 2004; Doyle and others 2006; Li and others 2008; Witte and others 2010).

Despite these demonstrated similarities in cellular responses to type I and III IFN signaling, and gene expression arrays that indicate no genes are uniquely induced by type III relative to type I IFNs (Zhou and others 2007), it has been shown *in vivo* that IFN- λ is an important component of innate antiviral immune responses in both intestinal and respiratory epithelial surfaces (Mordstein and others 2008; Khaitov and others 2009; Jewell and others 2010; Mordstein and others 2010; Pott and others 2011). This suggests that there are as-yet-undefined differences between type I and III IFN antiviral signaling. Marcello and others (2006), for example, suggest that the kinetics of type III IFN downstream signaling may be delayed relative to type I IFN signaling, allowing for a different role complementary to the type I IFNs.

We hypothesize 3 potential differences that could explain the seeming duplicate roles of type I and III IFNs: (1) Kinetics of responses are different for type I and III IFNs, (2) physical stability of type I and III IFNs are significantly different, allowing for different functional roles, and/or (3) mechanisms of downstream signaling differ between the 2 IFN types in ways that previous microarray analyses were not able to identify.

In this study, we tested these hypotheses by measuring the kinetics of type I and III IFN upregulation, stability, signaling, and antiviral response induction in cultured human lung epithelial (A549) cells in a highly quantitative manner. We also identified how signals activated by both IFN types interact by systematically measuring the extent of infection inhibition caused by different combinations of these molecules. We found that type I and III IFNs had similar upregulation kinetics in response to infection, but downstream signaling in response to type III IFN treatment was delayed 2–6 h relative to type I IFN signaling. Notably, we identified and classified functional synergy between type I and III IFNs, suggesting some yet-to-be-defined independence of their downstream signaling processes.

Materials and Methods

Cell lines and viruses

Human lung epithelial carcinoma cells (A549, ATCC CCL-185) were obtained from the American Type Culture Collection and cultured in RPMI medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) in a humidified incubator at 37°C and 5% CO₂. Baby hamster kidney (BHK-21) cells used for plaque assays were originally obtained from Isabel Novella (University of Toledo) and grown in minimal essential medium (MEM; Corning Life Sciences, Tewksbury, MA) with 10% FBS and 2 mM Glutamax I (Gibco). Cell lines were regularly tested for mycoplasma contamination.

VSV-DsRed2 reporter virus was created by reverse genetics, placing the DsRed2 (Clontech, Fitchburg, WI) gene at the fifth position of the VSV-Indiana genome and resulting in a genomic sequence N-P-M-G-DsRed2-L, as

described and previously validated (Voigt and others 2013). Similar synthesis and characterization of the VSV-M51R mutant virus used for stimulation of antiviral responses was described elsewhere (Voigt and others 2013).

Plaque assay

For quantification of infectious virus particles in infection supernates, samples were serially diluted 1:10 in MEM supplemented with 2% FBS and 2 mM Glutamax. BHK cells were plated at 18 h before the assay at a concentration of 5×10^5 cells/well in 6-well tissue culture plates and allowed to form monolayers under incubation. Cell monolayers were infected with 200 μ L virus dilution and incubated for 1 h with gentle rocking every 20 min. Virus suspension was then removed, cells were rinsed with phosphate-buffered saline (PBS), and cell monolayers were overlaid with 2 mL of MEM with 2% FBS and 2 mM Glutamax with 0.6% melted agar. The plates were cooled until agar solidified and incubated at 37°C, 5% CO₂ for ~24 h, until plaques appeared. Agar layers were then removed, and cells were fixed for 20 min with a 4% paraformaldehyde (PFA) solution containing 5% sucrose in PBS. The PFA solution was then removed, and cell layers were stained with 0.1% crystal violet in 20% ethanol to visualize plaques.

Western blotting

Cell monolayers were lysed using RIPA buffer (Sigma, St. Louis, MO) and frozen for Western blotting. Lysed cellular extracts were thawed and pipetted into a hard-shell 96-well plate, which was sealed, placed in a water bath, and sonified for 30 s at a 50% pulse to disrupt cellular membranes. Samples were then spun at 1,200 g for 10 min to pellet cell debris. Soluble protein content in supernates was measured by BCA assay (Pierce, Rockford, IL). Samples were diluted to equal concentrations, 4 $\times$ denaturing protein loading buffer with beta-mercaptoethanol was added (Licor, Lincoln, NE), and samples were boiled for 5 min to denature proteins. Twenty microliters of sample was loaded per lane of an 18-lane 10% polyacrylamide gel (SmartGel; Amresco, Solon, OH), and PrecisionPlus Protein Dual Xtra protein standards (BioRad, Hercules, CA) were used.

The gels were run at 120 V for 90 min in SmartGel running buffer (Amresco). Proteins were then transferred to PVDF membranes using a BioRad Semi-Dry transfer apparatus at 15 V for 30 min. Membranes were blocked in TBST with 5% BSA for 3 h at 4°C. Membranes were then incubated in primary mouse anti-Mx antibody (AM2061; Abgent, San Diego, CA) and mouse anti- β -actin antibody (A5441; Sigma) diluted 1:2,000 in blocking buffer overnight at 4°C with rocking. Membranes were then rinsed 3 $\times$ with TBST, and 1:5,000 dilution of Licor anti-mouse 800 nm fluorescent secondary antibody was added and incubated for 1 h at room temperature. Membranes were again rinsed 3 $\times$ and imaged on a Licor Odyssey infrared membrane scanner. Images were quantified with Licor Image Studio software.

Bronchial epithelial cell culture

Frozen stocks of human tracheal explants of nonidentifiable lung transplant donors were obtained from James Gern, University of Wisconsin School of Medicine and Public Health; these had been collected according to approval by

the University of Wisconsin–Madison Health Sciences institutional review board, using methods previously described (Schroth and others 1999). Stocks from 3 separate donors were cultured at 37°C and 5% CO₂ in monolayers in 75 cm² CellBind flasks (Corning Life Sciences) using bronchial epithelial growth medium (Lonza, Walkersville, MD). Bronchial epithelial cells (BECs) were then plated into 96-well tissue culture plates, grown to 80% confluence, pretreated for 24 h with 67 µL/well BEGM with 1,000× lower hydrocortisone levels, and supplemented with recombinant stock IFNs.

Cytokines and reagents

Universal type I IFN (human IFN- α A/D) and recombinant human IFN- β 1a (IFN- β) were purchased from PBL InterferonSource (Piscataway, NJ). Recombinant human IFN- γ , IFN- λ_1 , IFN- λ_2 , and IFN- λ_3 were obtained from Cell Signaling Technology, Inc. (Beverly, MA). IFN activity was determined by an antiviral bioassay (Voigt and others 2013). IFN stock activity levels were re-assayed during the course of these studies before each major experiment, to correct for degradation of biological activity in storage.

Conditioned media (CM) stocks were created by infection of A549 cell monolayers with VSV-M51R at a multiplicity of 5 pfu/cell (MOI 5) in RPMI media containing 2% FBS. The infection was allowed to progress for 24 h, and the supernates containing secreted antiviral compounds were then harvested. This CM was irradiated using 7,000 J/m² of UVC irradiation over 20 min to inactivate infectious virus and filtered through a 0.2 µm syringe filter before use. The net antiviral activity in U/mL of this CM solution was measured by antiviral bioassay before dilution and use.

Quantitative PCR

Cellular samples for mRNA quantification were harvested and stored in RNAprotect (Qiagen, Valencia, CA) at –20°C until RNA extraction and quantitative PCR (qPCR). Total RNA was extracted from cellular samples using the Qiagen RNeasy Mini kit. Random-primer (reverse transcription) was done with GoScript™ Reverse Transcription system (Promega, Madison, WI) according to the manufacturer's instructions. qPCR was then done on each sample for IFN- α , IFN- β , IFN- γ , IFN- λ_1 , and IFN- $\lambda_{2/3}$ using the following primers:

IFN- α For: 5'-AAATACAGCCCTTGTGCCTGG-3'
 IFN- α Rev: 5'-GGTGAGCTGGCATAACGAATCA-3'
 IFN- β For: 5'-AAGGCCAAGGAGTACAGTC-3'
 IFN- β Rev: 5'-ATCTTCAGTTTCGGAGGTAA-3'
 IFN- λ_1 For: 5'-CGCCTTGGAAGAGTCACTCA-3'
 IFN- λ_1 Rev: 5'-GAAGCCTCAGGTCCCAATTC-3'
 IFN- $\lambda_{2/3}$ For: 5'-AGTTCGGGCGCTGTATCCAG-3'
 IFN- $\lambda_{2/3}$ Rev: 5'-GAGCCGGTACAGCCAATGGT-3'
 all from (Ank and others 2006), and
 IFN- γ For: 5'-CTAATTATTCGGTAACCTGACTTGA-3'
 IFN- γ Rev: 5'-ACAGTTACAGCCATCACTTGGA-3'
 from (Stordeur and others 2002), and
 β -actin For: 5'-AAAGACCTGTACGCCAACAC-3'
 β -actin Rev: 5'-GTCATACTCCTGCTTGCTGAT-3'

Quantitative PCR was performed using the SsoFast SYBR Green Supermix by BioRad. qPCR conditions started with incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Ct values

were normalized to β -actin levels, and results were shown as fold induction over levels in mock-infected cells.

ELISA IFN assays

Cellular supernates from infected cells were assayed for secreted IFN protein using commercial ELISA assay sets following the manufacturer's instructions. IFN- λ_1 , IFN- λ_2 , and IFN- λ_3 were detected in samples diluted 1:2 and 1:4 in cell culture media using DuoSet ELISA development systems and the DuoSet ancillary reagent set (Nos. DY7246, DY1587, DY5259, and DY008; R&D Systems, Minneapolis, MN). IFN- α , IFN- β (diluted 1:2), and IFN- γ were detected using VeriKine Human Interferon ELISA kits (Nos. 41100-1, 41410-1 and 41500-1; PBL Assay Science, Piscataway, NJ).

Assay for cellular AVSs

Sixty-seven microliters per well of A549 cells were seeded into 96-well microtiter cell culture plates at a density of 2.5×10^5 cells/mL and cultured for 24 h before antiviral treatment. IFN was diluted in RPMI media supplemented with 2% FBS to the final concentrations listed. Culture media was removed from 96-well plates with confluent cell monolayers, and 67 µL/well of diluted IFN or mock-treatment control 2% RPMI was added and allowed to incubate for 24 h. The AVS of the cells was then tested by challenge with VSV-DsRed2 virus in 30 µL RPMI, 2% FBS, added to the existing IFN treatments in the wells for a final MOI of 5 pfu/cell. Infections were allowed to progress for 24 h, and the plates were then fluorescently imaged with a GE Typhoon FLA 9000 Biomolecular Imager at 555/580 nm (GE Healthcare, Pittsburgh, PA).

Stability of IFNs in vitro

Freshly-titrated recombinant IFN solutions (IFN- β , IFN- λ_1 , IFN- λ_2 , and IFN- λ_3 from mammalian sources, IFN- α and IFN- γ from *Escherichia coli*) were diluted in used, filtered cell culture supernates. These supernates were collected from A549 cell cultures 24 h after cellular subculture, irradiated, and syringe filtered, creating a solution that represents cellular environments partially depleted in nutrients and containing secreted cellular factors that may affect molecule half-lives (eg, degradation factors, proteases, etc.). Two dilutions of each recombinant IFN were made and independently assayed, with starting activities ranging from 10 to 400 U/mL.

IFN dilutions were aliquoted into 48-well microtiter plates, 300 µL/well. The plates were covered and allowed to incubate in humidified cell culture incubators at normal cell culture conditions of 37°C and 5% CO₂. Samples were removed in duplicate and immediately frozen approximately every 12 h for 140 h. The samples were then thawed and assayed for biological antiviral activity (Voigt and others 2013). The resulting activity measurements were normalized to starting concentration, averaged between the 2 different and independently assayed starting concentrations, and fit to an exponential decay and biological half-lives were calculated for each of the recombinant IFNs.

Synergy assay and analysis

We tested for synergy between binary combinations of type I, II, and III IFNs by treating A549 lung epithelial cells

in 96-well plates with an 8-dose gradient of one IFN, perpendicular to an 8-dose gradient of a second IFN, followed by 24 h of incubation to fully establish antiviral signaling. The AVS of each well of cells was then tested by challenge with our reporter RNA virus at MOI 5, incubated for 24 h, and red fluorescent protein (RFP) levels were quantified by a GE Typhoon FLA 9000 Biomolecular Imager at 555/580 nm. The images were analyzed as described next to determine the extent of viral inhibition, and these data were then used for drug-combination analysis to determine the type and degree of interaction between each treatment pair. IFN stocks were newly assayed for biological activity immediately before each experiment, and these levels were again confirmed in control wells during the actual synergy assays.

There are many different definitions of drug synergy, antagonism, and additivity, with each having different mathematical models for assessment. A schematic of different mechanisms of action is shown in Fig. 1. Greco and others (1995) provide a comprehensive review of different methods of assessing effects of drug combinations, from which we selected the methods of Loewe (additive) and Bliss (multiplicative) as the most suitable to identify and classify synergy in our system.

To categorize synergy occurring between different IFNs, we used the Loewe additivity model, where synergy is defined by greater inhibition of viral replication occurring when 2 IFNs are combined than would be expected by the strict sum of their individual effects. A model equation for studying deviation from Loewe drug additivity, modified from Hewlett, is as follows:

$$\left(\frac{D_{\text{IFN1}}}{\text{IC}_{50, \text{IFN1}}} \right)^{\alpha} + \left(\frac{D_{\text{IFN2}}}{\text{IC}_{50, \text{IFN2}}} \right)^{\alpha} = 1,$$

where $\text{IC}_{50, \text{IFN1}}$ and $\text{IC}_{50, \text{IFN2}}$ are the IC_{50} values for IFN1 and IFN2 alone, respectively; D_{IFN1} and D_{IFN2} are the treatment doses of each IFN in the mixture given to the cells; and

α represents the degree of interaction between IFN1 and IFN2 (Hewlett 1969; Sorensen and others 2007). By definition, the IC_{50} values for our IFN solutions were 1 U/mL.

We found the value of α using a standard least-squares method to fit the model to experimental data. If drug additivity is followed, isoboles are linear and the drug interaction parameter α has a value of 1. In antagonistic or competing drug combinations, higher doses of the drugs are necessary to reach a specified effect than a simple sum of individual outcomes, and the isobole is bent above the additive line, $\alpha > 1$. Synergistic effects between drugs result in lower drug doses achieving a set response than would be predicted by additivity, and the isobole is convex and $\alpha < 1$ is necessary to describe the data.

The final α parameters for each drug–drug combination were calculated as an average of the best-fit α values from the IC_{50} , IC_{60} , IC_{70} , and IC_{80} curves, with $\pm 95\%$ confidence intervals assuming approximate normal distributions. P values listed are from a 2-tailed Student's t test determining whether the calculated α value is statistically different than a value of 1.

After identifying the presence and absence of synergy in different IFN pairs, we then classified the type of synergy using the multiplicative definition of Bliss (Bliss 1939). Bliss proposed that for 2 drugs that act independently and have different modes of effective action, their combined effect can be predicted from dose-effect curves of the individual components as follows:

$$P_{\text{IFN1, dose1} + \text{IFN2, dose2}} = P_{\text{IFN1, dose1}} + (1 - P_{\text{IFN1, dose1}}) * P_{\text{IFN2, dose2}},$$

where $P_{\text{IFN1, dose1}}$ is the fractional effect of IFN1 treatment at dose 1, $P_{\text{IFN2, dose2}}$ is the fractional effect of IFN2 treatment at dose 2, and $P_{\text{IFN1, dose1} + \text{IFN2, dose2}}$ is the fractional effect of the drug combination. Essentially, IFN1 blocks $P_{\text{IFN1, dose1}}$ fraction of virus replication. Of the remaining replication

FIG. 1. Mechanisms of antiviral cytokine synergy. Three mechanisms for enhanced antiviral responses to treatment with 2 or more cytokines are as follows: (1) “cooperative action,” when multiple cytokine treatments enhance the activation of the same antiviral pathway, and (2) “independent action,” when multiple cytokine treatments activate 2 or more independent antiviral pathways, each of which contribute to blocking viral replication, and (3) “cooperative induction,” when cytokine treatments induce activation of antiviral pathways not induced by treatment with any one individual cytokine, as adapted from a recent review (Bartee and McFadden 2013).

that escapes IFN1 action, that is $(1 - P_{IFN1,dose1}) \cdot P_{IFN2,dose2}$ fraction of that is additionally blocked. Combined effects that follow this model are classified as synergy by independent action, or Bliss independence. Synergistic effects above and beyond this prediction are likely due to cooperative induction, where the combined treatment activates mechanisms or pathways that are silent during treatments by either drug alone.

Image analysis

Fluorescent scanned images were analyzed using JEX, a customized JAVA-based batch-processing image analysis platform incorporating much of the functionality of Image J (Rasband 1997–2012) that is available as shareware at <http://sourceforge.net/projects/jextools>. The mean fluorescent intensity of each well was extracted, and mean fluorescence well data (mean random fluorescence units [RFU]) for all fluorescent experiments were scaled to positive and negative controls as follows:

$$1 - \frac{\text{Sample mean RFU} - \text{uninfected mean RFU}}{\text{infected, untreated mean RFU} - \text{uninfected mean RFU}}$$

providing a calculated value for fraction of viral inhibition.

Statistical analysis

Error bars are given as 95% confidence intervals or standard deviation, as noted, assuming underlying distributions are normal. Comparisons between data sets were conducted using 2-tailed Student's *t* tests.

Results

Validation of an A549-based viral reporter assay for cellular AVSs

For detailed kinetic studies of AVS development in response to IFN signaling, an accurate and easy-to-use reporter of AVSs in human lung epithelial cells is necessary. We adapted a previously developed highly sensitive biological assay for type I, II, and III IFN to our purpose, using reduction in RFP signal from an RNA virus replication reporter to measure cellular AVSs.

To validate this reporter system for use in detecting generic cellular AVSs, A549 human lung epithelial cells were pretreated with a range of IFN- α or IFN- β concentrations for 24 h and assayed for levels of antiviral effector protein Mx1 (Western blot), inhibition of virus replication (titer reduction by plaque assay), and RFP assay readout (Fig. 2A, B). The reporter fluorescence signal correlated well with intracellular Mx1 protein levels and with viral titer inhibition over ~ 3 orders of magnitude in IFN concentration and viral replication (Fig. 2C and Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/jir).

We further compared IFN- β -induced AVSs in A549 adenocarcinoma human respiratory epithelial cells with those of primary human BECs, and we found similar inhibition of virus replication as determined from normalized RFP signal, similar IFN IC₅₀ values, and correlation between normalized reporter signals from the 2 cell types (Fig. 2D and Supplementary Fig. S2). The maximum virus growth appears lower in primary cells than in A549 cells. However, taken to-

gether, these results suggest that IFN- β -induced antiviral responses in the A549 cell line are similar to those of primary airway cells.

Type I and III IFNs are upregulated simultaneously after infection

To test whether viral induction of type III IFN has intrinsically different kinetics than induction of type I IFN, we measured cellular IFN mRNA levels and IFN protein secretion after infection with VSV-M51R, a mutant virus strain that abrogates the wild-type virus' ability to inhibit cellular translation. The mutant virus provides a convenient means to stimulate the intrinsic cellular antiviral response in the absence of virus-mediated host suppression. Infected cells were harvested at various times postinfection, cellular RNA were harvested and reverse transcribed, and the IFN- α , IFN- β , IFN- γ , IFN- λ_1 , and IFN- $\lambda_{2/3}$ mRNA levels relative to mock-infected cells were measured by quantitative PCR (Fig. 3A). Cellular supernates were also harvested at various times postinfection, and levels of secreted IFNs were measured by ELISA (Fig. 3B).

Figure 3A shows similar kinetic mRNA upregulation patterns for all 3 IFN types. Each showed initial upregulation between 4 and 6 h postinfection, and rose to a maximum fold induction by ~ 12 –16 h postinfection. Also notable were the relative levels of mRNA induction between the IFN types. Interestingly, in A549 cells, the type III IFNs—IFN- λ_1 and IFN- $\lambda_{2/3}$ showed the highest mRNA induction levels relative to uninfected cells, followed by the classical IFN- β response, the 10-fold lower IFN- α response, and, finally, a low-level (10-fold) induction of IFN- γ .

IFN protein levels (Fig. 3B) followed similar patterns, with initial levels detectable by 6 h postinfection, and rising to maxima ~ 16 h postinfection, slightly lagging behind mRNA maximal levels. High levels of type III IFNs were confirmed at the protein level, with IFN- λ s till an order of magnitude more highly expressed than IFN- β . IFN- α secretion was low, with a maximum of ~ 30 pg/mL relative to maxima of 2,100 pg/mL for IFN- β , 4,700 pg/mL for IFN- λ_1 , 15,000 pg/mL for IFN- λ_2 , and 11,000 pg/mL for IFN- λ_3 . Assays for IFN- γ were conducted, but no levels were detectable by ELISA.

Type I, II, and III recombinant IFNs have similar *in vitro* biological stability

Significantly different biological half-lives could allow type I and III IFNs to serve different functions in *in vivo* antiviral signaling, potentially altering the interpretation of *in vitro* experimental results from previous studies. Half-lives of purified recombinant IFNs under cell culture conditions were estimated and compared using the fluorescent virus-based IFN activity assay (Voigt and others 2013).

Exponential decay curves were fit to experimental data representing decay of 2 different starting concentrations of each IFN (10–400 U/mL), normalized to starting concentration (Fig. 4A), and half-lives for all tested IFNs exceeded 2 days (Fig. 4B). Significantly extended half-life values indicated for IFN- λ_1 and IFN- λ_3 do not accurately characterize biological half-lives of these molecules, as reflected in the poor R^2 values for these samples near assay limits. All

FIG. 2. A virus-based functional assay for cellular antiviral state. **(A)** The quantitative assay for induced cellular antiviral states depends on inhibition of fluorescence associated with replication by a red fluorescent protein (RFP) reporter virus. **(B)** Effects of interferon (IFN)- α treatments on Mx1 protein expression, reporter virus RFP expression, and virus titer. A549 lung epithelial cells were treated with different dilutions of IFN- α for 24 h. Half of the treated wells were analyzed for intracellular Mx1 protein by Western blot, while the other half were infected with reporter virus and used to measure virus titers and associated fluorescence. **(C)** Inhibition of RFP is correlated with expression of Mx1 protein and inhibition of virus titer. **(D)** Correlated inhibition of viral replication in A549 and primary human bronchial epithelial cells (BECs) in response to treatment with IFN- β . Monolayers of A549 and BECs were treated with IFN- β for 24 h; then, antiviral states were probed by challenge with the VSV-DsRed2 reporter virus for 24 h (A549 cells) or 48 h (BECs). Images are shown for 1 of 3 primary cell donors. Data points show averages and 95% confidence intervals of 4 biological replicates each, and data for BECs show the mean of 3 separate donor lines each with 4 biological replicates.

recombinant IFN biological half-lives are long relative to the replication cycles of most respiratory viruses, suggesting that differences in molecular stability do not contribute significantly toward different temporal roles for type I versus III IFNs. Further, the bioactivity of such IFNs should be stable over multi-day *in vitro* studies.

Type III IFN-induced development of cellular AVSs is rapid but delayed relative to type I IFN-mediated AVS induction

To quantify the kinetics of cellular AVS development in response to IFN signaling, we pretreated A549 cells with individual recombinant IFNs of known activity and probed the AVS at different times post-treatment by challenge with our reporter RNA virus (Fig. 5A). IFN stock activity was

always re-titrated just before experimentation to allow for accurate low-dose delivery of bioactive IFN; for typical specific activities of stocks on receipt, see Voigt and others (2013). Antiviral treatments used were dilutions of recombinant bioactive IFN- α , IFN- γ , IFN- λ_1 , and antiviral secretions collected from previously infected A549 cells, titrated for antiviral activity just before experimentation.

Cellular response times were dose dependent for both type I and III IFNs, with high IFN doses able to reduce viral titers with shorter pretreatment times than low doses. In cells treated with IFN- α , significant viral inhibition occurred even when infection and start of IFN treatment were concurrent. This base level of inhibition increased to a maximum level at 4 h post-treatment for doses of 64 and 16 U/mL, and a maximum level later, ~ 12 h post-treatment, for low doses of 4 and 1 U/mL.

FIG. 3. Upregulation of type I, II, and III IFN mRNA in response to virus infection. A549 human lung epithelial cells were mock infected or infected with VSV-M51R, a nonimmunosuppressive model RNA virus, at a multiplicity of 5. (A) Cells were collected at various points postinfection, and mRNA for IFN- α , IFN- β , IFN- γ , IFN- λ_1 , and IFN- $\lambda_{2/3}$ were measured by quantitative PCR. (B) Cellular supernates were collected at various times postinfection, and secreted IFN protein titers for IFN- α , IFN- β , IFN- γ , IFN- λ_1 , IFN- λ_2 , and IFN- λ_3 were determined by ELISA. Data are shown as means of biological replicates with each assayed in duplicate. Error bars are 95% confidence intervals assuming data are normally distributed.

FIG. 4. Kinetics of IFN degradation under cell culture conditions. Degradation of antiviral activity from: (A) A549 cell secretions [conditioned media (CM)], (B) type I IFNs, (C) type III IFNs, and (D) type II IFNs. Solutions of recombinant IFN- α , IFN- β , IFN- γ , IFN- λ_1 , IFN- λ_2 , and IFN- λ_3 were diluted in A549 cell culture supernatant and incubated under culture conditions (37°C, 5% CO₂) for indicated durations, then assayed for remaining biological antiviral activity. Error bars indicate 95% confidence intervals representing 2 experiments conducted on separate days, assuming normally distributed data. (E) Half-lives of antiviral activities for IFNs and CM.

FIG. 5. Kinetics of antiviral state development in A549 lung epithelial cells after treatment by type I, II, and III IFNs. (A) Development of antiviral cellular states after treatment with type I (IFN- α), type II (IFN- γ), type III (IFN- λ_1) recombinant IFNs, and cellular secretions, shown as a percentage of total inhibition of viral reporter. Data represent the mean of 4 experimental samples. Error bars indicate standard deviations of experimental data. (B) Cellular response times to activate an antiviral state after treatment with different IFNs and cellular secretions. Response times were based in each case on the time needed to reach a half-maximal response.

IFN- λ_1 induced similar but delayed patterns of AVS development. The type III response appeared to be delayed by ~ 2 –6 h relative to type I IFN responses. The apparent delay in response is shown by estimated times to half-maximum response (Fig. 5B). The magnitude of this delay is consistent with AVS upregulation in response to IFN- β , IFN- λ_2 , and IFN- λ_3 (data not shown). Cellular responses to cell-derived antiviral secretions mirrored the responses to type I IFN, as the apparent fastest-acting IFN in the secreted solution. AVSs arising from IFN- γ signaling showed distinctly different kinetic development behavior, with a slower, linear development of the AVS that was less dose dependent, and continued to develop beyond 14 h of pretreatment.

Loewe synergy between type I and III IFNs indicates divergent downstream signaling pathways

As an unbiased method to identify the presence of divergence in signaling networks, we tested for functional synergism between IFNs by examining the effects of binary combinations of type I, II, and III IFNs on virus replication.

A549 lung epithelial cells were treated with dose gradients of 2 separate IFNs, allowed to incubate for 24 h to fully establish antiviral signaling, and probed for AVSs with reporter virus. We tested all possible pairwise combinations of IFN- α , IFN- β , IFN- γ , IFN- λ_2 , and IFN- λ_3 , as well as single-drug responses.

Synergy between different classes of IFNs can be assessed by the Loewe additivity model, graphically represented by plotting antiviral activity isoboles or sets of concentration pairs that result in the same level of viral inhibition. For example, an IC_{50} isobole would represent all possible concentration pairs (concentration of drug A, concentration of drug B), which result in a net 50% viral inhibitory effect. Using this method, it is possible to graphically show the presence of synergy, antagonism, or additivity in drug combinations (Fig. 6A).

Figure 6B shows data and model fits for IFN combinations that resulted in IC_{50} , IC_{60} , IC_{70} , and IC_{80} inhibitory levels for all binary combinations of IFN- α , IFN- β , IFN- γ , IFN- λ_2 , and IFN- λ_3 . The resulting α drug interaction parameter is shown for each combination. P values indicate

FIG. 6. Loewe interaction between type I, II, and III IFN antiviral activation of cellular antiviral states. **(A)** Graphical depiction of additive ($\alpha=1$), synergistic ($\alpha < 1$), and antagonistic ($\alpha > 1$) interaction between 2 drug or cytokine treatments based on the Loewe interaction model. **(B)** Effects of different paired IFN treatments on the antiviral state, fit to the Loewe interaction model. Cells were treated with paired combinations of IFNs, over a range of concentrations, the resultant cellular antiviral state was measured after infection with reporter virus, and the data were used to estimate concentration pairs that resulted in 50%, 60%, 70%, and 80% viral inhibition. Best-fit model lines for IC₅₀, IC₆₀, IC₇₀, and IC₈₀ isoboles are also plotted (lines). Mean drug interaction parameters α were calculated along with 95% confidence intervals.

FIG. 7. Analysis of synergy by the Bliss independence model. **(A)** Graphical depiction of independent interaction behavior (red line) between 2 drug or cytokine treatments based on the Bliss independence model. Deviations from the Bliss model are shown when treatments: (1) compete, resulting in lower antiviral activity or higher virus growth (red points), or (2) cooperate, resulting in higher antiviral activity or lower virus growth (green points). **(B)** Effects of paired IFN treatments on the antiviral state, relative to the Bliss independence model (red line). This analysis employed the same data set as Fig. 6; virus growth data have been normalized to control samples that contain no IFNs. R^2 values representing goodness of fit of the Bliss model to the experimental data are shown, as well as the average % deviation of experimental values from the model. $R^2 > 0.5$ was considered good fit.

whether the calculated α value is statistically different than 1 and synergy is present. Analysis of control combinations indicated a lack of either Loewe synergy or antagonism between type I/I IFN- α /IFN- β ($\alpha=0.93\pm0.14$, $P=0.3668$) and type III/III IFN- λ_2 /IFN- λ_3 ($\alpha=0.91\pm0.28$, $P=0.28$) pairings, as expected. Within-class interactions between similar molecules binding to the same receptor would be expected to be additive, unless drug concentrations were high enough to saturate receptors, thus causing antagonism.

In contrast, IFN type I/II interactions (IFN- α /IFN- γ , IFN- β /IFN- γ) yielded α values of 0.44 and 0.39, respectively ($P<0.0005$), indicating the presence of Loewe (additive) synergy, consistent with previous reports. We also observed more novel examples of synergy between cytokine combinations. Strong synergy was found in type II IFN/type III IFN combinations (IFN- γ /IFN- λ_2 , IFN- γ /IFN- λ_3) with α values of 0.36 and 0.44 ($P<0.0005$), respectively. Intriguingly, synergy was also found in the type I/type III IFN combinations (IFN- α /IFN- λ_2 , IFN- α /IFN- λ_3 , IFN- β /IFN- λ_2 , and IFN- β /IFN- λ_3), with α values of 0.29, 0.55, 0.35, and 0.43, respectively ($P<0.001$), suggesting possible divergence between type I and III IFN signaling pathways.

Bliss independence analysis characterizes synergy types

We proceeded to characterize the observed synergy between type I, II, and III IFNs using a Bliss independence analysis (Fig. 7). Bliss independence describes interactions of drugs that model the synergy that occurs when 2 drugs work through independent mechanisms with similar final biological outcomes—in this case, inhibition of viral replication. Analysis of type I/I and III/III IFN combinations did not align with Bliss predictions, indicating that these combinations do not act independently and are competitive by nature, giving rise to lower combined inhibition (higher virus growth) than predicted by independent mechanisms.

Type II/III combinations showed synergy beyond simple pathway independence, similar to type I/II combinations. Finally, type I/III IFN combinations followed Bliss independence predictions, suggesting some independent action of the type I and III IFN signaling pathways, but different in nature than the cooperative induction of type I/II and II/III combinations.

Discussion

Antiviral responses to type I and III IFN signaling have been reported to be similar in downstream signaling and effect, and they are suggested to be redundant signaling mechanisms. The strong experimental evidence that IFN- λ is an important component of innate antiviral immune responses in intestinal and respiratory epithelia (Mordstein and others 2008; Khaitov and others 2009; Jewell and others 2010; Pott and others 2011) suggests that there may be unique features of the type III IFN system. To characterize the intrinsic cellular responses of human lung epithelial cells to type I and III IFNs, we used a VSV-DsRed2 virus reporter system to elucidate the kinetic development of cellular AVSs after treatment with IFNs. This fluorescent reporter assay was validated to accurately report on AVSs present in cells at the start of the assay, and it correlated well with intracellular Mx1 protein levels and viral titer reduction (Fig. 2).

Differing kinetics of type I and III IFN antiviral responses have previously been proposed as an explanation for the seemingly duplicate effects of these molecules (Marcello and others 2006). IFN-mediated antiviral responses can be temporally differentiated at 3 stages examined in this work: kinetics of cellular IFN upregulation, stability of the different IFN classes, and the downstream responses to IFN stimulation. IFN upregulation in response to virus infection showed no significant temporal differences in intracellular upregulation of type I, II, and III IFNs (Fig. 3).

Despite similar upregulation kinetics, the relative levels of mRNA induction and protein secretion were significantly different between the IFN types. Type III IFNs were secreted in large amounts by lung epithelial cells in response to viral infections, till an order of magnitude higher than the classic IFN- β response and 2 orders of magnitude above the IFN- α response. This is indicative of the important role that type III IFNs play in epithelial cell antiviral responses.

Interestingly, we also found low-level (10-fold over mock infected) IFN- γ mRNA induction in infected epithelial cells, though ELISA was not sufficiently sensitive to detect a corresponding protein secretion in cell supernates. Epithelial cell-derived IFN- γ is not canonically associated with direct antiviral effects. IFN- γ is predominantly produced by mononuclear cells, and it has a classic role in downstream immunity by activating macrophages and the inflammatory, cell-mediated antiviral and antigen-specific immune responses. Considering this, upregulation of IFN- γ in epithelial cells is not expected in a direct response to virus infection. However, low levels of IFN- γ released by epithelial cells during early stages of infection may be functionally important, acting cooperatively with other IFNs to directly induce cellular AVSs (Figs. 6 and 7).

Respiratory epithelial cells have been previously shown to secrete low levels of IFN- γ in response to *Mycobacterium tuberculosis* infection (Sharma and others 2007). Similarly, oral epithelial cells were shown to directly produce IFN- γ in response to *Candida albicans* at early infection stages (Rouabhia and others 2002), suggesting that low-level secretion of IFN- γ in epithelial cells may play a role in localized or early innate immunity against infections, and/or in priming cells for later responses.

We found that biological half-lives of recombinant IFNs *in vitro* were long, in the order of several days. While these IFN stability studies used recombinant IFNs, low-level IFN starting concentrations (10–500 U/mL), and were conducted *in vitro*, they suggest that these IFNs have similar and long-lasting biological effects during *in vitro* studies, and they suggest that differing biological stabilities cannot be responsible for synergies present between these IFNs.

Type I and III IFNs rapidly trigger cellular AVSs that significantly inhibit virus replication, even when cells are only exposed to IFN starting at infection (Fig. 5). Cellular response times were dose dependent for both type I and III IFNs, with high IFN doses able to induce AVSs more quickly than low doses. Notably, a dose of just 16 U/mL of IFN- α applied concurrently with infection resulted in a more than 50% inhibition of viral replication, a remarkably fast and effective response. Kinetic responses of cells to type III IFNs appeared to be delayed 2–6 h relative to type I IFN responses, suggesting possible differences in downstream signaling responses.

In contrast, the development of direct antiviral responses after IFN- γ treatment showed significantly slower and unique kinetics, likely reflecting the alternate functions of IFN- γ , for which direct antiviral inhibition is a secondary role. These differences in the cellular response kinetics to different IFNs suggest that the direct antiviral effectors downstream of IFN- γ are induced after a longer, more involved, or more indirect process than the type I and III IFN downstream antiviral signaling mechanisms.

To develop a more comprehensive picture of the intrinsic cellular antiviral landscape, we also measured cellular responses to pairwise IFN combinations. Combination treatment can elicit higher responses than would be expected from the sum of individual drug effects, and such synergy between type I and II IFNs against a number of both DNA and RNA viruses has previously been demonstrated (Larkin and others 2003; Sainz and others 2005; Peng and others 2008), reflecting the divergence of the type I and II IFN antiviral signaling networks.

We found a lack of Loewe synergy between the 2 type I IFNs tested, IFN- α and IFN- β , and indications of antagonism at higher doses, consistent with their acting through the same type I IFN receptor (Fig. 6). We also observed an interaction between type I and II IFNs in inducing functional cellular AVSs, which showed synergy beyond pathway independence (Fig. 7), suggesting that type I and II IFNs work by cooperative induction, acting together to upregulate pathways that neither upregulates alone, consistent with previous observations (Tan and others 2005; Sanda and others 2006; Peng and others 2008).

The observed antiviral synergy between type II and III IFNs is consistent with a previous report (Pagliaccetti and others 2008), though it has not been extensively studied. The existence of these synergistic effects is perhaps not surprising, as IFN- γ is associated with quite distinct pathways than those of type I IFNs, and if type III IFNs largely share pathways with type I IFNs, such synergy could be expected.

In contrast, the Loewe synergy found between type I and III IFNs (Fig. 6), also shown to be consistent with synergy by independent action (Fig. 7), was unexpected and intriguing. If the type I and III IFN signaling pathways were, indeed, overlapping, such synergy would be unlikely. Synergy reflects nonlinearities in signaling systems, such as cooperative induction of otherwise unstimulated pathways on co-treatment. Experimentally determined synergy between drugs applied to biological systems has been shown to indicate divergent pathways in signaling networks and can be used to refine network interaction models and predict novel drug targets for various systems, including virus/host interactions (Lehar and others 2007; Owens and others 2010).

Our results suggest that initial testing for the presence of synergy, using the Loewe criterion ($\alpha < 1$), followed by classification of synergy type by Bliss independence analysis can be a useful first step to indicate when pathways diverge between antiviral drug treatments (Jilek and others 2012; Laskey and Siliciano 2014). Synergy between type I and III IFNs is strong evidence that their downstream signaling pathways are not identical. Previous reports of similarities between type I and III IFN signaling were based on microarray studies, suggesting that post-transcriptional regulatory mechanisms may play a dominant role in these responses.

Type I–III IFN synergy may have interesting therapeutic implications. While type III IFNs are expressed by a broad

range of host cells, the type III IFN receptor, IFNLR1, is not expressed in all cell types and appears to be restricted to epithelial cells and leukocytes such as dendritic and T cells (Khaitov and others 2009; Megjugorac and others 2009). These differences in receptor distribution may function to confer selectivity of effect between type I and III IFNs.

Indeed, *in vivo* mouse studies suggest that type III IFNs function prominently on mucosal tissues, such as in the lungs and gastro-intestinal tract (Pulverer and others 2010) relative to type I IFNs. Type III IFNs may be considered an additional antiviral mechanism present on human mucosal surfaces, which are the most vulnerable to virus exposure and infection. Moreover, synergy between type I and III IFNs may serve to enhance this added antiviral function of mucosal epithelial surfaces, while helping minimize the negative effects of IFN on other tissues.

Type I IFNs used as antiviral therapeutics have many side effects, so harnessing this synergy by co-treatment with type III IFNs may be able to significantly reduce these negative effects of treatment. In addition, since many tissues, such as nervous tissues, do not have type III IFN receptors, use of type III IFNs, whether alone or in combination with small amounts of type I IFNs, may abrogate many harmful side effects seen with type I IFNs such as depression (a reported side effect of IFN- α treatment) and inflammation of many tissues.

We note that the IFN treatment levels necessary for observation of synergy effects are generally low and over a restricted range. Follow-up studies could use global studies of type I IFN-, type III IFN-, and combination type I/type III IFN-treated using a protein-based technique such as quantitative mass spectrometry to identify differences in signaling networks downstream of type I and III IFN stimulation, as well as effector proteins involved in producing the enhanced synergistic effect seen with co-treatment. Phosphoproteomics may also be used in combination with more traditional mass spectrometry, as many components of IFN signaling rely heavily on phosphorylation signals that would not typically be identifiable using other global measurement techniques.

In this work, we compared and contrasted type I, II, and III IFN antiviral signaling in human lung epithelial cells. We found that cells produced all 3 IFN types with similar kinetics after RNA virus infection, but the development of cellular AVSs downstream of type I, II, or III IFN treatment differed. Type I IFN-induced development of AVSs was rapid and sensitive; type III IFN responses showed delayed kinetics in AVS induction; and type II IFN treatment had a direct but slower and less effective antiviral effect. We further discovered synergistic effects in end-point antiviral responses to paired treatments of type I and III IFNs, which was consistent with a Bliss independence model reflecting synergy by independent action.

Together, these differences in the kinetics of antiviral activation and enhanced end-point responses reflect likely differences in the signaling by type I and III IFNs. Future work employing global signaling quantification techniques will serve to pinpoint and further characterize differences between signaling by these IFNs and the source of their synergistic effects.

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Author Disclosure Statement

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Address correspondence to:

Dr. John Yin

Systems Biology Theme

Wisconsin Institute for Discovery

330 N Orchard St.

Madison, WI 53715

E-mail: john.yin@wisc.edu

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