Pellino-1 Selectively Regulates Epithelial Cell Responses to Rhinovirus

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Pellino-1 has recently been identified as a regulator of interleukin-1 (IL-1) signaling, but its roles in regulation of responses of human cells to human pathogens are unknown. We investigated the potential roles of Pellino-1 in the airways. We show for the first time that Pellino-1 regulates responses to a human pathogen, rhinovirus minor group serotype 1B (RV-1B). Knockdown of Pellino-1 by small interfering RNA (siRNA) was associated with impaired production of innate immune cytokines such as CXCL8 from human primary bronchial epithelial cells in response to RV-1B, without impairment in production of antiviral interferons (IFN), and without loss of control of viral replication. Pellino-1 actions were likely to be independent of interleukin-1 receptor-associated kinase-1 (IRAK-1) regulation, since Pellino-1 knockdown in primary epithelial cells did not alter responses to IL-1 but did inhibit responses to poly(I·C), a Toll-like receptor 3 (TLR3) activator that does not signal via IRAK-1 to engender a response. These data indicate that Pellino-1 represents a novel target that regulates responses of human airways to human viral pathogens, independently of IRAK signaling. Neutralization of Pellino-1 may therefore provide opportunities to inhibit potentially harmful neutrophilic inflammation of the airways induced by respiratory viruses, without loss of control of the underlying viral infection.

Airway viral infections are a dominant trigger for exacerbations of asthma and chronic obstructive pulmonary disease (COPD) (10). Of the many viral pathogens that are associated with exacerbations of airway disease, the single RNA-stranded rhinoviruses are a ubiquitous and common trigger throughout life (10). Viral exacerbations of airway disease are associated with both interferon (IFN) generation, which is generally viewed as crucial for control of viral infection, and neutrophilic inflammation, which is often thought to be potentially counterproductive, since neutrophils have little role in the direct control of viral infection (8, 10, 16, 31) but are implicated in lung damage in a variety of settings. In particular, airway epithelial cells make type 1 and type 3 IFNs, which contribute to antiviral immunity (12). While induction of neutrophilic inflammation is classically dependent upon the activation of NF-κB and induction of CXCL8, IFN production is regulated by independent pathways that are dependent upon the interferon regulatory factor (IRF) transcription family (11, 31); therefore, the appealing possibility of manipulation of neutrophilic inflammation in a manner independent of IFN generation arises.

We have previously developed *in vitro* models of airway and vascular inflammation that have shown that responses to inflammatory stimuli mimicking bacterial or viral infection require interleukin-1 (IL-1) generation for maximal activation of chemokine production (3, 18, 19, 30). More recently, we have shown that knockdown of the IL-1/Toll-like receptor (IL-1/TLR) signaling adapter MyD88 in epithelial cells alters the response to rhinoviral infection such that CXCL8 production is reduced and rhinoviral replication is increased (29). Therefore, neutralization of the IL-1/TLR pathways at the level of shared adapters may be associated with unwanted consequences for control of viral infection and may not be the optimal way of selectively targeting virus-induced neutrophilic inflammation. In parallel with these studies, we have been exploring whether a more selective regulation of the responses to rhinovirus might be achieved through manipulation of the function of a poorly

understood protein, Pellino-1. We became interested in Pellino-1 when experiments predominantly performed in cell line systems showed that it regulates the function of interleukin-1 receptor-associated kinase-1 (IRAK-1) by ubiquitination (1, 9, 20, 21, 25) and thus might be important in the control of IL-1/TLR signaling. However, the roles of Pellino-1 in the regulation of responses to human pathogens in primary cells had not been explored. We hypothesized that Pellino-1 would regulate inflammatory responses to rhinovirus, potentially through the control of IL-1 signaling. Here, we found that Pellino-1 exerts selective control over primary epithelial cell innate immune responses to rhinovirus minor group serotype 1B (RV-1B), without impairing IFN generation, via a mechanism independent of the IL-1 signaling pathway. These data identify Pellino-1 as a new target with the potential to ameliorate virus-induced neutrophilic inflammation without impairing viral clearance.

MATERIALS AND METHODS

Materials. Cell culture media and reagents were obtained from Invitrogen (Paisley, United Kingdom) unless otherwise stated. Fetal calf serum (FCS) with endotoxin levels equal to or less than 0.5 endotoxin units (EU)/ml was obtained from PromoCell (Heidelberg, Germany). All primary epithelial cell culture media and supplements were supplied by PromoCell. Poly(I·C) was obtained from Invivogen (San Diego, CA), and tumor necrosis factor alpha (TNF-α) and IL-1β were obtained from Peprotech (Rocky Hill, NJ).

Cell line and primary cell culture. All cells were grown in a humidified incubator at 37°C with 5% CO_2 and were routinely tested for myco-

plasma. Cells of the immortalized epithelial cell line BEAS-2B (American Type Culture Collection [ATCC]; LGC Standards, Teddington, United Kingdom) were maintained in RPMI 1640 containing 2 mM L-glutamine supplemented with 10% FCS and antibiotics. HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS and antibiotics. Human embryonic kidney (HEK) 293 FT cells were maintained in DMEM supplemented with 10% FCS, 0.1 mM nonessential amino acids (NEAA), 2 mM L-glutamine, and antibiotics. Primary bronchial epithelial cells (PBECs) isolated from healthy human volunteers were purchased from PromoCell. PBECs were maintained in serum-free airway epithelial cell growth medium supplemented with bovine pituitary extract (BPE) (40 μg/ml), recombinant human epidermal growth factor (10 ng/ml), recombinant human insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), adrenaline (0.5 μg/ml), tri-iodo-L-thyronine (6.7 ng/ml), human transferrin (0.5 μg/ml), retinoic acid (0.1 ng/ml), and antibiotics. Media were replaced every 2 to 3 days, and cells were subjected to passage every 7 to 12 days using a PromoCell Detach kit.

Culture of epithelial cells from asthma patients. Primary bronchial epithelial cell (PBEC) cultures were obtained from bronchial brushings in 3 asthma patients by bronchoscopy using a fiberoptic bronchoscope as described previously (6). All subjects were nonsmokers ($\leq$10 pack years, no smoking in the last year), between 18 to 65 years of age, and free of other lung diseases. Patients were included on the basis of the presence of allergy (as determined by either skin or Phadiatop testing), a forced expiratory volume collected during the first second (FEV_1) > 80% of the predicted value, and a provocative methacholine concentration causing a 20% fall in FEV_1 (PC_{20} methacholine) < 8 mg/ml or PC_{20} histamine < 8 mg/ml. Subjects did not use inhaled corticosteroids during the last 4 weeks preceding the study. The Medical Ethics Committee of the University Hospital of Groningen approved the study, and signed informed consent was given by participants. Cells were cultured as described previously (7). Briefly, cells were grown in 2.5 ml of serum-free hormonally supplemented bronchial epithelium growth medium (BEGM; Lonza, Basel, Switzerland) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) in flasks coated with collagen (30 μg/ml), fibronectin (30 μg/ml), and bovine serum albumin (BSA) (10 μg/ml). Cells were subjected to passage using trypsin when 90% confluent, stored in liquid nitrogen, and used for experimentation in passage 2.

Transient transfection. Pellino-1 and receptor-interacting serine/threonine-protein kinase 1 (RIP1) were knocked down in epithelial cells by the use of a Dharmacon On-Target Plus SMARTpool short interfering RNA (siRNA) system (Thermo Scientific, Lafayette, CO) and Lipofectamine 2000 (Invitrogen) according to the manufacturers' protocols. Cells were grown in 12-well plates until fully confluent. Cells were washed twice in phosphate-buffered saline (PBS), and media were replaced with RPMI 1640 supplemented with 10% FCS but without antibiotics (BEAS-2B) or with serum-free airway epithelial cell growth medium without supplements (PBECs). siRNA (1 μM in Opti-MEM [Invitrogen]) and Lipofectamine were equilibrated at room temperature for 5 min, following which the two solutions were combined for a further 20 min. The complexed siRNA was added to cells and incubated for 4 h at 37°C with 5% CO_2. Cells were then washed in PBS before the addition of 1 ml of complete media (RPMI 1640 supplemented with 10% FCS and antibiotics for BEAS-2B cells or airway epithelial cell growth medium containing all supplements, excluding bovine pituitary extract, for PBECs) for recovery. Cells were incubated at 37°C with 5% CO_2 overnight before stimulation.

For transfection of epithelial cells from asthma patients, cells were seeded into coated 24-well plates at a density of 1×10^5/well, grown until ~70% confluence was reached, and transfected with Pellino-1 or scrambled siRNA by the use of Lipofectamine 2000. After 24 h, medium was replaced by bronchial epithelial basal medium (BEBM) supplemented with transferrin and insulin.

Viral culture and infection. Human rhinovirus minor group serotype 1B (RV-1B) was grown in Ohio HeLa cells (European Collection of Cell Cultures [ECACC]; Sigma-Aldrich, Paisley, United Kingdom), and stocks were prepared from HeLa lysates, yielding on average 2×10^7 50% tissue culture infective doses ($TCID_{50}$)/ml (29). Viral cytopathic effect (CPE) determinations were carried out in HeLa Ohio cells to determine $TCID_{50}$ values. Neutralization using serotype-specific antibody (Ab) (ATCC) was carried out to confirm RV-1B identity (29).

RV-1B was used to infect a confluent layer of BEAS-2B cells or PBECs in a 12-well plate. Cells were subjected to serum starvation overnight by incubation in RPMI 1640 supplemented with 2% FCS and antibiotics (BEAS-2B) or airway epithelial cell growth medium containing all of the supplements except bovine pituitary extract (PBECS). Cells were washed in PBS and infected with virus for 1 h at room temperature on a rotating platform to achieve $TCID_{50}$s of 0.5×10^7, 1×10^7, and 2×10^7. Virus was removed, cells were washed in PBS, and the media were replaced. Cells were incubated for a further 24 h, following which supernatants were removed for cytokine analysis or cell lysates were prepared for IFN gene expression or viral replication assays.

Virus CPE assay. Subconfluent Ohio HeLa cells in 96-well plates were exposed to serial dilutions of infectious supernatants. Development of a cytopathic effect (CPE) was visualized after 4 days. Assays were performed in eight replicate wells, and endpoint titers were defined by the highest dilution at which CPE was observed in 50% of the wells ($TCID_{50}$) (29).

Quantification of cytokines by ELISA. Cell-free supernatants were collected and stored at -80°C until use. CXCL8/IL-8, IL-6, CXCL10/IP-10, and CCL5/RANTES proteins were quantified by enzyme-linked immunosorbent assay (ELISA), using matched Ab pairs from R&D Systems (Abingdon, United Kingdom). All samples were above the limit of detection. Samples were diluted such that they ran in the linear range of a log/lin standard curve.

Quantification of cytokines by CBA. Cell-free supernatants were prepared and stored at -80°C until required. Cytokines were measured using a cytokine bead array (CBA) flex set multiplexed bead immunoassay (BD Biosciences) comprising antibodies to TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, IL-10, IL-1β, and IL-1α, and CX3CL1/fractalkine. Samples and standards were analyzed using a fluorescence-activated cell sorter (FACS) array (BD Biosciences).

RT-PCR and qPCR. RNA was prepared from cell lysates by the use of TRI reagent (Sigma) and converted to cDNA by the use of a high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Warrington, United Kingdom). Reverse transcriptase PCR (RT-PCR) was carried out using GoTaq Flexi DNA polymerase (Promega) and specific primers for Pellino-1, -2, -3a, and -3b. Quantitative PCR (qPCR) was carried out using primer-probe sets from Applied Biosystems for Pellino-1, Hs00221035_m1, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and Hs00182082_m1 or from Sigma-Aldrich for IFN-β, IFN-λ1, IFN-λ2/3, and RV-1B (26, 29). PCR master mix was obtained from Eurogentec (Southampton, United Kingdom), and reactions were carried out using an ABI 7900 automated TaqMan system (Applied Biosystems). For qPCRs for Pellino-1, rhinovirus copy number, and GAPDH determinations, samples were quantified against a standard curve of plasmids containing known copy numbers of target genes; for qPCRs of IFNs, relative expression values were calculated against an arbitrary standard (virus-infected BEAS-2B cDNA).

Western blotting. Cell lysates were prepared by resuspending cells in lysis buffer containing Triton-X plus 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease-inhibitor cocktail (both added just prior to lysis) and incubation on ice for 2 min followed by centrifugation at 10,000 rpm for 10 min at 4°C to remove insoluble material. Lysates were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) lysis buffer for 5 min and stored at -80°C until required. Samples were subjected to SDS-PAGE and membranes blotted with antibodies to RIP1 (BD Biosciences, San Diego, CA), Pellino-1/2 or I-κBα (Santa Cruz Biotechnology, Santa Cruz, CA), or actin (Sigma-Aldrich).

Statistical analyses. Data are presented as means ± standard errors of the means (SEM) unless otherwise stated. Data were analyzed by Student's
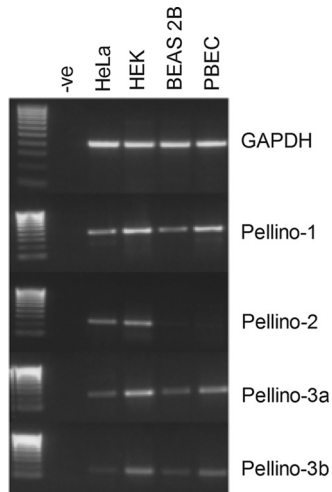
FIG 1 Pellino family mRNA expression in tissue cells. Pellino mRNA expression was explored using RT-PCR. HeLa, HEK 293FT (HEK), BEAS-2B, and PBEC cDNA was used to amplify Pellino-1, Pellino-2, Pellino-3a, or Pellino-3b or the loading control GAPDH, using specific primers. A negative control which excluded the template was also included (-ve). The figure shows representative gels from 2 independent experiments.

t test or analysis of variance (ANOVA) with an appropriate posttest as indicated in figure legends (Prism version 5.0d; GraphPad).

RESULTS

Data dominantly generated in cell line systems suggested a role for Pellino-1 in the regulation of IL-1 signaling. We hypothesized that Pellino-1 would regulate responses to viral infections in human epithelia and therefore explored Pellino-1 expression and function in human airway epithelial lines and primary cells.

Expression of Pellino in human lung epithelial cells. Epithelial expression of Pellino in humans has not been described. We therefore examined expression of Pellino-1, and the related family members Pellino-2, -3a, and -3b, by RT-PCR. Figure 1 shows that Pellino-1 and both isoforms of Pellino-3 are expressed in the human BEAS-2B epithelial cell line and also in PBECs. In contrast, HeLa epithelial cells and HEK cells expressed all known mammalian Pellino forms. We focused on the roles of Pellino-1 because of its potential to regulate IL-1 signaling. We further validated our RT-PCR data using qPCR and Western blotting. We determined that, in BEAS-2B cells, Pellino-1 expression could be readily measured by qPCR. Furthermore, using siRNAs, we could knock down Pellino-1 mRNA expression in BEAS-2B cells with a specific Pellino-1 siRNA but not with a scrambled control (Fig. 2). In PBECs, Pellino-1 mRNA expression was also detectable by qPCR and could be readily knocked down with siRNA (Fig. 2). We ex-
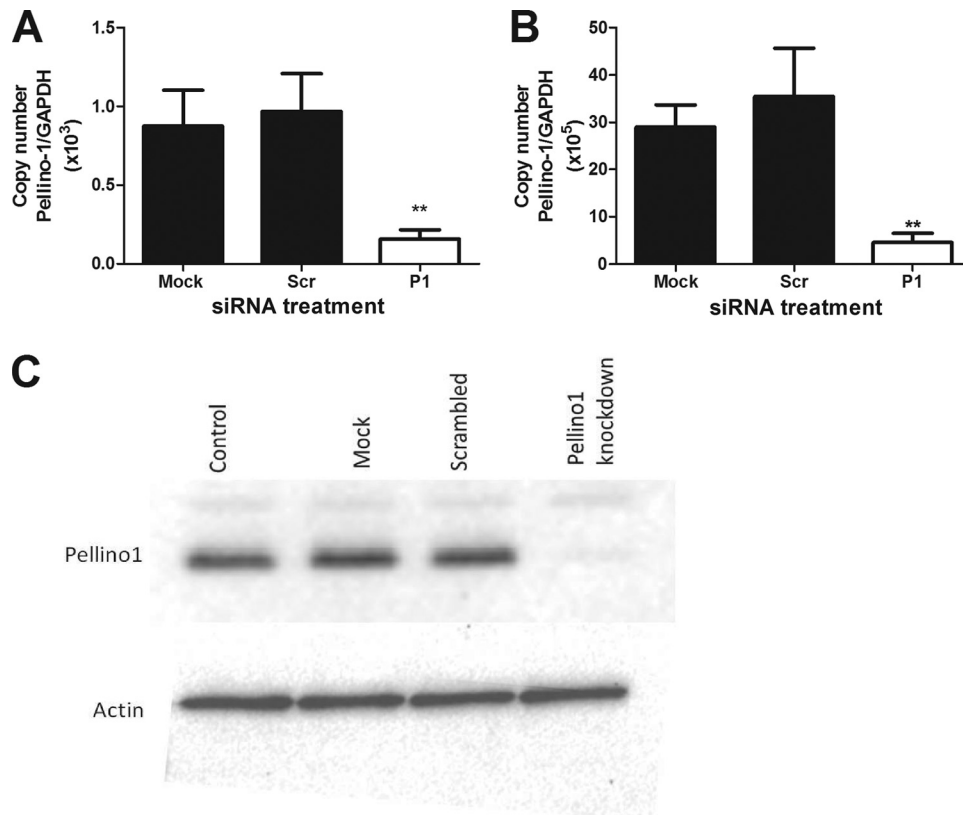

FIG 2 Transient Pellino-1 knockdown in BEAS-2B cells and PBECs. BEAS-2B cells and PBECs were transiently transfected with siRNA targeting Pellino-1 (P1) or a nontargeting scrambled siRNA (Scr). Pellino-1 mRNA expression levels in mock-, scrambled siRNA (Scr)-, and Pellino-1 (P1)-transfected BEAS-2B cells (A) and PBECs (B) were determined by qPCR. Pellino-1 copy numbers were normalized to the GAPDH copy number as a loading control, and data are expressed as means ± SEM of the results from 4 independent experiments (BEAS-2B cells) or from 3 independent donors (PBECs). Pellino-1 knockdown PBECs were lysed and immunoblotted using antibodies to either Pellino-1 or actin. Panel C shows a blot representative of 3 independent experiments performed with cells from a single donor. Statistical analysis was carried out by one-way ANOVA with Bonferroni's posttest, comparing Scr to P1 (**, $P < 0.01$).
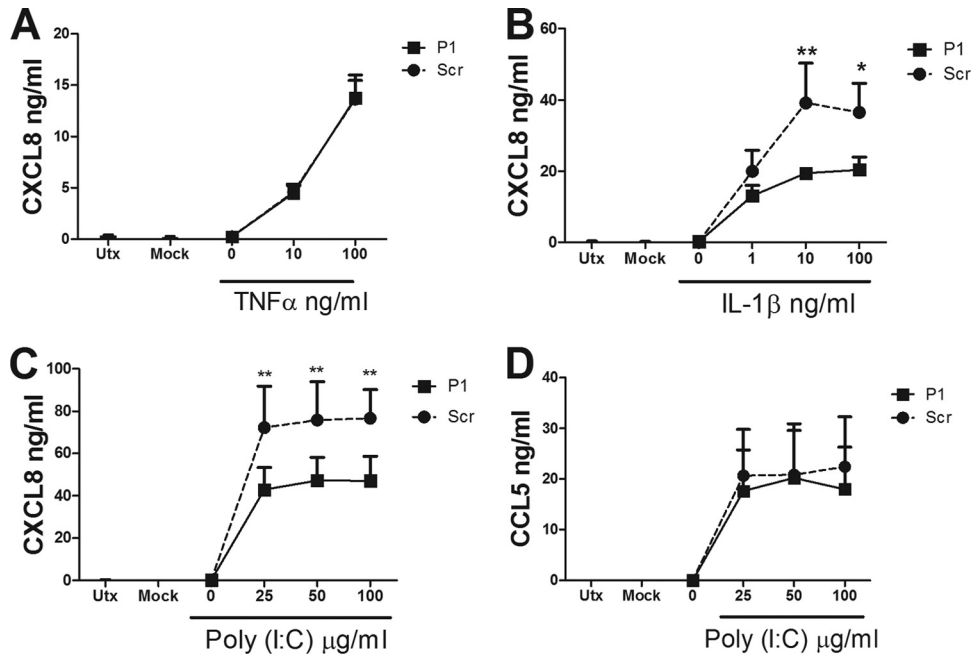
FIG 3 Cytokine generation in Pellino-1 knockdown BEAS-2B cells in response to TNF-α, IL-1β, and poly(I·C) stimulation. BEAS-2B cells were transiently transfected with siRNA targeting Pellino-1 (P1) or a nontargeting scrambled siRNA (Scr) and were treated for 24 h with media (Mock) or a response concentration of TNF-α (A), IL-1β (B), or poly(I·C) (C). Poly(I·C)-treated cells were also analyzed for CCL5 generation (D). Untransfected (Utx) cells treated with media only were also included. Supernatants were collected and analyzed for CXCL8 or CCL5 generation by ELISA. Data are expressed as means ± SEM of the results determined in 5 (CXCL8) or 4 (CCL5) independent experiments. Statistical analysis was carried out by one-way ANOVA with Bonferroni's posttest, comparing Scr to P1 for each concentration (*, $P < 0.05$; **, $P < 0.01$).

amined the properties of a number of commercial anti-Pellino-1 antibodies. An antibody from Santa Cruz with specificity against Pellino-1 and Pellino-2 gave clear staining of a band at the predicted size that was markedly reduced in cells treated with siRNA for Pellino-1. Pellino-2 is not expressed in airway epithelial cells (Fig. 1); thus, this knockdown of protein expression is consistent with the qPCR observations showing effective knockdown of Pellino-1 expression.

Roles of Pellino-1 in responses of the BEAS-2B epithelial cell line to proinflammatory stimuli. BEAS-2B cells comprise a well-characterized immortalized epithelial cell line that serves as a good representation of primary human airway epithelial cells (24) and that we have previously used to successfully study innate immune responses to bacterial and viral stimuli (18, 29). Cells were transfected with siRNAs to Pellino-1 or a scrambled control (in each experiment, Pellino-1 knockdown was confirmed by qPCR; data not shown). Induction of CXCL8 by TNF-α was unaffected by Pellino-1 knockdown (Fig. 3A). Other *in vitro* studies had indicated that Pellino-1 exhibited its principal function by regulation of IRAK-1 (1, 9, 14, 25, 28). In keeping with a role for Pellino-1 in the regulation of IRAK-1 function, induction of CXCL8 by IL-1β was significantly inhibited by Pellino-1 knockdown (Fig. 3B). Activation of IRAK-1 is critical to signaling of the IL-1R and almost all TLRs, and so, to examine the specificity of Pellino-1, we subsequently elected to investigate responses to a TLR agonist known to be independent of IRAK-1. We therefore stimulated cells with the double-stranded RNA mimic poly(I·C), which activates TLR3 and its adapter TRIF but does not use IRAK-1 to transduce a signal (9). To our surprise, the induction of CXCL8 by poly(I·C), but not the

induction of CCL5, was also impaired by Pellino-1 knockdown (Fig. 3C and D). We have previously shown that poly(I·C)-induced CXCL8 production is not impaired by MyD88 knockdown (29), so these data indicated that inhibition of the generation of CXCL8 by Pellino-1 knockdown was unlikely to be mediated by regulation of IRAK-1 function directly or via autocrine generation of IL-1.

Roles of Pellino-1 in proinflammatory responses of primary human bronchial epithelial cells. Having determined that Pellino-1 was expressed in a human epithelial line and regulated responses to both IL-1 and a mimic of viral infection, via a mechanism independent of IRAK-1, we elected to investigate the role of Pellino-1 in the control of responses of primary human airway epithelial cells. First, we determined that siRNA transfection of PBECs induced marked knockdown of Pellino-1 expression compared with cells transfected with a scrambled control (Fig. 2). We transfected PBECs with Pellino-1-targeting siRNA or the scrambled control and treated the cells with proinflammatory mediators whose signaling was dependent on or independent of IRAK-1. Figure 4 shows that, similarly to results seen in BEAS-2B cells, the induction of CXCL8 by TNF-α was unaffected by Pellino-1 knockdown. However, in contrast to results seen in BEAS-2B epithelial cells, the responses of PBECs to IL-1, a classical activator of human airway epithelial cells, were not affected by Pellino-1 siRNA pretreatment. Despite the lack of effect on IL-1 signaling, Pellino-1 knockdown markedly inhibited the responses of PBECs to poly(I·C) such that induction of CXCL8, but not induction of CCL5, was nearly completely inhibited. These data again reinforced the evidence showing that Pellino-1 could regulate re-
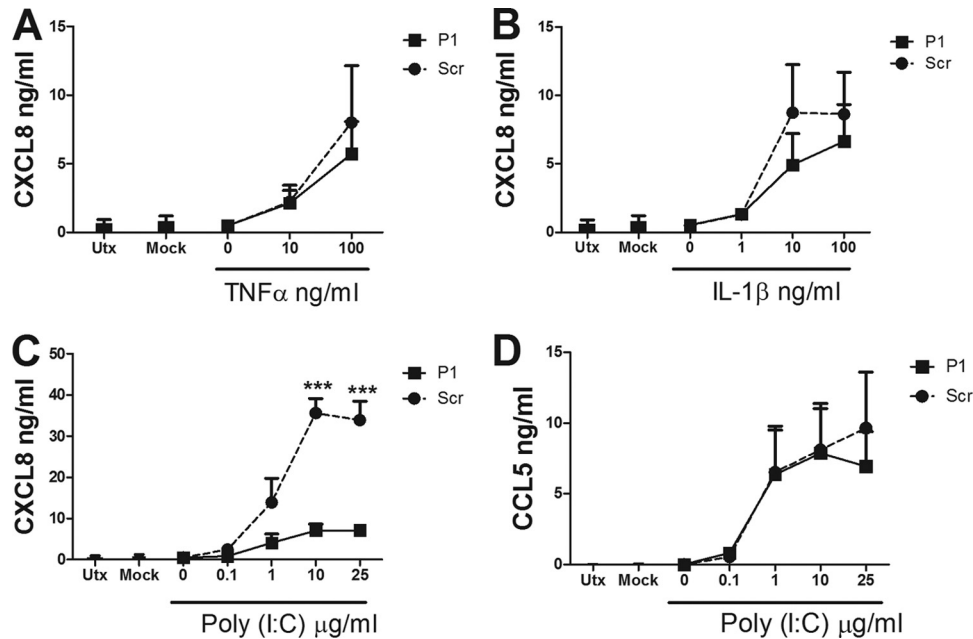
FIG 4 Cytokine generation in Pellino-1 knockdown PBECs in response to TNF-α, IL-1β, and poly(I·C) stimulation. PBECs were transiently transfected with siRNA targeting Pellino-1 (P1) or a nontargeting scrambled siRNA (Scr) and were treated for 24 h with media (Mock) or a response concentration of TNF-α (A), IL-1β (B), or poly(I·C) (C). Poly(I·C)-treated cells were also analyzed for CCL5 generation (D). Untransfected (Utx) cells treated with media only were also included. Supernatants were collected and analyzed for CXCL8 or CCL5 generation by ELISA. Data are expressed as means ± SEM of the results determined with samples collected from 3 independent donors. Statistical analysis was carried out by one-way ANOVA with Bonferroni's posttest, comparing Scr to P1 for each concentration (***, $P < 0.001$).

sponses of airway epithelial cells to important proinflammatory stimuli through control of a pathway that was independent of IRAK-1. Inhibition of responses to poly(I·C) was not confined to inhibition of CXCL8 production, since analysis of supernatants from poly(I·C)-challenged cells by cytokine bead array revealed that generation of several cytokines associated with innate immunity also appeared to be impaired by Pellino-1 knockdown, though these data did not reach statistical significance (Table 1).

TABLE 1 Cytokine generation in Pellino-1 knockdown PBECs in response to poly(I·C) stimulation[a]

Cytokine	Mean level (pg/ml) ± SEM		% decrease
	Scrambled	P1 knockdown	
TNF-α	250.9 ± 109.5	25.13 ± 8.636	90
GM-CSF	411.1 ± 87.13	81.41 ± 14.05	80.2
CCL2	14.73 ± 10.67	4.597 ± 2.015	68.8
CCL4	923.7 ± 457.7	412.4 ± 192.1	55.4
IL-6	4,025 ± 1,034	2,122 ± 326.3	47.3
CCL3	169.9 ± 83.27	94.55 ± 43.98	44.3
IL-1β	28.18 ± 15.37	25.36 ± 11.00	10
CX3CL1	364.0 ± 119.8	366.8 ± 72.98	−0.8
IL-10	1.130 ± 0.5730	1.253 ± 0.6326	−10.6
IL-1α	98.99 ± 59.17	100.7 ± 57.37	−21.1

[a] PBECs were transiently transfected with siRNA targeting Pellino-1 (P1) or a nontargeting scrambled siRNA (Scr) and were treated for 24 h with poly(I·C) (25 μg/ml). Supernatants were collected and analyzed for cytokine generation by cytokine bead array (except for IL-6, which was measured by ELISA). Data are expressed as means ± SEM or percent decrease of cytokine generation by P1 knockdown cells compared with the scrambled transfected control. Supernatants were collected from 3 independent donors. Differences between the results determined for Scr- and P1-targeted cells did not reach statistical significance (Student's t test).

The effects of Pellino-1 are not mediated by RIP1 in primary human bronchial epithelial cells. Concurrently with our studies, a Pellino-1 knockout mouse was generated. Similarly to our initial findings, the seminal study of this mouse showed that absence of Pellino-1 was associated with impaired induction of NF-κB-dependent cytokines in response to poly(I·C) (2). This study identified the protein RIP1 as a binding partner of Pellino-1 and led to the hypothesis that regulation of RIP1 function might be the point at which Pellino-1 regulates NF-κB activation (2). We therefore used siRNA to knock down RIP1 in PBECs and determined whether this recapitulated the phenotype seen in Pellino-1 knockdown cells. The data in Fig. 5 show that RIP1 knockdown did not alter CXCL8 induction in response to TNF-α or IL-1. Surprisingly, RIP1 knockdown was associated with enhancement of CXCL8 generation in response to poly(I·C). Thus, these data identify the Pellino-1 binding partner RIP1 as a negative regulator of induction of CXCL8 by poly(I·C) in human epithelial cells.

Differential regulation of NF-κB signaling in BEAS-2B and primary bronchial epithelial cells. In order to explore the signaling cascades potentially regulated by Pellino-1, we examined induction of NF-κB signaling in response to poly(I·C) and the control agonist IL-1, noting that CXCL8 production is very dependent on the NF-κB pathway. Treatment of BEAS-2B cells with IL-1 induced rapid activation of NF-κB, as determined by demonstration of a rapid loss of I-κBα (Fig. 6). Similarly, poly(I·C) stimulation of BEAS-2B cells also induced rapid NF-κB activation. In contrast, while IL-1 stimulation also activated NF-κB in PBECs, poly(I·C) did not induce I-κBα breakdown over short or long time courses despite being a potent inducer of CXCL8 production in these cells (Fig. 4). These data suggested that poly(I·C)
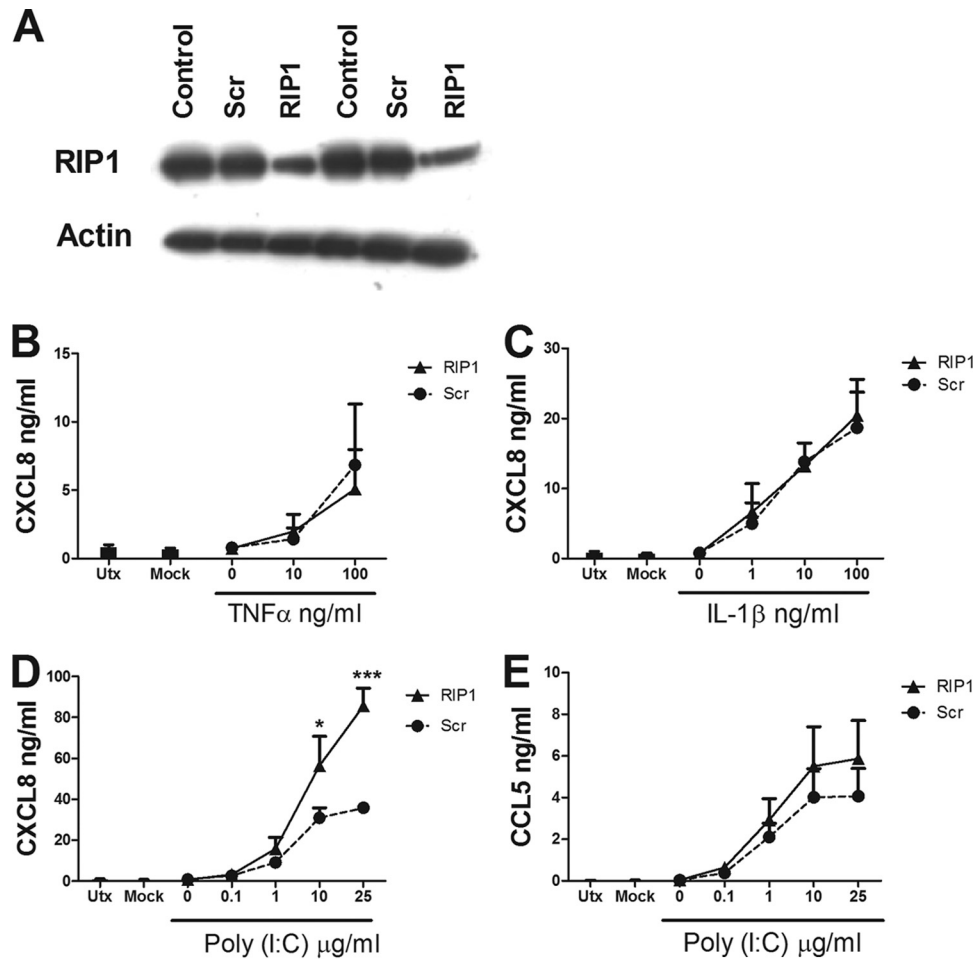
FIG 5 Cytokine generation in RIP1 knockdown PBECs in response to TNF-α, IL-1β, and poly(I·C) stimulation. PBECs were treated with media (Control) or transiently transfected with siRNA targeting RIP1 (RIP1) or a nontargeting scrambled siRNA (Scr). Cells were lysed and immunoblotted using antibodies against either RIP1 or actin. Panel A shows a blot representative of 2 independent experiments performed with samples from a single donor. Cells were treated for 24 h with media (Mock) or a response concentration of TNF-α (B), IL-1β (C), or poly(I·C) (D). Poly(I·C)-treated cells were also analyzed for CCL5 generation (E). Untransfected (Utx) cells treated with media only were also included. Supernatants were collected and analyzed for CXCL8 or CCL5 generation by ELISA. Data are expressed as means ± SEM of the results determined in 3 independent experiments performed with samples from a single donor. Statistical analysis was carried out by one-way ANOVA with Bonferroni's posttest, comparing Scr to P1 for each concentration (*, $P < 0.05$; ***, $P < 0.001$).

did not utilize the canonical NF-κB pathway and thus that the site at which Pellino-1 regulated NF-κB signaling must also lie outside this pathway, potentially operating through control of noncanonical NF-κB signaling.

Pellino-1 regulates responses of human bronchial epithelial cells to rhinovirus. Poly(I·C) is a mimic of viral infection, and our data presented above suggested that responses to viral infection may be regulated by Pellino-1. However, poly(I·C) stimulation does not recapitulate the full phenotype of viral activation of cells, since it does not replicate inside the cells. Roles of Pellino-1 in responses to human pathogens have not been described, and the roles of Pellino-1 have for the most part been studied in cell lines. Our data indicated that the role of Pellino-1 in the human airway might be to regulate responses to respiratory viruses. We therefore transfected PBECs with siRNA targeting Pellino-1, or a scrambled control, and infected these cells with various TCID$_{50}$s of the human pathogen rhinovirus RV-1B in accordance with previous work from our group (29). Strikingly, and similarly to results seen when PBECs were stimulated with poly(I·C), we found that Pel-

lino-1 knockdown reduced the induction of CXCL8 and IL-6 by rhinoviral infection, without impairing induction of the interferon-stimulated genes CCL5 and CXCL10 (Fig. 7).

Antiviral responses are preserved in Pellino-1 knockdown cells. Importantly, no increased rhinovirus-induced cell death was observed in Pellino-1 knockdown cells (data not shown), and these data, together with those demonstrating the preservation of the induction of CCL5/CXCL10 in virally infected cells, suggested that IFN production in response to viral infection was preserved. We confirmed this hypothesis by measuring rhinovirus-induced IFN-β and IFN-λ production by qPCR. No inhibition of production of these crucial antiviral defense proteins was caused by Pellino-1 knockdown (Fig. 8). Previously, we observed that MyD88 knockdown was associated with decreased responses to rhinoviral infection and increased viral replication (29), but here, and in keeping with our finding that Pellino-1 in primary human cells was not targeting IL-1 signaling pathways, Pellino-1 knockdown was not associated with increased viral replication, as measured by qPCR and measurement of active viral particle production (Fig. 9).
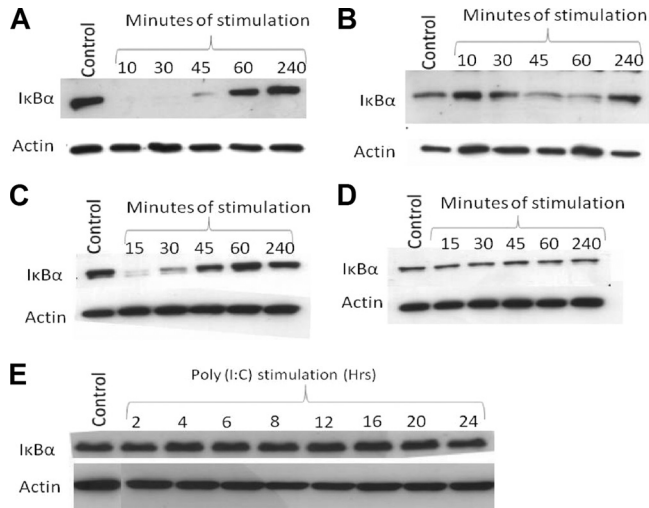
FIG 6 IκBα degradation in response to IL-1β and poly(I·C) stimulation in BEAS-2B cells and PBECs. BEAS-2B cells (A and B) and PBECs (C to E) were stimulated with either IL-1β (50 ng/ml) (A and C) or poly(I·C) (25 μg/ml) (B and D) for 10 or 15, 30, 45, 60, and 240 min, and the cells were lysed and immunoblotted for IκBα and actin control. Three independent experiments were carried out for each blot (PBECs were from a single donor). Panel E shows a blot representative of PBECs that were stimulated with poly(I·C) (25 μg/ml) for 2, 4, 6, 8, 12, 16, 20, and 24 h; the cells were lysed and immunoblotted for IκBα and actin control ($n = 2$ from a single donor). The actin samples represented in this blot were not loaded in the same order as those represented in the IκBα blot. In order to correctly vertically align the samples, the actin control lane was cut and pasted.

Pellino-1 regulates proinflammatory responses in airway epithelial cells derived from asthmatic patients. Having determined that Pellino-1 knockdown PBECs exhibit significantly reduced CXCL8 in response to viral stimuli [poly(I·C) and RV-1B], despite unchanged CCL5 production, we then wanted to investigate the effect of Pellino-1 knockdown on PBECs isolated from asthmatic patients. First, we determined that siRNA transfection of asthmatic PBECs induced marked knockdown of Pellino-1 expression compared with the results seen with cells transfected with a scrambled control (Fig. 10). We transfected asthmatic PBECs with Pellino-1-targeting siRNA or the scrambled control and treated the cells with proinflammatory mediators. Figure 10 shows that, similarly to results seen in normal PBECs, the induction of CXCL8 by TNF-α and IL-1β was unaffected by Pellino-1 knockdown. Also, Pellino-1 knockdown markedly inhibited the responses of asthmatic PBECs to poly(I·C) such that induction of CXCL8, but not induction of CCL5, was significantly reduced. These data again support the evidence that Pellino-1 could regulate responses of airway epithelial cells to important proinflammatory stimuli not only in normal PBECs but also in cells in the diseased state.

DISCUSSION

In this first study of the roles of Pellino-1 in the control of human pathogens, we have identified Pellino-1 as a novel, critical, and selective regulator of inflammatory responses to rhinovirus-1B in human epithelial cells. Targeting of Pellino-1 in these cells generates a specific defect in induction of non-IFN cytokines, without evidence of impaired antiviral responses, and suggests that Pel-
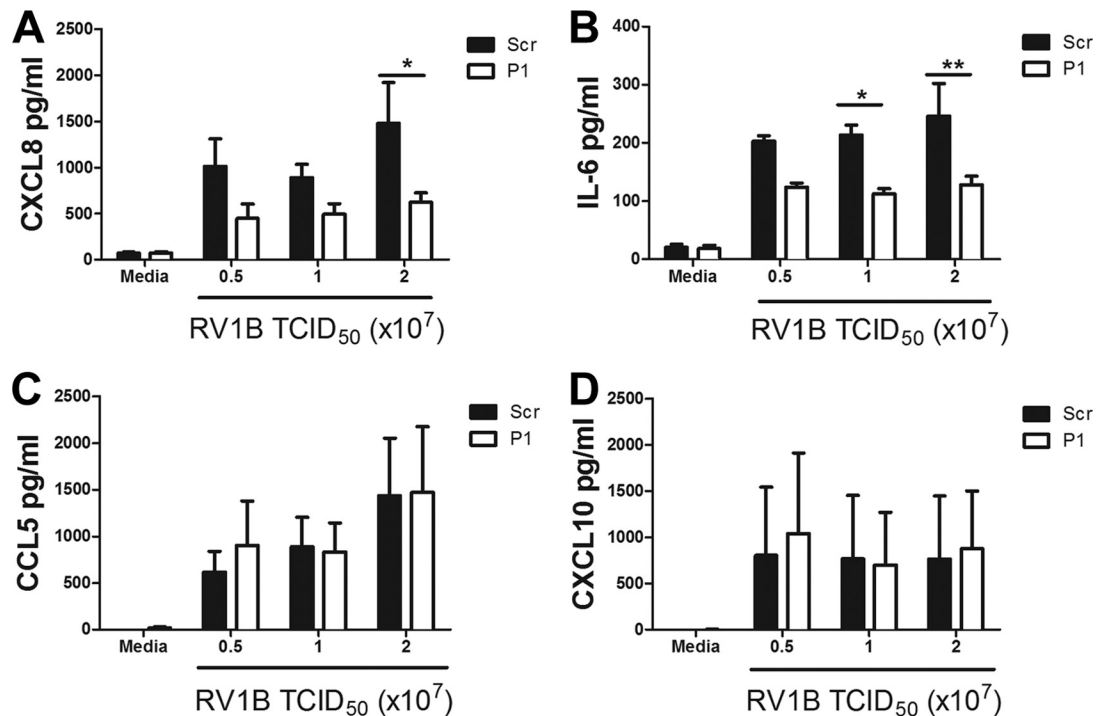

FIG 7 Cytokine generation in Pellino-1 knockdown PBECs in response to rhinovirus infection. PBECs were transiently transfected with siRNA targeting Pellino-1 (P1) or a nontargeting scrambled siRNA (Scr) and were infected with different TCID₅₀s of RV-1B for 1 h, following which supernatants were replaced with media and CXCL8 (A), IL-6 (B), CCL5 (C), and CXCL10 (D) generation was measured by ELISA after a further 24 h. Data are expressed as means ± SEM of the results determined in 3 independent experiments performed with samples from a single donor. Statistical analysis was carried out by two-way ANOVA with Bonferroni's posttest, comparing Scr to P1 (*, $P < 0.05$; **, $P < 0.01$).
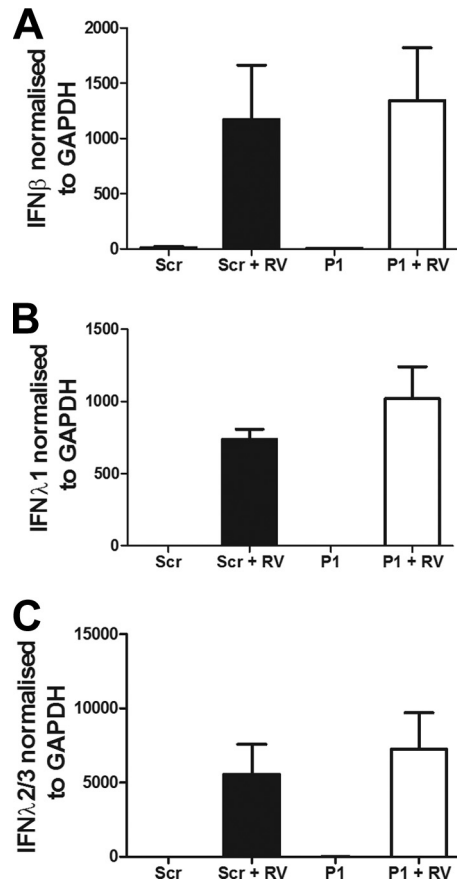
FIG 8 Interferon expression in response to rhinovirus in Pellino-1 knockdown PBECs. PBECs were transiently transfected with siRNA targeting Pellino-1 (P1) or a nontargeting scrambled (Scr) siRNA and were infected with different TCID$_{50}$s of rhinovirus1B (RV-1B) for 1 h, following which supernatants were replaced with media for a further 24 h. IFN-β (A), IFN-λ1 (B), and IFN-λ2/3 (C) mRNA expression was measured by qPCR. Data are expressed as arbitrary units of means ± SEM of IFN expression normalized to GAPDH expression.
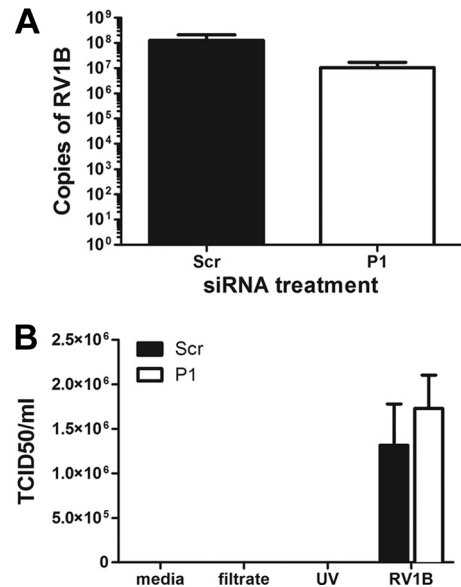

FIG 9 Viral replication in Pellino-1 knockdown PBECs. PBECs were transiently transfected with siRNA targeting Pellino-1 (P1) or a nontargeting scrambled (Scr) siRNA and were infected with rhinovirus 1B (RV-1B) at a TCID$_{50}$ of 2×10^7 for 1 h, following which supernatants were replaced with media for a further 24 h. RV-1B replication in cell lysates was measured by qPCR, and data are expressed as means ± SEM on a log$_{10}$ scale (A). Viral particle release into the supernatant was quantified using a viral CPE assay (B). Data were collected from 3 (qPCR) or 4 (CPE assay) independent experiments performed with samples from a single donor.

lino-1 may be a useful target in the control of airway inflammation.

We have previously shown, using *in vitro* models of airway and vascular inflammation (3, 18, 19, 29, 30), that responses to bacterial and viral stimuli are variously dependent upon IL-1 production and the MyD88 signaling pathway. We have shown that these networks are variously targetable by inhibition of TLR signaling with novel compounds targeting lipid rafts (22) or by inhibition of the IL-1/MyD88 pathway with IL-1 antagonists or relevant siRNAs (18, 19, 29, 30). However, inhibitors of the actions of apical cytokines such as IL-1 have the potential to be relatively blunt tools, and we have observed that MyD88 knockdown is associated with increased viral replication in epithelial cells in the control of viral infection (29). Accordingly, we sought to determine if other components of these pathways might yield more specific therapeutic targets for potential control of airway inflammation. Our interest was captured by Pellino-1. This protein has E3 ubiquitin ligase activity and is thought to activate IRAK-1 through K63 ubiquitination (1, 9, 20, 21, 25). Concurrently with our studies, a mouse knockout was generated that revealed a role for Pellino-1 not in IRAK-1 function but in control of TLR3-mediated activation of NF-κB (2). That study did not examine responses to native pathogens, only responses to the double-stranded RNA mimic poly(I·C), and concluded that Pellino-1 was likely mediating its effect through regulation of RIP1, a key protein bridging TLR3 and IκB kinase (IKK) complex activation (2). Very recently, Pellino-1 was shown to be phosphorylated by TBK1/IKKε (signaling pathways regulated by TLR3 and culminating in IFN generation) and was also shown to be an IRF3 gene target (27). Thus, Pellino-1 can regulated by the same pathways that culminate in IFN generation. However, there is an absence of information regarding the roles of Pellino-1 in the control of responses to pathogens in human cells and systems.

We observed that Pellino-1 was expressed in BEAS-2B epithelial cells and in primary human bronchial epithelial cells. Knockdown of Pellino-1 by the use of targeted siRNAs generated a phenotype of selective suppression of expression of cytokines such as CXCL8 and IL-6, with preservation of IFN generation and IFN-dependent cytokines such as CCL5 and CXCL10. Surprisingly, Pellino-1 appears to exert its actions in both normal and asthmatic primary human airway epithelial cells independently of regulation of IRAK-1, since knockdown of Pellino-1 did not impair IL-1 responses in PBECs but did impair responses to poly(I·C), which signals through TLR3 independently of IRAK-1 (9). These data are in part in keeping with work previously performed with the Pellino-1 knockout mouse, where poly(I·C) signaling was also found to be impaired (2). Further studies represented in Fig. 6 demonstrated that poly(I·C) and IL-1 were effective inducers of NF-κB activation in BEAS-2B cells, but in PBECs, poly(I·C) failed to induce activation of the canonical NF-κB pathway. We considered that poly(I·C) might be activating noncanonical NF-κB sig-
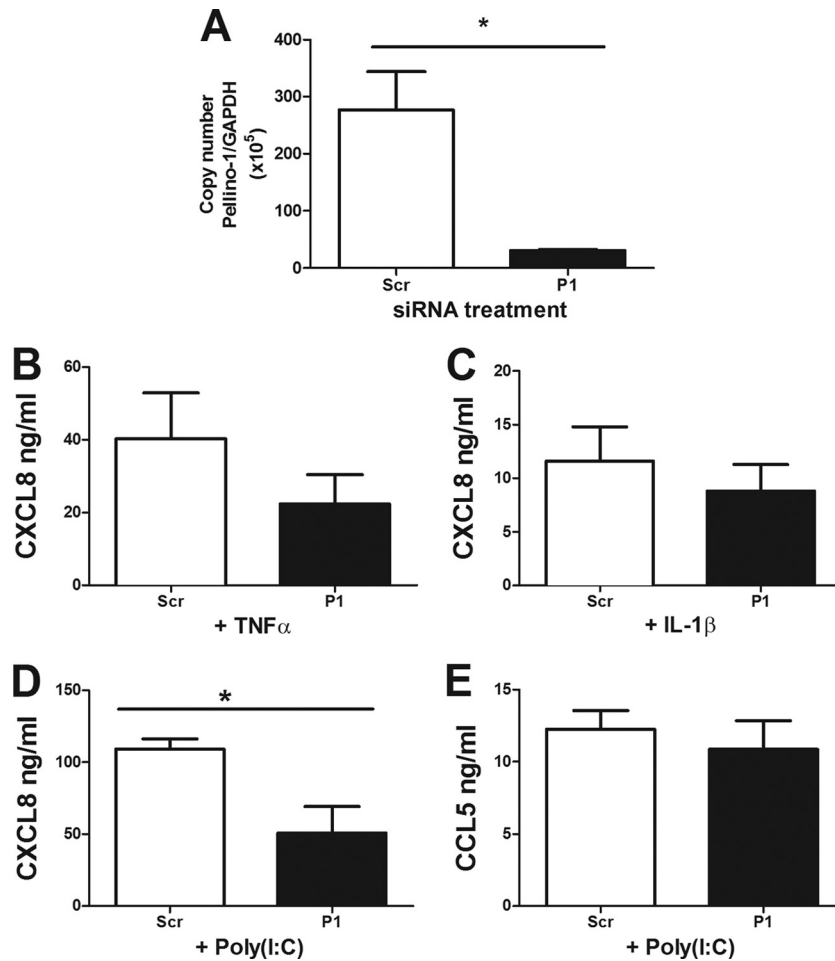
FIG 10 Cytokine generation in Pellino-1 knockdown PBECs isolated from asthmatic patients in response to TNF-α, IL-1β, and poly(I·C) stimulation. (A) PBECs isolated from asthmatic patients were transiently transfected with siRNA targeting Pellino-1 (P1) or a nontargeting scrambled siRNA (Scr), and Pellino-1 mRNA expression levels in Scr and P1 PBECs that had been stimulated with 10 μg/ml poly(I·C) were determined by qPCR. Pellino-1 copy numbers were normalized to GAPDH copy numbers as a loading control. (B to E) Scr- and P1-transfected PBECs were treated for 24 h with media (control) or a response concentration of TNF-α (B), IL-1β (C), or poly(I·C) (D and E). Supernatants were collected and analyzed for CXCL8 (B to D) or CCL5 (E) generation by ELISA. Data are expressed as means ± SEM of the results determined with samples collected from 3 independent patients. Statistical analysis was carried out using Student's *t* test (*, $P < 0.05$).

naling via NIK, which could therefore be the target for Pellino-1 regulation and which has proven roles in the responses of epithelial cells to airway viruses (4, 15). If correct, this hypothesis could also provide an explanation for the observation that CXCL8 production is inhibited by Pellino-1 knockdown to a greater degree in the primary cells than in BEAS-2B cells, since in the BEAS-2B cells, classical NF-κB signaling is likely to be preserved. To address this, we attempted NIK knockdown by siRNA but were unable to verify effective knockdown, and work to examine the potential role of noncanonical NF-κB signaling is ongoing in our group.

Chang and colleagues identified RIP1 as a Pellino-1 binding partner that lies between TLR3 and TRIF and is responsible for activation of cytokine production (2). We therefore knocked down RIP1 in PBECs to see if this would recapitulate the phenotype of Pellino-1 knockdown. To our surprise, RIP1 knockdown generated a phenotype opposite that which we expected, with enhancement of poly(I·C)-induced CXCL8 production. While these data indicate that RIP1 lies within the pathways regulating responses to poly(I·C) in primary airway epithelial cells, it does not

identify the point in the signaling pathways that is regulated by Pellino-1. Although RIP1 is usually thought to be involved in activation of NF-κB signaling via interaction with the TRIF signaling pathway (5, 17) and IKK complex (13), we note that RIP1 has also been shown very recently to negatively regulate activation of noncanonical NF-κB by TNF-α signaling (13) and also to negatively regulate the IFR3 induction downstream of RIG-I activation (23). In the latter study, activation of RIP1 was shown to render it a target for caspase 8, generating RIP1 fragments that inhibited IRF3 signaling (23). Our data raise the possibility that inhibitory actions of RIP1 may extend to both the IRF3- and NF-κB-dependent components of signaling responses to poly(I·C) through mechanisms as yet uncharacterized but potentially involving potentiation of signaling via noncanonical NF-κB (13).

Following identification of a role for Pellino-1 in the regulation of responses to the viral mimic poly(I·C), we conducted the first investigation of the roles of Pellino-1 in the responses to a human viral pathogen. We observed that Pellino-1 knockdown inhibited CXCL8 and IL-6 production in rhinovirus-infected cells without

inhibiting generation of IFNs or IFN-stimulated genes such as CCL5 and CXCL10 and without affecting either epithelial cell viability or rates of viral replication in infected cells. Pellino-1 knockdown had no effect on the small responses seen to UV-irradiated virus (data not shown). As yet, the target of Pellino-1 in PBECs by which cytokine production is modified remains unclear. Neutrophilic inflammation is common in viral exacerbations of airway disease, and neutrophils serve only very limited antiviral roles. These data, and particularly the significant reduction in CXCL8 in response to poly(I·C) in Pellino-1 knockdown PBECs isolated from asthmatic patients, indicate that therapeutic targeting of Pellino-1, by manipulating its expression or its activation by phosphorylation (27, 28) or by manipulation of its downstream target, may limit unwanted neutrophilic inflammation in the airways.

ACKNOWLEDGMENTS

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