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Parainfluenza type-4 infection of NCI-H292 airway epithelial-like cells amplifies the IL-8 response to TNF-α

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Abstract

The mechanisms by which viral airway infections potentiate airway inflammation are not fully understood. Expression of the pro-inflammatory mediators IL-8 and, to a lesser extent, IL-6 by respiratory syncytial virus (RSV)-infected airway epithelial cells has been studied in detail, but comparative data for other negative-sense single-stranded RNA viruses are missing. In addition, it is not known whether the IL-8 and IL-6 responses to a second stimulus is altered in virus-infected cells. Therefore, regulation of IL-8 and IL-6 expression by parainfluenza type 4 virus (PIV 4)-infected NCI-H292 cells was studied. PIV 4 rapidly induced sustained DNA-binding activities for NF-κB and AP-1, implicated in IL-8 gene transcription. NF-κB DNA-binding activity was biphasic and correlated with the biphasic IL-8 mRNA expression. The second phase of IL-8 mRNA expression, which appears due to viral replication, corresponded also with a transiently reduced IL-8 mRNA degradation. PIV 4-induced IL-6 mRNA expression was also biphasic and depended on a sustained C/EBP DNA-binding activity and a reduced IL-6 mRNA degradation. The dose-response curves for IL-8 and IL-6 with TNF-α as a stimulus are indicative of exaggerated responses by PIV 4-infected NCI-H292 cells. The sustained transcriptional activity and prolonged half life of IL-8 and IL-6 mRNA may at least in part, potentiate airway inflammation induced by parainfluenza type 4 and likely other negative-sense single-stranded RNA viruses.

Introduction

Viral infections of the respiratory tract can induce acute but transient inflammatory responses and airway hyperresponsiveness in healthy persons [1,2]. Also, exacerbations of asthma and chronic obstructive pulmonary disease (COPD) are associated with viral airway infections [3,4]. For atopic asthmatic subjects this was corroborated in a study with experimental rhinovirus-16 infection, where an increase in airway hypersensitivity was paralleled by an increased release of the pro-inflammatory mediator IL-8 [5]. These and other findings have led to the consideration that viral airway infection potentiates airway inflammation and, possibly indirectly, airway hyperresponsiveness.

The mechanisms by which viral airway infections flare up airway inflammation are not fully understood, and may be determined at large by the infecting viral species. The majority of respiratory viruses belong to the single-stranded RNA viruses, comprising both positive-sense RNA viruses (rhino- and corona-viruses) as well as negative-sense RNA...
viruses (respiratory syncytial, influenza and parainfluenza viruses) [6]. All of these single-stranded RNA respiratory viruses target airway epithelial cells (AEC), which are considered an important local source of pro-inflammatory mediators such as IL-8 and IL-6. The mechanism by which respiratory syncytial virus (RSV) induce pro-inflammatory mediators has been studied in great detail, particularly the induction of IL-8 in A549 cells, i.e. alveolar type II-like cells [7-14]. RSV induced both IL-8 and IL-6 [8,9]. IL-8 production was regulated transcriptionally and depended on the transcription factors NF-κB and C/EBP [10,11], or AP-1 instead of C/EBP [12]. No changes in IL-8 mRNA degradation were observed, at least not shortly after infection [7]. Surprisingly, NF-κB activity remained high, which was a consequence of viral replication [13,14]. In apparent contradiction with the sustained NF-κB activity, IL-8 mRNA expression was biphasic with a markedly reduced IL-8 mRNA expression 6 h after infection [7]. Whether the regulation of IL-8 expression by RSV is representative of that by the other negative-sense single-stranded RNA viruses is unknown. Given the similar pathophysiology evoked by negative-sense single-stranded RNA viruses, a detailed study into the regulation of IL-8 expression in airway epithelial cells infected with another negative-sense RNA-virus may identify relevant underlying mechanisms.

The increased airway inflammation during viral airway infection is, at least in part, explained by virus-induced IL-8 and IL-6 production of airway epithelial cells. Whether viral infection affect airway epithelial cells so that the regulation of the IL-8 and IL-6 response is modulated, is unknown. Our previous studies [15-17] into IL-8 and IL-6 mRNA and protein expression by NCI-H292 airway epithelial-like cells, have revealed that IL-8 and IL-6 mRNA degradation is an important means to limit IL-8 and IL-6 production. More importantly, a reduced IL-8 and IL-6 mRNA degradation resulted in exaggerated IL-8 and IL-6 responses to stimuli, as indicated by leftward-shifted and steeper dose-response curves [17, van Wissen et al, submitted]. Similar findings were obtained with Calu-3 cells, another polarized airway epithelial cell line, and in primary human bronchial epithelial cells [17]. Thus, a reduced IL-8 and IL-6 mRNA degradation in airway epithelial cells may potentiate airway inflammation. At present, there are no data to suggest that virus infection of airway epithelial cells reduce IL-8 mRNA degradation. A recent study with RSV, however, indicated that RANTES was induced at least in part by a reduced degradation of the encoding mRNA [18].

Here we report our studies into the mechanisms involved in IL-8 and IL-6 induction by the negative-sense RNA virus, parainfluenza type 4 (PIV 4). A second objective of the study was to determine whether infection of NCI-H292 cells with PIV 4 had an effect on IL-8 and IL-6 responses induced by a second stimulus. Like for RSV, the IL-8 and also the IL-6 mRNA
response to PIV 4 infection were biphasic, which may be due, at least in part, by modulation of IL-8 and IL-6 mRNA degradation. In addition, we show that viral infection results in an exaggerated IL-8 and, to a lesser extent, IL-6 response to a second stimulus.

Materials and Methods

Cell culture
NCI-H292 cells (CRL 1848; ATCC, Rockville, MD, USA), a human lung-derived mucoepidermoid adenocarcinoma cell line, was maintained as described previously [16].

Viral stock
Parainfluenza virus type 4a (strain M-25) was obtained from the ATCC (VR-1378). For experiments we have used both ATCC viral stocks as well as stocks prepared ourselves. To that end, 50 to 80% confluent NCI-H292 cells in 250 cm² culture flasks were washed with phosphate-buffered saline (PBS; pH 7.4), and exposed for 5 min to PBS containing 1.5 µg/ml of trypsin. Subsequently, medium was removed and cells were exposed to 5 ml of PBS containing 40 µl viral stock. After 1 h at room temperature, medium without FCS, but with 1.5 µg/ml of trypsin was added and left for 48 h. Then medium was removed and monolayers were washed 3 times with warm PBS. Three ml of culture medium without trypsin and FCS were added and cells were disrupted by three cycles of freezing and thawing. Cellular debris was removed by low speed centrifugation in a table centrifuge (3000 rpm, 10 min). Aliquots of clarified supernatants were stored at -80°C until use.

These stocks of virus were used throughout this study. Infection of NCI-H292 cells with PIV 4 induced a weak cytopathic effect (large multi-nucleated cells that became less adherent) but usually only after more than a week of infection. The induction of IL-8 and IL-6 shortly after infection were taken as internal controls for uniformity of the viral stocks. PIV 4 stocks were confirmed to induce the aggregation of guinea pig erythrocytes.

Experimental set up
NCI-H292 cells were grown to 50 to 80% confluency. Medium was removed, and cells were washed twice with warm PBS. Then, monolayers were exposed for 1 h at room temperature to PIV 4 (0.1 ml of the viral stock/cm²) in PBS. Subsequently, medium with FCS was added and experiments were carried out as indicated. Culture supernatants were stored at -20°C until IL-6 and/or IL-8 was determined. The cell layers were inspected by light
IL-8 and IL-6 induction by PIV 4 in NCI-H292 cells

Microscopy for morphological aspects. None of the conditions resulted in shedding of adherent cells or morphological changes. Each condition was tested in triplicate and experiments were carried out at least three times unless specified otherwise.

In some experiments, NCI-H292 cells were exposed to ultraviolet (UV)-irradiated viral stocks, in which virus at 0-4°C was exposed for 5 to 30 min at 5 cm distance from a 240 nm UV source.

**Assays for IL-8 and IL-6 protein and mRNA**

IL-8 and IL-6 cytokines were quantified by ELISA, as described [15,16]. IL-6, IL-8 and GAPDH mRNA were assessed on Northern and dot blots as described [15,16]. mRNA stability experiments were performed using the transcription inhibitor Actinomycin D (Boehringer Mannheim, Mannheim, Germany) at a final concentration of 10 μg/ml.

**Detection of PIV 4 antigen**

NCI-H292 cells exposed to PIV 4 were scraped with a rubber policeman from the tissue-culture plate and brought onto a microscope slide. After air drying, cells were fixed in acetone at room temperature for 10 min. Aspecific binding sites were blocked with 1% (v/v) normal goat serum in PBS. After 10 min, cells were washed twice with PBS and incubated with 1 in 10 dilution of mouse anti-parainfluenza 4 (MAB877; Chemicon, Veenendaal, The Netherlands) for 30 min at 37°C. After two wash steps with PBS, cells were incubated for 30 min with goat anti-mouse IgG-FITC (Fab) 

**Transfection**

NCI-H292 cells at 70% confluence were transfected transiently with 3 μg of chloramphenicol acetyltransferase (CAT) reporter vectors using the calcium/phosphate precipitation method as reported before [15,16]. Forty hours after transfection, medium was removed and the monolayers were infected with PIV 4 or not. After 8 h, monolayers were washed twice with cold PBS and cytoplasmic proteins extracted using an hypotonic cell lysis buffer containing Triton X-100. Cell debris was removed by centrifugation. Soluble protein was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). CAT protein was determined with ELISA (Boehringer Mannheim) and expressed to total protein.

The series of 5' deleted IL-6 promoter CAT constructs were obtained from Drs. K. Wong and W.M. Rom (Bellevue Hospital Center, New-York) [15]. The IL-8 promoter CAT
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constructs were obtained from Drs N. Mukaida and K. Matsushima (Cancer Research Institute, Kanazawa, Japan) [16].

Nuclear extract preparation and EMSAs

Nuclear extracts were prepared as described before [15,16]. Protein recovery was measured with the Bio-Rad Protein Assay kit (Hercules, CA, USA). Four μg of nuclear extracts (in 10 μl) were incubated 15 min at room temperature with 1 μl of radiolabeled oligonucleotide probes (~0.25 ng, ~25000 cpm), 7 μl of a buffer composed of 20 mM Hepes pH 8.0, 50 mM KCl, 0.5 mM EDTA, 1 mM DTT, 0.1% (v/v) NP40, 0.5 mM PMSF, 1 mg/ml bovine serum albumine, 5% (v/v) glycerol and 2 μl of 1 mg/ml poly(dl-dC)-poly(dl-dC) (Pharmacia, Uppsala, Sweden). Reaction mixtures were separated on 4% non-denaturing polyacrylamide gels at 230 V at room temperature in 0.5 X TBE buffer. After drying, gels were exposed to X-ray films. Oligonucleotides used in EMSA were the following: NF-κB: 5'-ATCGTGGAATTTCCTCTGAC-3'; AP-1: 5'-GTGTGATGACTCAGGTTTGG-3', distal C/EBP: 5'-TCACATCGCAATCTTT-3'; proximal C/EBP: 5'-AAGATTTATCAAATGTG-3'.

Results

PIV 4 induces IL-8 and IL-6 release by H292 cells

Studies by Castells and colleagues [19] have shown that parainfluenza viruses readily infect and replicate in NCI-H292 cells. In the present study we used parainfluenza type 4, which causes no cytopathic effects during the first 96 h after exposure to NCI-H292 cells (not shown).

NCI-H292 cells were exposed to serial dilutions of the PIV 4 stock (see Material and Methods), and IL-8 and IL-6 release over 24 h were determined (Figure 1). Exposure to PIV 4 induced the release of both IL-8 and IL-6 in a dose-dependent manner. Exposure to heat-inactivated PIV 4 (30 min, 56°C) did not increase the release of IL-8 and IL-6 as compared to cells not exposed to virus. The PIV 4 stock was obtained by freeze-thawing of infected NCI-H292 cells, and thus lysate also contains cytoplasmic contents. Lysate of non-infected cells, as well as supernatant from a PIV 4 stock which was cleared from viral particles by centrifugation (90 min at 50,000 x g), did not induce IL-8 and IL-6 above basal levels (data not shown). These results indicate that live PIV 4 triggers the induction of IL-8 and IL-6, but this does not imply that NCI-H292 cells were actually infected by PIV 4.
IL-8 and IL-6 induction by PIV 4 in NCI-H292 cells

**Figure 1.** Production of IL-8 and IL-6 by NCI-H292 cells exposed to various doses of PIV 4. NCI-H292 cells were exposed to PIV 4 and heat-inactivated PIV 4 (HI PIV, 1 in 10 dilution tested only). 24 h after initial exposure, supernatants were collected and IL-8 and IL-6 were determined by ELISA. Values represent the mean ± SD of four experiments. *, p<0.05 as compared to control medium.

NCI-H292 cells exposed to PIV 4 displayed cytopathic effects, such as multi-nucleated cells, usually one week after exposure. Furthermore, at 24 h but not at 1 h after exposure to PIV 4, PIV 4-antigen was detected in NCI-H292 cells by immunofluorescence (Figure 2). This indicates that PIV 4 indeed infected and replicated in NCI-H292 cells.

In subsequent experiments we used a 1 in 10 dilution of PIV 4 stock, unless indicated otherwise. IL-8 and IL-6 release by H292 cells exposed to PIV 4 was followed over time (Figure 3). PIV induced a constant release of IL-8 and IL-6 greater than that of the control at 6 and 24 h. This continuous release was sustained for at least 32 h.
Figure 2. Time-dependent detection of PIV 4 antigen in NCI-H292 cells. NCI-H292 cells were exposed to PIV 4 (1 in 10 dilution) for 1 h (A) and 24 h (B). Cells were stained for PIV 4 antigen as described in Material and Methods. Isotype controls were negative.
**Biphasic IL-8 and IL-6 mRNA expression upon infection with PIV 4**

To assess whether the continuously increased IL-8 and IL-6 release was paralleled by increased IL-8 and IL-6 mRNA steady state levels we determined the respective mRNAs in PIV 4-infected cells over time (Figures 4A and B). PIV 4 induced a fast but transient increase of IL-8 and IL-6 mRNA levels, peaking at 1 h. At 4 h after initial exposure, IL-8 and IL-6 mRNA levels dropped to near basal levels. Subsequently, IL-8 and IL-6 mRNA levels increased transiently, peaked around 8 h and returned to basal levels between 16 to 24 h after initial exposure. The maximal mRNA fold-induction for the second peak was lower than for the first peak, especially for IL-6 mRNA (n = 3). Heat inactivation of PIV 4 (HI PIV) prior to exposure almost fully abolished IL-8 and IL-6 mRNA induction, in agreement with no increased IL-8 and IL-6 production by heat-inactivated PIV 4 (Figure 1). UV irradiation disables replication of single-stranded RNA viruses. Exposure of NCI-H292 cells to UV-irradiated PIV 4 showed a normal first peak of IL-8 mRNA, but a markedly reduced second peak (Figure 4C).

Together these findings indicate that IL-8 and IL-6 mRNA expression are regulated by two events. The first event appears related to initial interactions between PIV 4 and NCI-H292 cells, and which is lost by heat-inactivation of the virus. The second event appears related to viral replication as both heat-inactivation and UV-irradiation markedly reduce the second peak of mRNA expression.
Figure 4. Time course of IL-8 and IL-6 mRNA expression in NCI-H292 cells infected with PIV 4. NCI-H292 cells were exposed to PIV 4 or heat-inactivated (HI PIV; 1 in 10 dilution). Total RNA was isolated at different time points and analysed. Northern blots were hybridized with IL-6, IL-8 and GAPDH probes (panel A). Signals obtained after hybridization were quantified using a Phospholmager. IL-8 and IL-6 mRNA levels normalized to GAPDH were expressed relative to that of resting cells (t = 0) (panel B). Similar results were obtained in three separate experiments. Panel C: As above, but NCI-H292 cells were exposed to PIV 4 and PIV 4 exposed to UV irradiation for 5, 15 and 30 min (see Materials and Methods).
Effect of PIV 4 on transcriptional activity of IL-8 and IL-6 promoters

The effect of PIV 4 on IL-8 and IL-6 gene transcription was assessed by transient transfection of NCI-H292 cells with CAT reporter gene constructs containing various truncated and mutated portions of human IL-8 and IL-6 promoters. Transfected cells were exposed for 8 h either to medium or to PIV 4, after which CAT expression was determined in cell lysates. As shown in Figure 5A for the IL-6 promoter, the maximal response to PIV 4 as obtained with the full length construct -1158-CAT was also found with -225-CAT construct, but lost with the -109-CAT construct. This indicated that the AP-1 site (-282/-276)-CAT was not involved in PIV-mediated IL-6 gene induction. And secondly, the relevant transcriptional sites were positioned in between position -224-CAT and -109-CAT from the IL-6 promoter. Disruption of the distal (-153/-145) or the proximal C/EBP (-83/-75) sites in the -224-CAT construct dramatically reduced CAT recovery, indicating that these two C/EBP sites played a major role in IL-6 gene transcription.

For the IL-8 promoter, the maximal response to PIV 4 was observed with the full length construct -546-CAT and the deletion construct -133-CAT. Further deletion of the AP-1 site in -94(Δ-70/-51)-CAT still resulted in CAT expression, although at a reduced level compared to that for the -546-CAT and -133-CAT constructs. CAT expression was abolished using -85-CAT and when the C/EBP site or the NF-κB site from -94(Δ-70/-51)-CAT were mutated. This indicated that a minimal element containing the NF-κB and the C/EBP sites was required for IL-8 gene transcription. Disruption of the NF-κB site in -133-CAT revealed that an intact NF-κB site was essential for transcription. In contrast, mutation in the C/EBP site of -133-CAT had no effect, whereas disruption of the AP-1 site slightly reduced CAT expression by PIV 4. This suggested that AP-1 was also involved in IL-8 gene activation, but that the C/EBP site could replace the AP-1 site when it was missing.

These findings indicate that PIV 4-induced IL-8 gene expression is dependent on NF-κB and either AP-1 or C/EBP, the latter two in order of dominance. PIV 4-induced IL-6 gene expression is strictly dependent on C/EBP. It is worth noting that PIV 4 increased CAT expression for both IL-8- and IL-6-promoter constructs only 2 to 3 times as compared to that in cells without exposure to PIV 4. This indicates that PIV 4 induces a modest increase of gene transcription only.
PIV 4 induces nuclear AP-1, C/EBP and NF-κB activities

Band shift assays with nuclear extracts obtained from NCI-H292 cells exposed to PIV 4 were performed to estimate relative levels of the relevant transcription factors. Specific DNA-protein complexes were detected in uninfected cells using oligonucleotides specific for NF-κB, C/EBP and AP-1 sites (Figure 6). Exposure to PIV 4 rapidly increased (t = 12 min) all of these complexes, particularly NF-κB and C/EBP (5-10 times), and to a lesser extent AP-1 (2-3 times). DNA-protein complexes were also high at 24 h after PIV exposure, which is surprising given that IL-8 and IL-6 mRNA expression had returned to basal levels (Figure 3).
IL-8 and IL-6 induction by PIV 4 in NCI-H292 cells

Figure 6. Increased NF-κB, C/EBP and AP-1 activation in the nucleus of NCI-H292 cells in response to PIV 4 infection. Nuclear extracts were prepared from NCI-H292 cells infected with PIV 4 (1 in 10 dilution) for different times. EMSA were performed with oligonucleotides specific for NF-κB, AP-1 and C/EBP sites as indicated in Material and Methods. Specific retarded complexes (indicated by an arrow) were identified by competition with unlabelled specific oligonucleotide (100x and 1x excess, 2 lanes on the right, respectively), but not with an irrelevant oligonucleotide (not shown). These results are representative of 4 to 6 experiments.

Only the levels of NF-κB appeared to parallel the induction of the second IL-8 mRNA peak between 4 to 8 h after exposure to PIV 4. The amount of C/EBP even decreased between 4 to 8 h after exposure to PIV 4, whereas that of AP-1 did not change. This suggests, particularly for IL-6 mRNA, that other than transcriptional processes are implicated in the induction of the second mRNA peak. Given the rapid degradation of IL-6 and IL-8 mRNA, modulation of mRNA degradation may have a large impact on mRNA steady state levels.

IL-8 and IL-6 mRNA stability in NCI-H292 cells exposed to PIV 4

To determine mRNA stability, mRNA synthesis was blocked in NCI-H292 cells with actinomycin D at various time points after exposure to PIV 4. Subsequently, IL-8 and IL-6 mRNA levels were followed over time (Figure 7).

The decrease of IL-8 and IL-6 mRNA was rapid at 1 h and 4 h after exposure to PIV 4, with an estimated half life for both mRNAs of 30 min. Six h after exposure to PIV 4, both
IL-8 and IL-6 mRNA were more stable, with a half life of more than 2 h and 4 h, respectively. Only for IL-8 mRNA we could reliably determine the decrease at 8 and 11 h after viral exposure. Whereas IL-8 mRNA is still more stable at 8 h, at 11 h the half life is similar to that at 1 h after exposure.

This transient stabilization of IL-8 mRNA and probably also for IL-6 mRNA, parallels the second peak of mRNA expression. This is intriguing, as earlier studies have shown that stabilization of IL-6 and IL-8 mRNA results in exaggerated IL-6 and IL-8 responses as reflected by leftward-shifted and steeper dose-response curves [17].

**Figure 7.** Modulation of IL-8 and IL-6 mRNA stability in NCI-H292 cells infected with PIV 4. NCI-H292 cells were infected with PIV 4 (1 in 10 dilution). At different times after infection (1, 4, 6, 8 or 11 h), actinomycin D (Act, 10 μg/ml) was added to the cultures and RNA recovered after an additional 0, 0.5, 1, 2 or 4 h of incubation. The extracted RNA was subjected to northern blot analysis. Blots were hybridized with IL-6, IL-8 and GAPDH probes. A typical result for IL-8 is shown in panel A. Signals obtained after hybridization were quantified using a Phospholmager. IL-8 and IL-6 mRNA levels normalized to GAPDH were expressed relative to the corresponding level before adding actinomycin (t = 0) and plotted as a function of time (panel B).
Exaggerated IL-8 and IL-6 responses by PIV 4-infected NCI-H292 cells

We analysed whether PIV 4-infected NCI-H292 cells exhibited an exaggerated response during the phase of mRNA stabilization (around 5 h after initial exposure). As a control we took NCI-H292 cells exposed to PIV 4 for 16 h. Figure 8 shows that 5 h after the addition of PIV 4, the IL-8 and IL-6 responses are exaggerated in response to TNF-α. At 16 h, the dose response curves by infected and noninfected NCI-H292 cells are very similar, although in some experiments we found a slightly exaggerated response by infected T cells.

Figure 8. IL-8 and IL-6 release by PIV 4-infected NCI-H292 cells exposed to a concentration range of TNF-α. NCI-H292 cells were either exposed to PIV 4 (1 in 10 dilution; – – –), or to no virus (O—O) for 5 (A,C) and 16 h (B,D), after which cells were exposed for 8 h to TNF-α. IL-8 (top panels) and IL-6 (lower panels) in supernatants were determined by ELISA. These results are representative of three experiments.

Discussion

Comparison of the mechanism by which parainfluenza type 4 (PIV 4) induces IL-8 expression in NCI-H292 cells with that of respiratory syncytial virus (RSV) in A549 cells reveals similarities, but also intriguing differences. Both of these negative-sense single-
stranded RNA viruses trigger the same transcription factors for IL-8 gene transcription, although there are contradictory results for RSV on the contribution of C/EBP and AP-1 [10,12]. NF-κB DNA-binding activity was induced within 12 to 15 min by both RSV [10] and PIV 4. With PIV 4, we found a biphasic regulation of the NF-κB DNA-binding activity, with a rapid increase and decrease within 30 min after the initial exposure, followed by a second and sustained increase up to 24 h after the initial exposure. Data from separate studies with RSV showed a similar biphasic NF-κB DNA-binding activity, with a transient peak of DNA-binding activity within 2 h after initial exposure [10], and again higher NF-κB DNA-binding activity at 24, 48 and 72 h [13,14]. The DNA-binding activities of the cooperative transcription factors AP-1 and C/EBP (NF-IL6) were increased upon exposure to PIV 4 at 12 min after initial exposure, and remained high (upto 24 h), with an apparent small peak for C/EBP DNA-binding activity at 4 h. For RSV, an increased C/EBP DNA-binding activity was found at 3 h [20], or 12 h [21] and was sustained for at least 36 h [20,21]. In contrast, in another study no increased C/EBP DNA-binding activity was found [10]. At 3 h, an increased AP-1 DNA-binding activity was found upon exposure to RSV [12].

Taken together, in both RSV and PIV 4 infection, early and sustained activation of NF-κB is a key feature. The sustained NF-κB DNA-binding activity, and indeed of AP-1 and C/EBP, in case of the PIV 4 infection are in apparent contradiction with the reduced IL-8 (and IL-6) mRNA steady state levels at 24 h. This indicates that either the transcription factors do not facilitate transcription, or there is transcription, but IL-8 (and IL-6) mRNA degradation has been increased. In contrast to PIV 4 infection, RSV infection still gives rise to increased steady state levels of IL-8 mRNA at 24 h. This difference may relate to differences in viral replication and cytopathic effects between PIV 4 and RSV. A major reason for selecting parainfluenza type 4, was that PIV 4 does not induce cytopathic effects during the first days after infection. Previous studies have indicated that microtubular rearrangements in airway epithelial cell can modify IL-8 expression [24]. Whether cytopathic effects give rise to similar modulation of IL-8 expression is unknown as yet and, if so, whether this is relevant in RSV infection, requires further investigation.

Another intriguing similarity with respect to regulation of NF-κB DNA-binding activity is the biphasic expression, which appears to parallel IL-8 mRNA expression in RSV- and PIV 4-infected cells (see ref. 7 and present study). A similar biphasic mRNA expression in RSV-infected cells has been found for ICAM-1 [22]. Both earlier studies [7,13] and the present study, indicate that the second IL-8 mRNA peak is related to viral replication. The origin of the first peak is unclear but may reflect the initial interaction of virus and airway epithelial cells. Exposure to heat-inactivated PIV 4, but not UV-irradiated PIV 4, resulted in a reduced
first IL-8 mRNA peak, which point to initial interactions as the triggering event of the first IL-8 mRNA peak. We cannot exclude, however, that epithelial-derived factors in our PIV 4 stocks triggered the first IL-8 mRNA peak, as was indeed suggested for the biphasic IL-8 mRNA and NF-κB DNA-binding activity in RSV infection [23].

The present study clearly indicates that IL-8 mRNA degradation was modulated during PIV 4 infection. IL-8 mRNA degradation in RSV-infected cells has not been studied in such detail as here. This reduced degradation of IL-8 mRNA is likely to contribute to the second peak of IL-8 mRNA during PIV 4 infection.

IL-6 is also induced by exposure to virus and shares a number regulatory mechanisms with IL-8. At large, the IL-6 promoter contains potential binding sites for the same transcription factors implicated here in IL-8 gene transcription by PIV 4. In addition, both IL-8 and IL-6 mRNA contain destabilizing motifs in their 3'-untranslated region, which target these mRNAs for rapid degradation. Despite the presence of NF-κB and AP-1 binding sites in the IL-6 promoter, IL-6 gene transcription induced by PIV 4 appears to depend solely on C/EBP. We can not exclude that this is specific for the NCI-H292 cells, but this finding underlines that despite similarities in promoter regions, different transcription factors may be used for gene transcription. In line with this, RSV infection of nasal epithelial cells induced IL-8 but not IL-6 [25]. Parallel to the reduced degradation of IL-8 mRNA we found a reduced IL-6 mRNA degradation. In fact, a reduced degradation of IL-6 mRNA clearly underlies the appearance of the second IL-6 mRNA peak as there was no modulation of C/EBP DNA binding activity. It is tempting to speculate that the reduction of IL-8 and IL-6 mRNA degradation is mediated by the destabilizing AUUUA repeats. If so, this reduction of mRNA degradation may also apply to other AUUUA repeat-containing mRNAs and which encode a large number of epithelial mediators that are implicated in viral airway infections.

Our previous studies in IL-8 and IL-6 regulation have underlined the relevance of mRNA degradation in limiting both the IL-8 and IL-6 response. A reduced IL-8 and IL-6 mRNA degradation resulted in exaggerated responses as reflected by leftward-shifted and steeper dose-response curves [17; M. van Wissen et al., submitted]. Given the observed modulation of IL-8 and IL-6 mRNA degradation we looked into IL-8 and IL-6 production as a function of the TNF-α dose. We found exaggerated responses for IL-8 and to a lesser extent IL-6, particularly at 5 h after exposure to PIV 4. We can conclude that viral infection of NCI-H292 cells amplifies the IL-8 and IL-6 response. This may be due to the reduced mRNA degradation, but for IL-8 we cannot exclude a role for an increased transcriptional activity. It remains to be determined whether similar amplified responses occur upon infection with
other viral species. If so, it may be envisaged that this amplification could play an important role in the potentiation of airway inflammation with viral airway infection. Whether similar mechanisms are relevant in vivo remains to be studied.

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