Stimulation of the RIG-I/MAVS Pathway by Polyinosinic:Polycytidylic Acid Upregulates IFN-β in Airway Epithelial Cells with Minimal Costimulation of IL-8

Nurlan Dauletbaev, Maria Cammisano, Kassey Herscovitch and Larry C. Lands

*J Immunol* published online 17 August 2015
http://www.jimmunol.org/content/early/2015/08/16/jimmunol.1400840

---

Supplementary Material  http://www.jimmunol.org/content/suppl/2015/08/16/jimmunol.1400840.DCSupplemental

Subscription  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Stimulation of the RIG-I/MAVS Pathway by Polyinosinic: Polycytidylic Acid Upregulates IFN-β in Airway Epithelial Cells with Minimal Costimulation of IL-8

Nurlan Dauletbaev,* Maria Cammisano,* Kassey Herscovitch,* and Larry C. Lands†

Pharmacological stimulation of the antiviral cytokine IFN-β in the airways may help to counter deleterious virus-induced exacerbations in chronic inflammatory lung diseases (asthma, chronic obstructive pulmonary disease, or cystic fibrosis). Polyinosinic-polycytidylic acid [poly(I:C)] is a known inducer of IFN-β but also costimulates an inflammatory response. The latter response is undesirable given the pre-existing airway inflammation in these diseases. The objective of our study was to identify conditions for poly(I:C) to selectively upregulate IFN-β in airway epithelial cells without a concomitant inflammatory response. The inflammatory response was gauged by production of the chemokine IL-8. Using cell lines and primary airway epithelial cells (both submerged and well-differentiated), we observed that pure poly(I:C) stimulated IFN-β mainly through the TLR3/TRIF pathway and IL-8 through an unidentified pathway. The magnitude of the IL-8 response stimulated by pure poly(I:C) matched or even exceeded that of IFN-β. Furthermore, this IL-8 response could not be pharmacologically downregulated without affecting IFN-β. In contrast, we show that stimulation of the RIG-I/MAVS pathway, such as when poly(I:C) is delivered intracellularly in a complex with liposomes or via nucleofection, selectively stimulates IFN-β with low IL-8 costimulation. The magnitude of IFN-β stimulation by liposome-encapsulated poly(I:C) is markedly diminished in well-differentiated cells. In conclusion, it is feasible to augment IFN-β production in airway epithelial cells without excessive costimulation of IL-8 if the RIG-I/MAVS pathway is stimulated, such as via liposomal delivery of poly(I:C). Better cytoplasmic delivery vehicles are needed to efficiently stimulate this pathway in well-differentiated cells. The Journal of Immunology, 2015, 195: 000–000.

Any patients with chronic inflammatory lung diseases, such as asthma, chronic obstructive pulmonary disease, or cystic fibrosis, suffer from deleterious exacerbations subsequent to upper respiratory viral infections (a.k.a. the common cold). These exacerbations can lead to poorer disease control, diminished response to standard therapies, and hospitalization (1–3). Although the clinical problem and associated economic burden have been recognized, no specific preventive or therapeutic measures exist to control or abate these exacerbations. Because of a multitude of viruses and their subtypes that cause the common cold, a universal vaccine is not available, prompting pursuit of alternative strategies, including stimulation of innate antiviral immunity in the airways.

Respiratory viruses primarily infect airway epithelium. Infected airway epithelial cells normally mount efficient antiviral defenses, including production of the antiviral cytokine IFN-β. Secreted IFN-β stimulates, in an autocrine and paracrine manner, the transcription of numerous IFN-responsive genes, whose combined effects lead to timely and efficient viral clearance. It is a subject of current debate whether production of IFN-β in virus-infected airway epithelial cells or airway secretions from patients with chronic lung diseases is deficient. Some studies did not observe such IFN-β deficiency (4–7). Yet, others report either intrinsic impairment of the IFN-β response to respiratory viruses (6, 8–11) or negative modulation of this response by pathologic factors associated with diseased airways (12–16). Importantly, stimulation of IFN-β production or exogenous supplementation of this cytokine facilitates virus clearance even in cells with normal IFN-β responses (5, 17, 18). Therefore, augmentation of IFN-β production in the airways of patients with chronic inflammatory lung diseases may help to counter viral exacerbations.

Polyinosinic-polycytidylic acid [poly(I:C)], a synthetic analog of viral dsRNA, is a well-established inducer of IFN-β (19). Poly(I:C) is primarily sensed by three intracellular receptors: TLR3, the endosome-located sensor that signals through adapter Toll/IL-1R domain–containing adapter–inducing IFN-β (TRIF); and two cytoplasmic receptors, retinoic acid–inducible gene I (RIG-I) and melanoma differentiation–associated protein-5 (MDA-5), which both signal through mitochondrial antiviral-signaling protein (MAVS) (20). Signals from these receptors activate the transcription factor IFN regulatory factor 3 (IRF3), which upregulates transcription of IFN-β.

The major drawback of poly(I:C) as a potential antiviral immunostimulant is that it costimulates inflammatory responses (21) and can cause cytotoxicity (22). Both latter effects are undesired in the context of pre-existing airway inflammation in patients with chronic...
inflammatory lung diseases. Thus, it is frequently discussed whether airway epithelium in patients with cystic fibrosis is intrinsically hyperinflammatory (23–25). Therefore, immunostimulating strategies should be specifically tailored as not to exacerbate the pre-existing airway inflammation, such as that seen in cystic fibrosis airways.

The objective of this study was to determine the optimal conditions under which poly(I:C), either alone or in combination with other therapeutics, can stimulate IFN-β in airway epithelium without overly provoking the inflammatory response. The latter was gauged by upregulation of the neutrophil chemoattractant IL-8. Our studies were done in a panel of airway epithelial cell lines and primary airway epithelial cells. We demonstrate that poly(I:C), when used as a pure compound to stimulate cells, upregulates IFN-β mainly through the TLR3/TRIF pathway and IL-8 through an unidentified pathway. This IL-8 costimulation cannot be downregulated by current therapies without affecting IFN-β production. In contrast, we show that stimulation of the RIG-I/MAVS pathway, such as when poly(I:C) is delivered intracellularly in a complex with liposomes, selectively stimulates IFN-β with low IL-8 costimulation.

Materials and Methods

Cells

As a model cell, we used immortalized airway epithelial cell lines and primary normal human bronchial epithelial (NHBE) cells. The cell lines were a kind gift of Dr. Dieter Gruenert and expressed either wild-type (1HAEo and 1HBE14o) or delF508 mutant Cystic Fibrosis Transmembrane Regulator gene (CFT290a and CFB4E10 ). The cell lines with mutant Cystic Fibrosis Transmembrane Regulator gene were included to test whether their IFN-β and IL-8 responses to poly(I:C) were similar to healthy cells. Primary NHBE cells (n = 2; passages 1–2) were purchased from Lonza (Walkersville, MD) or were provided by the Primary Cell Airway Biobank (n = 4; passage 2) at McGill Cystic Fibrosis Translational Research Centre (Montreal, QC, Canada). The latter cells were obtained from discarded portions of transplant lungs under approval by the Human Ethics Board of Centre Hospitalier de l'Université de Montréal. Written informed consents were given by the donors under a protocol approved by the Institutional Review Board of the Research Ethics Office of McGill University.

Reagents

Cell culture flasks and plates were either from Fisher Scientific (Ottawa, ON, Canada) or Greiner (Mississauga, ON, Canada). Transwells were from Corning (Tewksbury, MA). Cell culture media and supplies were from Life Technologies (Burlington, ON, Canada), with the exception of bronchial epithelial growth medium (BEGM) and bronchial air liquid interface medium (B-ALI) culture media (Lonza) and FBS (Wisent, Saint-Jean-Baptiste, QC, Canada). The endotoxin-free, high m.w. poly(I:C) was used as a pure compound to stimulate cells, upregulates IFN-β mainly through the TLR3/TRIF pathway and IL-8 through an unidentified pathway. This IL-8 costimulation cannot be downregulated by current therapies without affecting IFN-β production. In contrast, we show that stimulation of the RIG-I/MAVS pathway, such as when poly(I:C) is delivered intracellularly in a complex with liposomes, selectively stimulates IFN-β with low IL-8 costimulation.

Cell, cell stimulation, and associated downstream assays

Cell lines were mostly tested under proliferating (i.e., submerged) conditions. The cells were seeded onto culture plates and grown until 90% confluency. Then cell culture medium was switched to antibiotic-free MEM supplemented with 2% FBS and 200 mM t-glutamine, and cells were stimulated as described later. Cell supernatants for IFN-β and IL-8 ELISAs were aliquoted and stored at −80°C. IL-8 ELISAs were carried out according to manufacturer instructions. In some unstimulated supernatants, the levels of IFN-β were below the lowest level of detection. For the purpose of quantification, those samples were assigned values that were equal to 50% of the lowest level of detection of IFN-β ELISA. In contrast with IFN-β, IL-8 values were readily quantifiable in all cell lines and under all tested conditions. Cells were lysed in TRIZol for the purposes of mRNA quantification; these lysates were also stored at −80°C. After RNA isolation and reverse transcription, mRNA expressions were quantified by qPCR (26, 27).

To assure reproducibility of the results across cell lines, we conducted primary outcomes (IFN-β and IL-8, both protein and mRNAs) in all four cell lines. Most mechanistic studies with pharmacological agents and inhibitors were also done in all four cell lines. The studies involving nucleofection (described later) were done using CFT290a and 1HAEo cell lines because of high nucleofection efficiency in these cell lines. Some studies were conducted in 16HBE14o and CFB4E10 cells because of their IFN-β production levels. Therefore, mechanistic studies were conducted in parallel in at least one healthy airway epithelial cell line and at least one cell line expressing mutant Cystic Fibrosis Transmembrane Regulator gene.

In some experiments, we tested the effects of poly(I:C) on well-differentiated CFB4E10 cells grown as previously described (26). In brief, cells were plated onto Transwells and grown until confluency. After reaching 100% confluency, apical fluid was aspirated, and cells were grown at the air–liquid interface. After 14 d at the air–liquid interface, basolateral culture medium was replaced with antibiotic-free MEM (2% FBS, 200 mM t-glutamine), and the cells were stimulated apically with different concentrations of poly(I:C) dissolved in 200 μl of the earlier experimental medium. Apical fluid was required to quantify apical production of IFN-β and IL-8. After 24 h, both apical and basolateral fluids were sampled and stored at −80°C pending IFN-β and IL-8 ELISAs. The cells were lysed in 80 μl lysis buffer (Qiagen) or RNA lysing buffer (Zymo Research), and lysates were stored at −80°C until RNA isolation, reverse transcription, and qPCR.

Primary NHBE cells were tested either submersed or well-differentiated. The former cells were grown on 24-well plates in complete BEGM to 70–80% confluency. Then they were incubated for 18 h in BEGM devoid of antibiotics, hydrocortisone, and retinoic acid. Retinoic acid was removed because it significantly upregulated basal IL-8 secretion in NHBE cells, which could have confounded IFN-β responses in our studies. After incubation for 18 h, NHBE cells were stimulated in the same retinoic acid–free BEGM, and cell supernatants and lysates were collected as described earlier. For experiments with well-differentiated culture, NHBE cells were seeded onto Transwells and grown until confluency in complete BEGM supplemented both apically and basolaterally. Then apical supernatant was aspirated, and basolateral medium was replaced with complete B-ALI culture medium. After 14 d at the air–liquid interface, primary NHBE cells were tested as described in the Results.

Nucleofection for NF-κB reporter and siRNA studies

Nucleofection of CFT290a and 1HAEo cell lines was conducted using Ingenio electroporation solution (Mirus Bio, Madison, WI) and Nucleofector II device (Lonza), as previously done by us (26, 27). In brief, 1 million cells were pelleted by centrifugation and resuspended in 100 μl Ingenio electroporation solution. For NF-κB reporter studies, cells were nucleofected using program T-020 in the presence of 1 ng NF-κB inducible firefly luciferase and 0.1 ng constitutive Renilla luciferase reporters, resuspended in culture medium and grown for 24 h before stimulation. For siRNA studies, cells were nucleofected in the presence of negative control or specific siRNA (TLR3, MDA-5, RIG-I, or MAVS siRNA: 200 nM; MAPK14 siRNA: 1 μM), resuspended in culture medium, and grown for 48 h before qPCR (to determine silence efficiency) or stimulation with poly(I:C). In all cells, nucleofection achieved at least 70% silencing efficiency with all genes of interest.

Activation of IRF3

Activation of IRF3 was documented by nuclear translocation (immuno- staining) and phosphorylation of Ser763 (Western blot). For nuclear translocation experiments, cells were stimulated for 1 h with poly(I:C) dissolved from Daltex substrates (Daltex Scientific, Mississauga, ON, Canada). Gel electrophoresis and transfer supplies were as indicated in our recent publication (28). All other supplies were from Sigma-Aldrich (Mississauga, ON, Canada).

Activation of IRF3

Activation of IRF3 was documented by nuclear translocation (immuno- staining) and phosphorylation of Ser763 (Western blot). For nuclear translocation experiments, cells were stimulated for 1 h with poly(I:C) dissolved from Daltex substrates (Daltex Scientific, Mississauga, ON, Canada). Gel electrophoresis and transfer supplies were as indicated in our recent publication (28). All other supplies were from Sigma-Aldrich (Mississauga, ON, Canada).
cells were sequentially stained overnight at 4°C with rabbit anti-IF3 Ab (1:50 dilution in PBS, 1% BSA), 45 min at room temperature with a goat anti-rabbit Ab, Alexa Fluor 488 conjugate (1:500 dilution in the same buffer), and 5 min with DAPI as nuclear counterstain (300 nM in 100 mM sodium phosphate buffer, pH 7.0). The coverslips were mounted using Fluorescent Mounting Medium. The slides were viewed using laser confocal microscopy LSM 700 (Zeiss; North York, ON, Canada), with 405 and 488 laser excitations for, respectively, DAPI and Alexa Fluor 488 signals. The images were obtained with a Plan Apochromat 63×/1.4 oil immersion objective. For each field, three to five z-series optical sections were collected, and a combined image was prepared using Zeiss Zen 2010 image software.

The Western blot analysis of IRF3 phosphorylation was done as follows. The cells were grown on 35-mm culture dishes until 95–100% confluency. Then the cells were stimulated with pure or liposome-encapsulated poly(I:C) as in the immunostaining experiments. After stimulation, cells were washed twice with ice-cold PBS. After this, cells were lysed with ice-cold lysis solution [radioimmunoprecipitation assay buffer (50 mM Tris, pH 8.0, 150 mM 0.9% NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS)] supplemented with protease inhibitors and freshly prepared phosphatase inhibitors (50 mM NaF, 10 mM sodium pyrophosphate, and 2 mM sodium orthovanadate). After cell lysis, the lysates were centrifuged at 12,000 × g (10 min at 4°C) to pellet insoluble fractions. The supernatants were aliquoted in prechilled 0.5-ml microtubes and frozen at −80°C pending analysis. After quantification of total protein content with BCA protein kit, 60 μg total protein was denaturated for 5 min at 95°C in the presence of SDS and 100 mM DTT and loaded on a gel. After electrophoretic separations, samples were transferred onto a PVDF membrane, blocked for 1 h at 4°C with 5% BSA in TBST, stained overnight with rabbit monoclonal anti-phospho IRF3 (phospho-Ser386; 1:1000 dilution in TBST), and washed rigorously with TBST. Chemiluminescent bands were documented using ChemiDoc MP system. After that, primary and secondary Abs were removed, and membranes were subsequently reprobed with rabbit polyclonal Ab against IRF3 (1:1000 dilution in 5% nonfat dry milk/TBST) and rabbit mAb against α-tubulin (1:1000 dilution in 5% BSA/TBST). The staining with secondary Ab and chemiluminescence detection was done as described earlier.

**Statistical analysis**

The data are presented as mean ± SEM of fold changes over basal, or percent of basal, or percent of appropriate control to enable a better comparability between cell lines or donors. Statistical analyses were conducted using GraphPad Prism (GraphPad Software, La Jolla, CA) and included descriptive statistics, parametric or nonparametric one- or two-way ANOVA comparisons with Tukey or Dunn post hoc tests, or Mann–Whitney U test. The IC50 were calculated as described previously (26). The level of statistical confidence was set at p < 0.05.

**Results**

**Pure poly(I:C) stimulates IFN-β and IL-8**

We first studied cytotoxic, IFN-β–stimulating, and IL-8–provoking effects of poly(I:C) incubated for 24 h as a pure compound (1–100 μg/ml) with normal and diseased airway epithelial cell lines. We observed that pure poly(I:C) at the tested concentration range did not cause cytotoxicity (Fig. 1A), but stimulated both IFN-β and IL-8 production (Fig. 1B, 1C, respectively). The IFN-β–stimulating potential of poly(I:C) was both dose dependent and cell line specific, with responses ranging from 1.27- to almost 13-fold across cell lines (Fig. 1B). In contrast with IFN-β, IL-8 production in the above cell lines stimulated with pIC. Cells were stimulated as above, and IFN-β levels in cell supernatants were quantified by ELISA and expressed as fold of basal cells. *p < 0.05, †p < 0.001 versus basal. *p < 0.01, ‡p < 0.001 versus 1 μg/ml pIC, †‡p < 0.001 versus basal.

Upon analyses of the magnitudes of IFN-β mRNA and protein upregulation, it was noticeable that mRNA upregulation did not clearly predict the magnitude of protein response. For example, the magnitude of IFN-β mRNA upregulation in CFBE410− cells was comparable with that in HAEo− cells (Fig. 2B), even though the former cell line secreted twice as much IFN-β when stimulated with pure poly(I:C) (Fig. 1B). Interestingly, the discrepancy between the magnitudes of upregulation of IFN-β mRNA versus secreted protein has also been noticed in virus-infected cells (12), indicating that poor correlation of IFN-β mRNA and secreted protein is not limited to cells stimulated with poly(I:C). In contrast with IFN-β, IL-8 showed a better agreement between the peak magnitudes of its mRNA and the magnitude of upregulation of protein production (Fig. 2B versus 1C).

**Pleiotropic inhibition of signaling pathways involved in the upregulation of IL-8 by pure poly(I:C) also inhibits IFN-β**

The preceding experiments demonstrated that poly(I:C) as a pure compound stimulates both IFN-β and IL-8 production. We then...
asked whether it would be feasible to selectively suppress undesired IL-8 responses without affecting IFN-β production. For this, we first assessed the involvement of the major inflammatory signaling pathways in the IL-8 response to poly(I:C). We specifically focused on transcription factor NF-κB and MAPK (ERK, p38, and JNK), which majorly determine IL-8 production (29).

To test NF-κB transactivation, we transfected CFT290− and 1HAEo− cells with NF-κB luciferase reporter and stimulated them with pure poly(I:C) for 0–8 h. In preceding experiments, this stimulation timeframe led to detectable IL-8 in cell supernatants, indicating the completion of the initial signaling events. Our experiments demonstrated a mild and transient NF-κB transactivation (CFT290−: Supplemental Fig. 1A; 1HAEo−: data not shown). Specifically, NF-κB transactivation by poly(I:C) was much less prominent than the one stimulated by IL-1β (Supplemental Fig. 1A), which stimulates NF-κB via classical activation pathway (30). We subsequently asked whether the difference in the magnitude of NF-κB transactivation between pure poly(I:C) and IL-1β could have been caused by activation of the noncanonical NF-κB activation pathway by the former. The noncanonical NF-κB activation pathway is mediated by the kinase MAP3K14 (also known as NF-κB–inducing kinase) (31, 32). We, therefore, silenced MAP3K14 in 1HAEo− cells and stimulated them with pure poly(I:C). To exclude potential effects of MAP3K14 silencing on the classical NF-κB activation pathway, we stimulated parallel MAP3K14 silenced cells with IL-1β. MAP3K14 silencing did not affect IL-8 production in 1HAEo− cells stimulated with pure poly(I:C) or IL-1β (Supplemental Fig. 1A), which led us to conclude that MAP3K14 (and likely the noncanonical NF-κB activation pathway) is not involved in IL-8 upregulation by pure poly(I:C). The observations made in 1HAEo− cells were also reproduced in CFT290− cells (data not shown).

We next tested the involvement of ERK, p38 MAPK, and JNK in IL-8 upregulation by pure poly(I:C). Using the chemical inhibitor U0126, we observed that ERK was involved in IL-8 upregulation in all four tested cell lines (Supplemental Fig. 1B). By contrast, p38 MAPK involvement, tested using the chemical inhibitor SB203580, was more cell line specific (Supplemental Fig. 1B). Therefore, our data confirm, uniformly for ERK and partially for p38 MAPK, previous reports on the role of these two MAPKs in upregulation of IL-8 by poly(I:C) (21, 33, 34). As for JNK, which under some conditions upregulates IL-8, our studies documented a less prominent involvement of this kinase in poly(I:C)-stimulated cells. Specifically, JNK inhibition downregulated IL-8 only at very high concentrations, with IC₅₀ of the JNK inhibitor SP600125 in CFT290− and 1HAEo− cells being, respectively, at 9.6 and 9.8 μM (IC₅₀ curves not shown).

Because both NF-κB and ERK were found to be involved in IL-8 upregulation by poly(I:C), we next asked whether their combined inhibition would downregulate IL-8 without affecting IFN-β. The kinase receptor interacting protein 1 (RIP1) is known to activate both NF-κB and ERK signaling pathways (35). We next tested whether RIP1 inhibition could specifically (i.e., without affecting IFN-β) modulate IL-8 responses in poly(I:C)-stimulated cells. Because IFN-β production was more readily quantifiable in CFBE41o− and 16HBE14o− cell lines in preceding experiments, we used these two cell lines in the RIP1 inhibition studies.

As expected, inhibition of RIP1 by the inhibitor necrostatin-5 markedly downregulated IL-8 (Supplemental Fig. 2A). However, IFN-β was also significantly downregulated (Supplemental Fig. 2A). This indicated that pleiotropic inhibition of the signaling pathways stimulated by pure poly(I:C) does not allow selective modulation of IL-8 production without affecting IFN-β responses.

The inability of pleotropic anti-inflammatory compounds to selectively downregulate IL-8 in cells stimulated with pure poly(I:C) was further highlighted in experiments with the corticosteroid budesonide. In our previous studies, corticosteroids effectively downregulated IL-8 (26, 27). These anti–IL-8 effects of corticosteroids involve suppression of NF-κB transactivation (27, 36, 37), also via ERK (38, 39). In this study, we preincubated all four tested cell lines with low and clinically relevant doses of budesonide before stimulation with pure poly(I:C). We observed that budesonide effectively downregulated the poly(I:C)-stimulated IL-8 in all cell lines, exhibiting IC₅₀ in the subnanomolar range (Supplemental Fig. 2B). We then tested whether budesonide...
would affect IFN-β responses to pure poly(I:C). Similar to RIP1 inhibition experiments, we tested CFBE410- and 16HBE140- cells as high IFN-β producers. In these cell lines, budesonide, even at low doses, efficiently downregulated IFN-β stimulated by pure poly(I:C) (Supplemental Fig. 2B).

**TLR3 is important for IFN-β, but not IL-8, stimulated by pure poly(I:C)**

Having witnessed the limitations of pleiotropic pharmacological modulation of IL-8 stimulated by pure poly(I:C), we next attempted a more targeted inhibition of this chemokine. First, we asked how poly(I:C) is sensed intracellularly. Because external substances are normally uptaken by endosomes, it was plausible that TLR3, the endosomal receptor for dsRNA, would act as the primary sensor of pure poly(I:C).

We first found that TLR3 mRNA expression levels were in good agreement with the levels of IFN-β production stimulated by pure poly(I:C). Specifically, 16HBE140- cells, the top IFN-β producers, expressed significantly higher levels of TLR3 mRNA, compared with the other three cell lines (Fig. 3A). To further verify TLR3 involvement in IFN-β and IL-8 responses, we inhibited the TLR3 adaptor TRIF. Confirming the prominent role of TLR3 in the IFN-β response to pure poly(I:C), TRIF inhibition nearly abolished IFN-β production in 16HBE140- cells (Fig. 3B). In cell lines with lower expression of TLR3 (CFTE290- , 1HAeo-, and CFBE410-; Fig. 3A), TRIF inhibition also downregulated IFN-β response to pure poly(I:C), albeit with a lesser magnitude (Fig. 3B).

Interestingly, TRIF inhibition, although markedly downregulating IFN-β production in poly(I:C)-stimulated 16HBE140- cells, did not affect the associated IL-8 response in this cell line (Fig. 3C). Furthermore, TRIF inhibition only marginally downregulated, or caused no effect on, IL-8 upregulation by pure poly(I:C) in the remaining three cell lines (Fig. 3C).

In line with the earlier observation, TLR3 siRNA silencing only marginally downregulated IL-8 stimulated by pure poly(I:C) in CFTE290- and 1HAeo- cells (Fig. 3D).

**Cells with low expression of TLR3 sense pure poly(I:C) by a combination of TLR3 and RIG-I: relevance to IFN-β, but not IL-8, production**

The preceding experiments demonstrated that IFN-β upregulation by pure poly(I:C) seems to be majorly dependent on TLR3 in cells that express high levels of this receptor (16HBE140- cell line), whereas cells with innately low levels of TLR3 appear to sense pure poly(I:C) only partially by TLR3. We therefore asked whether MDA-5 and RIG-I, the cytoplasmic sensors of dsRNA, could serve as receptors alternatively or complementary to TLR3 in the latter cells. To verify potential involvement of MDA-5 and RIG-I, we next silenced MAVS, which is the adaptor to both receptors.

We observed that MAVS silencing greatly diminished the IFN-β response to pure poly(I:C) in both CFTE290- and 1HAeo- cells (Fig. 4A). Because this observation confirmed the involvement of cytoplasmic receptors in these cells, we next silenced MDA-5 and RIG-I. Surprisingly, MDA-5 silencing potentiated IFN-β response to pure poly(I:C) (Fig. 4B). In contrast, RIG-I silencing led to a diminished IFN-β response (Fig. 4C). Therefore, RIG-I, but not MDA-5, silencing recapitulated IFN-β downregulation seen in MAVS silencing experiments (Fig. 4C versus 4B versus 4A, respectively).

We next tested the impact of silencing of the earlier signaling proteins on IL-8 response to pure poly(I:C). siRNA silencing of MAVS, and both MDA-5 and RIG-I, did not diminish, but even potentiated the poly(I:C)-stimulated IL-8 production (Fig. 4D–F), which excluded their involvement in IL-8 upregulation by pure poly(I:C).

**Phosphoinositide 3-kinase is not involved in IL-8 upregulation by pure poly(I:C)**

Because neither TLR3 nor its combination with RIG-I seemed to be the primary sensors for IL-8 upregulation by pure poly(I:C), this could indicate an existence of an alternative receptor. We next asked whether PI3K, which was implicated in IL-8 production in rhinovirus-infected cells (40), might be the receptor responsible for upregulation of this chemokine by pure poly(I:C). To address this, in
a subsequent experiment, we irreversibly inhibited PI3K with the chemical inhibitor wortmannin. However, PI3K inhibition did not affect IL-8 stimulated by pure poly(I:C) in CFTE290−, 1HAEo−, and CFBE41o− cells (Supplemental Fig. 3). Experiments with 16HBE14o− cells showed a similar trend but were not evaluated because of a dose-dependent detachment of 16HBE14o− cells on wortmannin (data not shown). Thus, our experiment ruled out PI3K as the IL-8 defined by innate expression of TLR3. For instance, CFBE41o− cells express lower levels of TLR3 mRNA than 16HBE14o− cells (Fig. 3A), yet the IFN-β response to liposome-encapsulated poly(I:C) in CFTE290− and 1HAEo− cells was nucleofected and stimulated as above. IFN-β in cell supernatants was quantified by ELISA. Data are expressed as percent of remaining IFN-β relative to neg siRNA and shown as mean ± SEM of three to four independent experiments. *p < 0.05 versus neg siRNA + 10 μg/ml pIC. (B) MDA-5 silencing potentiates IFN-β stimulated by pIC. CFTE290− and 1HAEo− cells were nucleofected and stimulated as above. IFN-β in cell supernatants was quantified by ELISA. Data are expressed as percent of remaining IFN-β relative to neg siRNA and shown as mean ± SEM of three to four independent experiments. *p < 0.05 versus neg siRNA + 10 μg/ml pIC. (C) RIG-I silencing significantly downregulates IFN-β stimulated by pIC. CFTE290− and 1HAEo− cells were nucleofected and stimulated as above. IFN-β in cell supernatants was quantified by ELISA. Data are expressed as percent of remaining IFN-β relative to neg siRNA and shown as mean ± SEM of three to four independent experiments. *p < 0.05, #p < 0.001 versus neg siRNA + 10 μg/ml pIC.

A desired antiviral immunostimulant for chronic inflammatory lung diseases should potently upregulate IFN-β with minimal costimulation of IL-8 in airway epithelial cells, the main target of respiratory viruses. Because poly(I:C) as a pure compound and CFBE41o− cells express lower levels of TLR3 mRNA than 16HBE14o− cells (Fig. 3A), yet the IFN-β response to liposome-encapsulated poly(I:C) in CFTE290− and 1HAEo− cells was nucleofected and stimulated as above. IL-8 in cell supernatants was quantified by ELISA. Data are expressed as percent of remaining IL-8 relative to neg siRNA and shown as mean ± SEM of three to four independent experiments.

**FIGURE 4.** Involvement of MAVS, MDA-5, and RIG-I in upregulation of IFN-β and IL-8 by pure poly(I:C). (A) MAVS silencing significantly downregulates IFN-β stimulated by pure poly(I:C). One million CFTE290− and 1HAEo− cells were nucleofected with 200 nM negative control (neg siRNA) or MAVS siRNAs, cultured for 48 h, and stimulated for 24 h with 10 μg/ml pure poly(I:C) (pIC). IFN-β in cell supernatants was quantified by ELISA. Data are expressed as percent of remaining IFN-β relative to neg siRNA and shown as mean ± SEM of three to five independent experiments. *p < 0.05, †p < 0.01 versus neg siRNA + 10 ng/ml pIC. (B) MDA-5 silencing potentiates IFN-β stimulated by pIC. CFTE290− and 1HAEo− cells were nucleofected and stimulated as above. IFN-β in cell supernatants was quantified by ELISA. Data are expressed as percent of remaining IFN-β relative to neg siRNA and shown as mean ± SEM of three to four independent experiments. *p < 0.05 versus neg siRNA + 10 μg/ml pIC. (C) RIG-I silencing significantly downregulates IFN-β stimulated by pIC. CFTE290− and 1HAEo− cells were nucleofected and stimulated as above. IFN-β in cell supernatants was quantified by ELISA. Data are expressed as percent of remaining IFN-β relative to neg siRNA and shown as mean ± SEM of three to four independent experiments. *p < 0.05, †p < 0.001 versus neg siRNA + 10 μg/ml pIC.

**Liposome-encapsulated poly(I:C) stimulates IFN-β with minimal costimulation of IL-8**

We evaluated cell viability and production of IFN-β and IL-8 in cells stimulated with liposome-encapsulated poly(I:C), used at a range of concentration of 0.1–1.0 μg/ml. We observed no cytotoxicity at the tested concentrations (Fig. 5A). We further witnessed that liposome-encapsulated poly(I:C) stimulated IFN-β production in all four cell lines at magnitudes exceeding those stimulated by pure poly(I:C) (Fig. 5B versus 1B).

Furthermore, based on these observations, we concluded that IFN-β response to liposome-encapsulated poly(I:C) was not directly defined by innate expression of TLR3. For instance, CFBE41o− cells express lower levels of TLR3 mRNA than 16HBE14o− cells (Fig. 3A), yet the IFN-β response to liposome-encapsulated poly(I:C) was much greater in the former cell line (Fig. 5B versus 1B).

In a complex with liposomes, poly(I:C) induced IFN-β production via TLR3-independent receptors. In a complex with liposomes, poly(I:C) stimulated low levels of IL-8 (Fig. 5C). Therefore, a more favorable ratio of IFN-β versus IL-8 responses (i.e., a predominant IFN-β upregulation with low costimulation of IL-8) was yielded, compared with pure poly(I:C) (Fig. 6A, 6B, respectively).

We next studied how the IFN-β response to liposome-encapsulated poly(I:C) is defined at the transcriptional level. This experiment revealed two important differences between liposome-encapsulated poly(I:C) and pure compound. First, IFN-β mRNA expression was in better agreement with secreted protein levels in cells stimulated by liposome-encapsulated poly(I:C) than when
stressed with pure poly(I:C). For instance, CFBE410− cells exhibited prominent IFN-β mRNA expression levels and highest IFN-β production (Fig. 6C versus 5B), whereas 1HAEo− cells were low producers of both IFN-β mRNA and protein (Fig. 6C versus 5B). Second, IFN-β mRNA stimulated by liposome-encapsulated poly(I:C) increased toward the 8 h of stimulation in most tested cell lines (Fig. 6C), whereas pure poly(I:C) caused a transient mRNA peak at 2 h in all cell lines (Fig. 2B).

To exclude potential confounding effects of liposomes, we used nucleofection to transfer pure poly(I:C) directly (i.e., without a vehicle) into the cytoplasm. We observed potent IFN-β responses, whereas IL-8 production still remained negligible (data not shown). Interestingly, 1HAEo− cells, which were low responders to liposome-encapsulated poly(I:C) in the previous experiment (Fig. 5B), exhibited a very robust IFN-β response when nucleoected with pure poly(I:C) (data not shown). This suggests that the magnitude of IFN-β upregulation by liposome-encapsulated poly(I:C) may be primarily defined by the efficiency of liposomal uptake. Furthermore, nucleoection of pure poly(I:C) confirmed that cytoplasmic delivery of this compound stimulates negligible IL-8.

**Liposome-encapsulated poly(I:C) is sensed by the RIG-I/MAVS pathway**

We next sought to define the signaling pathways that upregulate IFN-β in airway epithelial cells stimulated with liposome-encapsulated poly(I:C). Our preceding experiments showed a dissociation between TLR3 expression levels and the magnitude of IFN-β upregulation by liposome-encapsulated poly(I:C). To confirm noninvolvement of TLR3 in the sensing of liposome-encapsulated poly(I:C), in the next set of experiments we siRNA silenced TLR3 before cell stimulation with this form of poly(I:C). As expected, TLR3 silencing did not diminish IFN-β upregulation by liposome-encapsulated poly(I:C) (Fig. 7A). On the contrary, IFN-β production was even potentiated by liposome-encapsulated poly(I:C). To confirm involvement of TLR3 in the sensing of liposome-encapsulated poly(I:C), in this set of experiments we siRNA silenced TLR3 before cell stimulation with this form of poly(I:C). As expected, TLR3 silencing did not diminish IFN-β upregulation by liposome-encapsulated poly(I:C). On the contrary, IFN-β production was even potentiated by liposome-encapsulated poly(I:C). We next verified the potential involvement of the cytoplasmic receptors MDA-5 and RIG-I. To this end, we first siRNA silenced...
MAVS, the adaptor to both of these receptors. MAVS silencing significantly attenuated IFN-β response, confirming the cytoplasmic sensing of liposome-encapsulated poly(I:C) (Fig. 7B). Subsequent experiments excluded MDA-5, and identified RIG-I, as the receptor for liposome-encapsulated poly(I:C), because IFN-β production was significantly diminished only upon silencing of the latter receptor (Fig. 7C, 7D, respectively). This finding was interesting, because MDA-5 is most commonly defined as a sensor for high m.w. poly(I:C), such as the one used in the present studies, whereas RIG-I is believed to sense low m.w. poly(I:C) (42).

Importantly, none of the siRNA conditions diminished IL-8 upregulation by liposome-complexed poly(I:C) (Fig. 7E–H).

**Pure and liposome-encapsulated poly(I:C) differentially activate IRF3**

Having observed differential receptor involvement in IFN-β upregulation by pure versus liposome-encapsulated poly(I:C), we then asked whether these receptors would differentially activate IRF3, the principal transcription factor upregulating IFN-β.

We first assessed activation of IRF3 by examining nuclear translocation of this transcription factor in 16HBE14o- cells. Basal cells did not show nuclear translocation of IRF3 (Fig. 8A). In contrast, stimulation for 2 h with pure poly(I:C) led to extensive IRF3 nuclear translocation and fading of cytoplasmic IRF3 staining (Fig. 8A). Subsequent time points saw a rapid decrease of nuclear translocation of IRF3 (Fig. 8A). When 16HBE14o- cells were stimulated with liposome-encapsulated poly(I:C), the kinetics of IRF3 nuclear translocation was different from that seen with pure poly(I:C). Thus, increased IRF3 nuclear translocation was observed toward the later time points (Fig. 8A). Overall, IRF3 nuclear translocation was coherent with IFN-β mRNA upregulation by respective forms of poly(I:C) (Figs. 2B, 6C).

We next documented nuclear translocation of IRF3 caused by either form of poly(I:C) in CFBE41o- cells. In contrast with 16HBE14o- cells, IRF3 nuclear translocation was less prominent in CFBE41o- cells stimulated with pure poly(I:C) (Fig. 8A). The lesser extent of IFN3 nuclear translocation in CFBE41o- cells was coherent with smaller IFN-β mRNA upregulation stimulated in CFBE41o- cells by pure poly(I:C) (Fig. 2B). When CFBE41o- cells were stimulated with liposome-encapsulated poly(I:C), IRF3 increasingly translocated into the nucleus during later time points, especially at 8 h of stimulation (Fig. 8A). We were surprised, however, that the extent of this IRF3 translocation was relatively modest compared with a very substantial upregulation of IFN-β mRNA by liposome-encapsulated poly(I:C) at the 8-h time point (Fig. 6C). We therefore wanted to assess global IFR3 activation to complement the cell-based assessment by immunostaining and tested IRF3 phosphorylation (Ser386) by Western blot analysis in both cell lines.

**FIGURE 7. Involvement of TLR3, MAVS, MDA-5, and RIG-I in the upregulation of IFN-β and IL-8 by liposome-encapsulated poly(I:C).**

(A) TLR3 silencing potentiates IFN-β stimulated by liposome-encapsulated poly(I:C). One million CFTE29o- and 1HAEo- cells were nucleofected with 200 nM negative control (neg siRNA) or TLR3 siRNAs, cultured for 48 h, and stimulated for 24 h with 1.0 μg/ml liposome-encapsulated poly(I:C) (lipo-pIC). IFN-β in cell supernatants was quantified by ELISA. Data are expressed as percent of remaining IFN-β relative to neg siRNA and shown as mean ± SEM of three to five independent experiments.

(B) MAVS silencing significantly downregulates IFN-β stimulated by poly-pIC. CFTE29o- and 1HAEo- cells were nucleofected with neg or MAVS siRNAs, and stimulated as above. IFN-β in cell supernatants was quantified by ELISA. Data are expressed as percent of remaining IFN-β relative to neg siRNA and shown as mean ± SEM of three to five independent experiments.

(C) MDA-5 silencing potentiates IFN-β stimulated by poly-pIC. CFTE29o- and 1HAEo- cells were nucleofected with neg or MDA-5 siRNAs and stimulated as above. IFN-β in cell supernatants was quantified by ELISA. Data are expressed as percent of remaining IFN-β relative to neg siRNA and shown as mean ± SEM of three to five independent experiments.

(D) RIG-I silencing significantly downregulates IFN-β stimulated by poly-pIC. CFTE29o- and 1HAEo- cells were nucleofected with neg or RIG-I siRNAs and stimulated as above. IFN-β in cell supernatants was quantified by ELISA. Data are expressed as percent of remaining IFN-β relative to neg siRNA and shown as mean ± SEM of three to four independent experiments. *p < 0.05, &p < 0.01 versus neg siRNA + 1.0 μg/ml lipo-pIC.
In 16HBE14o− cells, IRF3 phosphorylation in basal cells was almost undetectable but was substantially increased in cells stimulated for 2 h with pure poly(I:C) (Fig. 8B). Therefore, the difference of IRF3 phosphorylation at 2 h of stimulation versus basal levels greatly exceeded the changes at other time points, as well as fluctuations of the level of total protein (Fig. 8B). The results of this Western blot analysis were consistent with both the kinetics of IRF3 nuclear translocation (Fig. 8A) and IFN-β mRNA upregulation (Fig. 2B) stimulated by pure poly(I:C). In contrast with pure poly(I:C), the liposome-encapsulated form caused a more modest phosphorylation of IRF3 in 16HBE14o− cells (Fig. 8B), with the extent of phosphorylation increasing toward the later time points (Fig. 8B). This was largely consistent with both IRF3 translocation (Fig. 8A) and upregulation of IFN-β mRNA (Fig. 6C) seen in this cell line.

The studies in CFBE41o− cells stimulated with pure poly(I:C) demonstrated a modest phosphorylation of IRF3, with the peak seen at 2 h of stimulation (Fig. 8B). This was largely in line with the kinetics of IRF3 nuclear translocation (Fig. 8A) and IFN-β mRNA (Fig. 2B) on pure poly(I:C). In contrast with pure compound, liposome-encapsulated poly(I:C) caused a time-dependent increase in IRF3 phosphorylation in CFBE41o− cells, with the peak observed at 8 h of stimulation (Fig. 8B). This, as well, was consistent with the kinetics of IFN-β mRNA kinetics on liposome-encapsulated poly(I:C) (Fig. 6C).

Therefore, we concluded that different forms of poly(I:C) differentially stimulate IRF3 activation, which explained well the differential IFN-β mRNA upregulation seen across cell lines.

Stimulation of primary NHBE (submerged and well-differentiated) and well-differentiated CFBE41o− cells with pure or liposome-encapsulated poly(I:C)

Preceding experiments, conducted in immortalized airway epithelial cell lines, demonstrated a more favorable response pattern (potent IFN-β and low IL-8 responses) in cells stimulated with liposome-encapsulated poly(I:C). In the next set of experiments, we verified the transferability of our observations onto primary NHBE cells.

We first stimulated submerged primary NHBE with pure poly(I:C) (1–100 μg/ml) or with liposome-encapsulated poly(I:C) (0.1–1.0 μg/ml). In these cells, liposome-encapsulated poly(I:C) demonstrated a favorable ratio of IFN-β and IL-8 responses (Fig. 9).

Because the majority of airway epithelium consists of well-differentiated cells, it is important that experimental findings are applicable to these cells as well. To this end, we first grew CFBE41o− cells for 2 wk at the air–liquid interface. The air–liquid interface culture allows polarization and differentiation of airway epithelial cells. We then stimulated these cells apically with either pure or liposome-encapsulated poly(I:C). Pure poly(I:C) did not cause any upregulation of IFN-β, either in apical or in basolateral supernatants (Fig. 10A). This was not due to the apical mucous layer, because cell rinsing with hypertonic saline did not rectify the lack of cell stimulation (data not shown). In contrast with pure poly(I:C), liposome-encapsulated poly(I:C) caused mild, but significant, dose-dependent, and polarized (i.e., apical, not basolateral) IFN-β upregulation in well-differentiated CFBE41o− cells (Fig. 10A). Interestingly, the magnitude of IFN-β upregulation to liposome-encapsulated poly(I:C) was markedly lower in well-differential CFBE41o− cells compared with

FIGURE 8. Kinetics of IRF3 activation in 16HBE14o− and CFBE41o− cells. (A) IRF3 nuclear translocation in 16HBE14o− and CFBE41o− cells. Cells were stimulated for 0–8 h with 10 μg/ml pure poly(I:C) (pIC) or 1.0 μg/ml liposome-encapsulated poly(I:C) (lipo-pIC). IRF3 was immunostained using primary anti-IRF3 Ab and Alexa Fluor 488–conjugated secondary Ab (pseudocolored green). Cell nuclei were counterstained with DAPI (pseudocolored red). IRF3 translocated in the nucleus changes the nucleus color to orange. Original magnification ×630. (B) IRF3 phosphorylation in 16HBE14o− and CFBE41o− cells. Both cell lines were stimulated as above.

Total cell lysates were used in Western blot detection of IRF3 phosphorylation (Ser386), total IRF3 levels, and α-tubulin (gel-loading control).

Downloaded from http://www.jimmunol.org/ by guest on April 30, 2017
the stimulation when this cell line was grown under submerged conditions and actively proliferated (Fig. 10A versus Fig. 5B). Importantly, in well-differentiated CFBE410- cells stimulated with either form of poly(I:C), IL-8 production, both apical and basolateral, was not upregulated (Fig. 10B).

We then tested IFN-β and IL-8 responses in well-differentiated primary NHBE cells. Before these experiments, we attempted to equilibrate primary cells, grown at the air–liquid interface for 2 wk, in experimental B-ALI culture medium devoid of retinoic acid, hydrocortisone, and antibiotics. This is because the presence of retinoic acid greatly upregulates basal and stimulated IL-8 production, impeding quantification of this chemokine by ELISA. However, the cells lost polarization after several hours of incubation in the retinoic-acid–free B-ALI culture medium (data not shown). Therefore, we conducted subsequent experiments in B-ALI containing retinoic acid, but no hydrocortisone or antibiotics. With this culture medium, the air–liquid interface culture remained intact. However, the drawback of the presence of retinoic acid in experimental culture medium was that apical IL-8 remained excessively high and unquantifiable even after significant dilution (data not shown). Therefore, we were not able to reliably quantify apical IL-8. Because this was combined with very low levels of secreted IFN-β, we did not feel confident to assess the IFN-β/IL-8 response ratios, such as was done with submerged NHBE. To overcome this, we stimulated well-differentiated NHBE cells apically with either 1 μg/ml pure or liposome-encapsulated poly(I:C), and quantified expression of IFN-β versus IL-8 mRNAs in cell lysates. As Supplemental Fig. 4 demonstrates, only stimulation with liposome-encapsulated poly(I:C) led to a small, but significant, upregulation of IFN-β mRNA. Furthermore, IL-8 mRNA upregulation by liposome-encapsulated poly(I:C) tended to be lower than that stimulated by pure poly(I:C), although neither change was significant in comparison with basal cells (Supplemental Fig. 4).

To summarize, pure and liposome-encapsulated poly(I:C) have been found to signal through different pathways (Fig. 11). Following cell stimulation with liposome-encapsulated poly(I:C), a more favorable IFN-β/IL-8 response ratio was achieved.

**Discussion**

The experiments in this report were conducted to evaluate whether it would be feasible to preferentially stimulate IFN-β, without excessive costimulation of IL-8 responses, in airway epithelial cells, the primary target of respiratory viruses. As the IFN-β–stimulating agent, we used poly(I:C), a synthetic analog of viral dsRNA. Such targeted immunostimulation may help to boost antiviral immunity in the airways of patients suffering from postviral exacerbations of underlying lung disease, such as cystic fibrosis. Prior animal studies favorably assessed the ability of different formulations of poly(I:C) to augment antiviral defenses and facilitate viral clearance in the airways (43–48). However, potential inflammagenic effects of such immunostimulation have not been extensively addressed. This aspect is important because collateral stimulation of inflammatory responses in the airways of patients with chronic diseases may negate beneficial effects of immunostimulation because of worsening of pre-existing airway inflammation. Thus, the primary objective of our study was to achieve the most favorable pattern of IFN-β and IL-8 responses (i.e., high IFN-β and low IL-8) to poly(I:C) in healthy and cystic fibrosis airway epithelial cells.

Most previous studies used poly(I:C) as a pure compound. Our studies demonstrate that pure poly(I:C) stimulates IFN-β production via the TLR3/TRIF pathway in proliferating cells that innately express high levels of TLR3. Cells with low expression levels of TLR3 were found to sense pure poly(I:C) via a combination of TLR3 and the cytoplasmic RIG-I (Fig. 11). However, stimulation of proliferating epithelial cells with pure compound brings about a high and undesired IL-8 response, which involves signaling through an unidentified receptor (Fig. 11). Potential candidates for this receptor may include protein kinase R (49), protein kinase D (50), or DHX33 RNA helicase (51). Our attempts to dampen the epithelial IL-8 response at the level of transcription factors and MAPK inevitably led to suppression of the IFN-β response. Inhibition of upstream signaling proteins (e.g., at receptor/adaptor levels) seems to warrant a more targeted modulation of cell responses to pure poly(I:C). We did not test whether selective suppression of inflammatory pathways

**FIGURE 9.** Stimulation with liposome-encapsulated poly(I:C) yields a beneficial IFN-β/IL-8 response ratio in primary human bronchial epithelial cells. Primary human bronchial epithelial (NHBE) cells were stimulated for 24 h with different concentrations of pure poly(I:C) (pIC) or liposome-encapsulated poly(I:C) (lipo-pIC). IFN-β and IL-8 in cell supernatants were quantified by ELISA and expressed as fold of basal cells. Data are mean ± SEM of three independent experiments. *p < 0.05 lipo-pIC versus pure pIC.

**FIGURE 10.** Upregulation of apical IFN-β production, but not IL-8, in well-differentiated CFBE410- cells by liposome-encapsulated poly(I:C). (A) Liposome-encapsulated, but not pure, poly(I:C) stimulates apical IFN-β production in well-differentiated CFBE cells. The cells were grown for 14 d at the air–liquid interface and stimulated apically for 24 h with 200 μl culture medium containing 1–100 μg/ml pure poly(I:C) (pIC; left panel) or 0.1–1.0 μg/ml liposome-encapsulated poly(I:C) (lipo-pIC; right panel). Cells stimulated only with culture medium served as control cells (basal). IFN-β production in apical and basolateral supernatants was quantified by ELISA and expressed as fold of basal cells. Data are mean ± SEM of three independent experiments. (B) Neither liposome-encapsulated nor pure poly(I:C) stimulates IL-8 production in well-differentiated CFBE cells. The cells were grown and stimulated apically as above. Cells stimulated only with culture medium served as control cells (basal). IL-8 production in apical and basolateral supernatants was quantified by ELISA and expressed as fold of basal cells. Data are mean ± SEM of six independent experiments.
is feasible at posttranscriptional levels, via, for example, microRNA. Further studies in both earlier areas may yield potentially important and clinically pertinent results. For example, identification and pharmacological inhibition of the receptor that recognizes viral dsRNA and upregulates IL-8 may lead to a selective downregulation of the undesired inflammatory response without negative consequences for antiviral immunity. Such an approach may be more advantageous compared with the pleotropic effects of inhaled corticosteroids, the drugs often used during viral exacerbations of chronic lung diseases.

In this study, we did not pursue those approaches and instead tested whether stimulation of cytoplasmic pathways recognizing dsRNA would yield more favorable responses from airway epithelial cells. These pathways (such as the RIG-I/MAVS pathway; Fig. 11) can be stimulated by liposome-encapsulated poly(I:C). With this approach, we obtained a much more favorable pattern of IFN-β and IL-8 responses, with potent IFN-β and low IL-8 upregulation. Others also observed higher specificity of cytoplasmic antiviral pathways for IFN-β stimulation in other cell types (52–55). This confirms the suitability of stimulation of, for example, the RIG-I/MAVS pathway for upselective regulation of IFN-β. This stimulation can be exploited by future studies to develop targeted and safe antiviral immunostimulation for chronic inflammatory lung diseases.

Importantly, we demonstrate that cells expressing mutant Cystic Fibrosis Transmembrane Regulator gene are fully capable of upregulating IFN-β. Furthermore, we show that this upregulation can be achieved in cystic fibrosis cells without excessive IL-8 production. The latter observation is important because innate inflammatory responses of cystic fibrosis airway epithelium may be exaggerated (23–25).

Notably, several commercial formulations of poly(I:C) exist with potentially different effects on immune cells (56, 57). The availability of various formulations of poly(I:C) may be advantageous, because its molecular size and chemical modifications play an important fine-tuning role in stimulating innate cell responses and may allow for a greater versatility in different pathologic conditions.

An important finding was that liposomal delivery of poly(I:C) is less efficient in well-differentiated airway epithelial cells. Thus, well-differentiated CFBE41o− cells secreted markedly less IFN-β, compared with their response to liposome-encapsulated poly(I:C) under submerged conditions. These results are not surprising given that well-differentiated cells are resistant to liposomal transfection. Although not directly tested by us, we believe that this could be because of a diminished endosomal uptake from apical surfaces of well-differentiated airway epithelium. Well-differentiated cells were also reported to resist apical uptake of other nucleic acids (58). Nonetheless, we believe that one should not be discouraged by the present inability to stimulate IFN-β in well-differentiated epithelial cells. Recent studies indicate that liposomal transfection of well-differentiated cells is feasible but requires utilization of various cofactors to increase the efficiency of liposomal uptake (58). Therefore, future studies should specifically focus on improvement of liposomal delivery of poly(I:C) to well-differentiated airway

**FIGURE 11.** Proposed signaling cascades for IFN-β and IL-8 upregulation by pure and liposome-encapsulated poly(I:C). Both pure poly(I:C) and liposome-encapsulated poly(I:C) are uptaken by the endosomes. In cells with innately high levels of TLR3, pure poly(I:C) is retained in the endosomes and signals through the TLR3/TRIF pathway to stimulate IFN-β via IRF3. Pure poly(I:C) further stimulates an unidentified receptor [marked with a question mark (?) symbol] to upregulate IL-8 via NF-κB. NF-κB also plays some role in the IFN-β stimulation by pure poly(I:C). In cells with low levels of TLR3, some pure poly(I:C) escapes the endosomes to stimulate the cytoplasmic RIG-I/MAVS pathway and upregulate IFN-β. However, a much more potent stimulation of the RIG-I/MAVS pathway is achieved by liposome-encapsulated poly(I:C). Poly(I:C) complexed with liposomes mostly escapes the endosomes, and thereby avoids stimulation of TLR3/TRIF and the unidentified IL-8 upregulating receptor. Once in the cytoplasm, poly(I:C) activates the cytoplasmic RIG-I/MAVS pathway to upregulate IFN-β via IRF3. This stimulation maximizes IFN-β upregulation, whereas costimulation of IL-8 remains low. Therefore, the IFN-β/IL-8 response ratio is more favorable with liposome-encapsulated poly(I:C).
epithelium. It may not require many cells to achieve a substantial upregulation of IFN-β secretion. As our immunostaining experiments show, a seemingly low number of CFBE410 with translocated IRF3 appears to be responsible for a very robust upregulation of IFN-β mRNA and protein. IFN-β stimulates its own production in an autostimulation manner, and having even a few responder cells may be sufficient to stimulate antiviral responses in the vicinity.

Our study tested the IFN-β response to pure or liposome-encapsulated poly(I:C) in noninfected cells. The rationale for this was as follows. Rhinoviruses, the major cause of the common cold and associated exacerbations, normally infect only a small fraction of epithelial cells (59). Therefore, most cells targeted by liposome-encapsulated poly(I:C) will be uninfected. Stimulation by liposome-encapsulated poly(I:C) may prime their antiviral defenses to prevent further spread of viral infection in the airways. Furthermore, anecdotal evidence and experimental rhinovirus infections (1, 60) indicate that liposome-encapsulated poly(I:C) may prime uninfected cells (59). Therefore, most cells targeted by liposome-encapsulated poly(I:C) may prime their antiviral defenses to prevent further spread of viral infection in the airways. Furthermore, anecdotal evidence and experimental rhinovirus infections (1, 60) indicate that liposome-encapsulated poly(I:C) may prime uninfected cells.

In conclusion, liposomal delivery of poly(I:C) into the cytoplasm of airway epithelial cells effectively upregulates antiviral IFN-β with minimal costimulation of IL-8 by stimulating the RIG-I/MAVS pathway. Future studies should address the suitability of this IFN-β stimulation for facilitating clearance of respiratory viruses in airway epithelial cells and also in their well-differentiated state.

Acknowledgments
We thank Dr. Dieter Gruenert (University of California, San Francisco, San Francisco, CA) for the kind gift of airway epithelial cell lines used in this study; Elizabeth Waring, Michele Iskandar, Robert Reiss, and Mithun Das for help with preliminary experiments; and Dr. Dao Nguyen (Research Institute of McGill University Health Centre) for the use of the confocal microscope.

Disclosures
The authors have no financial conflicts of interest.

References


