Influenza A mutant viruses with altered NS1 protein function provoke caspase-1 activation in primary human macrophages, resulting in fast apoptosis and release of high levels of interleukins 1β and 18

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Several NS1 mutant viruses of human influenza A/PR/8/34 (H1N1) virus were tested for their ability to induce pro-inflammatory cytokines in primary human macrophages. The findings revealed a pronounced difference in the virus-induced cytokine pattern, depending on the functionality of the NS1 protein-encoded domains. The PR8/NS1–125 mutant virus, which encodes the first 125 aa of the NS1 protein, thus lacking the C-terminal domains, induced significantly higher amounts of beta interferon, interleukin (IL) 6, tumour necrosis factor alpha and CCL3 (MIP-1α) when compared with the A/PR/8/34 wild-type virus. However, this mutant virus was as efficient as wild-type virus in the inhibition of IL1β and IL18 release from infected macrophages. Another group of viral mutants either lacking or possessing non-functional RNA-binding and dimerization domains induced 10–50 times more biologically active IL1β and five times more biologically active IL18 than the wild-type or PR8/NS1–125 viruses. The hallmark of infection with this group of mutant viruses was the induction of rapid apoptosis in infected macrophages, which correlated with the enhanced activity of caspase-1. These results indicated that the NS1 protein, through the function of its N-terminal domains, might control caspase-1 activation, thus repressing the maturation of pro-IL1β-, pro-IL18- and caspase-1-dependent apoptosis in infected primary human macrophages.

INTRODUCTION

Influenza A virus is a common pathogen that can cause severe disease in humans and animals. The severity of infection in humans depends on the ability of the virus to spread from the initial site of infection to the lower respiratory tract, escaping the factors of innate immunity. At the cellular level, the innate immune response to viruses relies on the execution of two cellular programmes: cell suicide (apoptosis) and cell survival (Iordanov et al., 2001). The first programme involves protein kinase R (PKR) and RNase L (Balachandran et al., 1998; Castelli et al., 1998), resulting in self-elimination of the infected cells, thus disabling further spread of virus. The second programme is associated with the production and secretion of pro-inflammatory cytokines by an infected cell. This pathway requires a post-translational modification of transcription factors such as NF-κB, ATF-2/c-Jun and interferon (IFN)-regulatory factors (IRFs) (Adcock, 1997; Barnes et al., 2002; Hanada & Yoshimura, 2002; Lisowska & Witkowski, 2003; Maniatis et al., 1998) and serves the purpose of preparing neighbouring cells for virus invasion. Double-stranded RNA (dsRNA), together with viral proteins that accumulate at various stages of virus replication, is the major trigger for cellular antiviral programmes (Jacobs & Langland, 1996; Kim et al., 2002; Sareneva et al., 1998; Servant et al., 2002). In addition, it is thought that the switch from one antiviral programme to another might be determined not only by the properties of the virus, but also by the origin and specialization of infected target cells.

The major target for influenza virus is the ciliated epithelial cells; later on in the course of infection, mononuclear cells also become involved (Kaufmann et al., 2001). It has been shown that virus-infected epithelial cells produce only a limited number and amount of pro-inflammatory cytokines and chemokines in response to influenza A virus infection (Adachi et al., 1997; Matsukura et al., 1996). In contrast, influenza A virus-infected monocytes/macrophages are capable of producing higher quantities and a broader spectrum of different pro-inflammatory cytokines and chemokines, e.g. IFN-α/β, interleukin (IL) 1β, IL6, tumour necrosis factor alpha (TNF-α), IL18, CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL2 (MCP-1), CCL7
Influenza A virus is a negative-strand RNA virus belonging to the family Orthomyxoviridae. The genome of influenza A virus consists of eight RNA segments that encode 10–11 proteins, depending on the viral strain. Among them, the NS1 and PB1-F2 proteins have been shown to interfere with the antiviral measures of the host’s innate immunity, thus enabling effective replication of the virus in the host. The PB1-F2 protein does this by way of promoting the apoptotic death of immunocompetent cells (Chen et al., 2001). The NS1 protein accomplishes this interference by way of multiple mechanisms, employing several functional domains. First, the dimerized NS1 protein, via its N-terminal RNA-binding domain (RBD), can bind to dsRNA, thus preventing the activation of PKR (Bergmann et al., 2000; Hatada et al., 1999; Lu et al., 1995) and several transcription factors, such as IRF-3 (Talon et al., 2000) and NF-κB (Wang et al., 2000). As a consequence, virus-induced apoptosis is delayed (Takizawa et al., 1996) and the induction of type I IFNs is inhibited in primary infected cells (Talon et al., 2000; Wang et al., 2000). Secondly, the NS1 protein, through the function of its effector domain (ED), is able to downregulate the formation and nuclear export of cellular mRNAs that interfere with the cellular 3′-end processing and splicing machineries, thus inhibiting the maturation of cellular pre-mRNAs (Chen et al., 1999; de la Luna et al., 1995; Krug et al., 2003; Noah et al., 2003). Thirdly, the NS1 protein is able to selectively enhance the translation of viral mRNAs, but not cellular mRNAs (de la Luna et al., 1995). Thus, the NS1 protein can antagonize the production of cellular proteins at several levels – transcriptional, post-transcriptional and translational (Chen et al., 1999; de la Luna et al., 1995; Krug et al., 2003; Noah et al., 2003; Talon et al., 2000; Wang et al., 2000).

As the NS1 protein is considered to be an IFN antagonist (Egorov et al., 1998; Garcia-Sastre et al., 1998; Noah et al., 2003), we hypothesized that the NS1 protein might also be involved in the control of the production of other pro-inflammatory cytokines. For this reason, we investigated cytokine release in primary human macrophages infected with various influenza A virus NS1 mutants. Here, we have demonstrated that mutant viruses lacking the entire NS1 gene or expressing the non-dimerized (non-functional) NS1 protein were strong inducers of IL1β/IL18 production in virus-infected macrophages. At the same time, an NS1 mutant virus encoding a functional N-terminal RNA binding/dimerization domain and lacking the C-terminal ED was able to antagonize IL1β and IL18 production, but failed to inhibit the production of IFN-β, TNF-α, IL6 and CCL3 (MIP-1α). Our data indicate that influenza A virus NS1 protein controls the production of pro-inflammatory cytokines in infected macrophages through the function of its N- and C-terminal domains. Moreover, we demonstrated that the N-terminal domains of the NS1 protein are essential for preventing caspase-1 activation, thus inhibiting the caspase-1-dependent post-translational processing of pro-IL1β//pro-IL18 in primary human macrophages.

### METHODS

**Isolation of monocytes and generation of macrophages.** To generate macrophages, peripheral blood monocytes were obtained from leukocyte-rich buffy coats of healthy donors. Mononuclear cells were purified by centrifugation through a Ficoll-Paque gradient (Amersham Biosciences). Monocytes were isolated by a magnetic-activated cell sorting (MACS) separation system. Mononuclear cells were stained first with a biotinylated anti-CD14 mAb (Ansell), washed twice with washing buffer (0.5% BSA, 25 μM EDTA in PBS), stained with streptavidin-coated microbeads (Miltenyi Biotec), washed again twice and separated by positive selection using LS columns and a MidiMACS separation unit (Miltenyi Biotec). Separated monocytes (1–5 × 10⁶ per well; 97% purity) were cultured in RPMI 1640 medium supplemented with 10% FCS (HyClone SH30071) and 20 ng granulocyte–macrophage colony-stimulating factor (GM-CSF) ml⁻¹ (Stratmann Biotech AG) in six-well plates for 7 days. Macrophages were re-fed every 2 days with fresh culture medium. On day 7, cells were harvested, replated with the desired cell density and used in experiments. Isolated monocytes and generated macrophages were identified by their typical morphology and CD14 expression, as assessed by flow cytometry using an anti-CD14–phycoerythrin mAb (Becton Dickinson).

**Viruses, cells and cell infection.** Vero cell-adapted influenza A/PR/8/34 (H1N1) virus (PR8 wt), together with NS1 mutant viruses [PR8/NS1–125, PR8/delNS1 and PR8/NS1del40–80; obtained as described previously (Egorov et al., 1998)], were used in this study (Fig. 1). All of the tested viruses were propagated in Vero cells (WHO certified, obtained from ATCC), which were infected at a low m.o.i. of 0.001 and incubated at 37°C for 48 h in Dulbecco’s modified Eagle’s medium/Ham’s F15 (Biochrom F4815) supplemented with a protein-free additive (proprietary formulation; Polymun Scientific) containing 1 μg trypsin ml⁻¹ (Sigma). Infectious titres of generated viral stocks were assessed by plaque assays

![Image](image.png)

**Fig. 1.** Schematic map of the NS1 protein for A/PR/8/34 (PR8 wt) and NS1 mutant viruses. The positions of the RNA-binding domain, the effector domain and the binding sites for the 30 kDa cleavage and polyadenylation specificity factor (CPSF) and PABII are adapted from Krug et al. (2003). The generation of the indicated NS1 mutant viruses was described previously (Egorov et al., 1998).
utilizing Vero cells. Harvested macrophages were washed twice with RPMI 1640 medium and seeded into six-well plates (1 × 10⁶ cells per well) and infected with wild-type or NS1 mutant viruses at an m.o.i. of 2. After 1 h virus adsorption at 37 °C, the inoculum was removed and cells were washed twice with RPMI 1640 medium and incubated in RPMI 1640 medium supplemented with 20 ng GM-CSF ml⁻¹ at 37 °C. Cells and cell-culture supernatants were harvested at various time points post-infection (p.i.) and further processed for testing by ELISA, Western blotting, Quantikine, apoptosis and caspase-1 activity assays.

**Virus replication in primary human macrophages.** Harvested macrophages were washed twice with RPMI 1640 medium, seeded into 24-well plates (2 × 10⁵ cells per well) and infected with wild-type or NS1 mutant viruses (at an m.o.i. of 0-01). After 1 h virus adsorption at 37 °C, the inoculum was removed and the cells were washed twice with RPMI 1640 medium and incubated in RPMI 1640 medium supplemented with 20 ng GM-CSF ml⁻¹ and 0-7 µg trypsin ml⁻¹ at 37 °C. Supernatants were harvested at 2, 24, 48 and 72 h.p.i. and assayed for the presence of infectious virus particles by plaque assays utilizing Vero cells.

**Cytokine ELISAs.** The amounts of IFN-β, IL6, TNF-α, CCL3 (MIP-1α), IL1β and IL18 in the culture supernatants were determined at 18 h.p.i. by using quantitative cytokine-specific ELISA kits purchased from PBL (IFN-β) or R&D [IL6, TNF-α, CCL3 (MIP-1α), IL1β, IL18], following the manufacturer’s instructions.

**Isolation of RNA and assessment of IL1β-specific mRNA.** Total RNA was extracted from virus-infected macrophages at 5 h.p.i. by using TRizol reagent (Invitrogen) following the manufacturer’s instructions. The obtained RNA was quantified with an Agilent 2100 Bioanalyzer using an RNA6000 Nano Assay Kit (Agilent Technologies) plus RNA 6000 ladder marker (Ambion). Equal amounts of RNA (2 µg per sample) were assayed for the presence of IL1β- or β-actin-specific mRNA by using Quantikine kits (R&D). Samples and a manufacturer-supplied calibrator (standard) were hybridized with the IL1β- or β-actin-specific biotin-labelled oligonucleotide probe and the digoxigenin-labelled detection probe in microplates. The hybridization solution was then transferred to streptavidin-coated microplates, the specific mRNA–probe hybrid was captured and unbound material was washed away. Intensive washing was followed by incubation with anti-digoxigenin alkaline phosphatase conjugate. Excess conjugate was washed away and substrate was added. The development of the colour reaction, proportional to the amount of IL1β- or β-actin-specific mRNA, was enhanced by the addition of an amplifier solution. The intensity of the colour reaction was determined by using a microplate reader and the concentration of IL1β- or β-actin-specific mRNA in each sample was calculated from calibration curves.

**Western blot analysis.** Virus-infected macrophages were lysed at 5 h.p.i. and 20 µg protein per lane was analysed by 4–20% gradient SDS-PAGE (Novex, using the Tris/glycine buffer system. After separation, proteins were transferred from the gel to PVDF membranes (Millipore) by using a Novex electrotransfer apparatus at 400 mA for 2 h. The membranes were blocked with PBS containing 0-1% Tween 20 (TPBS) and 5% non-fat milk. Blots were stained for 2 h at room temperature with rabbit anti-human IL1β mAb (R&D) or polyclonal rabbit anti-human caspase-1 antibody (Santa Cruz). Intensive washing of the membranes with TPBS was followed by staining (1 h at room temperature) with anti-rabbit peroxidase conjugate (Sigma). Visualization of the IL1β- and caspase-1-specific protein bands was carried out by using an ECL PLUS kit (Amersham Biosciences).

**Fluorometric caspase-1 activity assay.** Day 7 macrophages (1 × 10⁶) were infected with wild-type or NS1 mutant viruses (at an m.o.i. of 2). At 5 h.p.i., cells were lysed and tested for caspase-1 activity by using a caspase-1 fluorometric kit (R&D). All reagents used in the assay were supplied by the manufacturer. The enzymatic reaction for caspase-1 was performed in 96-well flat-bottomed microplates. Cell lysates (100 µl) and supernatants were mixed with 100 µl 2 × reaction buffer supplemented with 1 µl 1 M dithiothreitol and 10 µl of the caspase-1 fluorogenic peptidic substrate WEHD-AFC. Subsequently, the microplate was incubated at 37 °C and 2 h later analysed with a fluorescence-detecting microplate reader. Cleavage of the peptidic substrate by caspase-1 led to the release of the fluorochrome, which, when excited at a wavelength of 400 nm, emitted fluorescence at 505 nm. The detected fluorescence signal was proportional to the level of caspase-1 enzymic activity in the cell lysates.

**Measurement of the extent of virus-induced apoptosis.** The extent of apoptosis induced in virus-infected macrophages at 2, 4 and 6 h.p.i. was assessed by using a Cell Death Detection ELISAPLUS kit (Roche) according to the manufacturer’s instructions. This assay is based on a quantitative sandwich–enzyme immunoassay principle, utilizing mouse mAbs directed against DNA and histones, respectively, and allows the specific determination of mono- and oligonucleosomes of degraded DNA in the cytoplasmic fraction of cell lysates from apoptotic cells.

**Inhibition of virus-induced apoptosis by using a PKR inhibitor, universal caspase inhibitor and caspase-1 inhibitor.** Macrophages were left untreated or were pre-treated for 30 min with 2 mM PKR inhibitor (2-aminoenurine), 25 µM universal caspase inhibitor (Z-VAD-FMK) or 25 µM caspase-1-specific inhibitor (Z-WEHD-FMK) and then infected with the PR8/NS1del40–80 virus (at an m.o.i. of 2). The ratio of infected to uninfected control cells (apoptosis death index) was determined at 6 h.p.i. by using a Cell Death Detection ELISAPLUS kit as above.

**Inhibition of PKR.** Macrophages were left untreated or were pre-treated for 30 min with 4, 3, 2 or 1 mM PKR inhibitor (2-aminoenurine) and subsequently infected with wild-type or NS1 mutant viruses at an m.o.i. of 2) in the presence or absence of 2-aminoenurine. Cell-culture supernatants were collected at 18 h.p.i. and assayed for the presence of IL1β by ELISA.

**Inhibition of caspase-1, -8, -9 and -3.** Macrophages were left untreated or were pre-treated for 30 min with 25 µM caspase-1 inhibitor (Z-WEHD-FMK), caspase-8 inhibitor (Z-IETD-FMK), caspase-9 inhibitor (Z-LEHD-FMK) or caspase-3 peptidic inhibitor (Z-DEVD-FMK) (all from R&D). Subsequently, cells were infected with wild-type or NS1 mutant viruses (m.o.i. of 2) in the presence or absence of each caspase inhibitor. Cell-culture supernatants were collected at 18 h.p.i. and assayed for the presence of IL1β and IL18 by ELISA.

**RESULTS**

Here, we tested whether the influenza A virus NS1 protein might play a role in the regulation of pro-inflammatory cytokines, such as IFN-β, IL6, TNF-α, CCL3 (MIP-1α), IL1β and IL18, in virus-infected primary human macrophages. Day 7 macrophages were infected with several mutants of the A/PR/8/34 virus that encoded NS1 proteins containing deletions either in the C-terminal (PR8/NS1-125) or N-terminal (PR8/NS1del40–80) part (Fig. 1). The PR8/NS1-125 mutant virus represented a group of viruses that encode the functional N-terminal half of the NS1 protein, and thus are able to bind dsRNA. These viruses have been shown to be IFN-resistant (Kittel et al., 2004). A second group of
mutant viruses was represented by the PR8/delNS1 virus, which completely lacks the NS1 gene, and the PR8/NS1del40–80 virus, which encodes the NS1 protein lacking the dimerization and RNA-binding activities (non-functional NS1 protein). Viruses of this group have been shown to be fully attenuated in IFN-competent cells.

**NS1 protein is required for virus replication in primary human macrophages**

We found that the PR8 wt virus could replicate in human macrophages infected at an m.o.i. of 0.01, giving a yield in the range of 10^4–10^5 p.f.u. ml^{-1}. In contrast, none of the NS1 mutant viruses tested was able to replicate in macrophages. Infection of macrophages at an m.o.i. of 2 resulted in a striking difference in the onset of virus-induced apoptosis. Macrophages infected with PR8/delNS1 or PR8/NS1del40–80 virus developed apoptosis at 6 h p.i. (Fig. 2), whereas macrophages infected with wild-type or PR8/NS1–125 mutant virus showed signs of apoptosis at 12 h p.i. (data not shown).

**NS1 mutant viruses induce two different cytokine patterns in primary human macrophages**

In order to evaluate cytokine production, day 7 macrophages were infected with PR8 wt or NS1 mutant viruses (at an m.o.i. of 2) in the absence of trypsin. Cell-culture supernatants were harvested 18 h p.i. and analysed for the presence of IFN-β, IL6, TNF-α, CCL3 (MIP-1α), IL1β and IL18 by using ELISA kits. The PR8 wt virus was fully competent to inhibit the release of all cytokines tested. The PR8/NS1–125 virus was also able to repress the production of IL1β and IL18, but failed to inhibit the release of IFN-β, IL6, TNF-α or CCL3 (MIP-1α). On the other hand, viruses encoding non-functional NS1 proteins (PR8/delNS1 and PR8/NS1del40–80) induced significantly higher amounts of IL1β and IL18 than the PR8 wt and PR8/NS1–125 viruses (Fig. 3). The surprisingly low production of IFN-β, IL6 and TNF-α (Fig. 3) induced by the PR8/NS1del40–80 and PR8/delNS1 viruses could be attributed to the rapid cell death of macrophages infected with these two mutant viruses (Fig. 2). Inhibition of apoptosis by the universal caspase inhibitor Z-VAD-FMK resulted in a dramatic increase in cytokine release, reaching the levels that were induced by the PR8/NS1–125 mutant virus (data not shown).

Our results implied that removal of C-terminal domains of the NS1 protein was associated with the loss of functions responsible for inhibition of IFN-β, IL6, TNF-α and CCL3 (MIP-1α) production in human macrophages. However, inhibition of IL1β and IL18 production by primary human macrophages could be related to the function of the N-terminal part of the NS1 protein. We thus further investigated the possible mechanisms of NS1 protein-mediated regulation of IL1β and IL18.

**NS1 protein restricts the production of IL1β in influenza A virus-infected macrophages at the post-translational level**

We investigated whether the enhanced production of IL1β by macrophages infected with the PR8/delNS1 or PR8/NS1del40–80 virus could be attributed to enhanced expression of the IL1β gene. High background levels of IL1β-specific mRNA were detected in uninfected macrophages, presumably due to the presence of GM-CSF in the culture medium. All of the tested viruses enhanced the accumulation of IL1β-specific mRNA to a similar extent (Fig. 4a). These data suggested that, under our experimental conditions, the NS1 protein did not interfere with IL1β production at either the transcriptional or post-transcriptional level. We next assessed the accumulation and processing of pro-IL1β protein in macrophages by Western blotting using anti-IL1β mAbs. As shown in Fig. 4(b), infection of macrophages with any of the tested viruses did not result in enhanced accumulation of pro-IL1β protein. However, the biologically active 17 kDa form of IL1β was detected only in macrophages infected with the PR8/delNS1 or PR8/NS1del40–80 mutant virus (Fig. 4b).

**NS1 protein prevents proteolytic processing of pro-caspase-1 into its active form**

Caspase-1 is a key enzyme for the post-translational processing of pro-IL1β and pro-IL18. Therefore, the extent of caspase-1 activation was investigated in virus-infected...
macrophages. Lysates of infected macrophages were obtained at 5 h p.i. and analysed for the presence of the active and inactive forms of caspase-1 by Western blot analysis. Virus-infected macrophages, as well as uninfected cells, expressed equal amounts of the 45 kDa pro-caspase-1 protein. However, the markedly enhanced cleavage of pro-caspase-1 into its mature form (20 kDa) was observed in macrophages infected with PR8/delNS1 or PR8/NS1del40–80 virus, but not in cells infected with wild-type or the PR8/NS1–125 virus (Fig. 5a). These data correlated with the measurement of caspase-1 activity in a functional assay utilizing the caspase-1-specific fluorometric substrate WEHD-AFC. Macrophages infected with the PR8/NS1del40–80 virus displayed 90 % higher caspase-1 activity than macrophages infected with wild-type virus (Fig. 5b). Treatment of PR8/delNS140–80 virus-infected macrophages with a caspase-1-specific inhibitor blocked the release of IL1β (by 91 %) and IL18 (by 80 %) (Fig. 6), as well as resulting in the development of early apoptosis (see Fig. 8). Surprisingly, production of IL1β/IL18 was also markedly reduced by caspase-9 (by 90 %), caspase-3 (by 70 %) and caspase-8 (by 70 %) inhibitors (Fig. 6).

**PKR inhibitor prevents IL1β release and development of fast apoptosis in influenza A virus-infected macrophages**

It has previously been shown that influenza virus-induced apoptosis involves the activation of PKR and is associated with the activation of several caspases (Balachandran et al.,
Fig. 4. Expression of IL1β mRNA and proteolytic processing of pro-IL1β into its mature form in primary human macrophages infected with PR8 wt or NS1 mutant viruses. Day 7 macrophages were infected at an m.o.i. of 2 with the wild-type or NS1 mutant viruses. Total RNA was extracted at 5 h p.i. and tested for the presence of IL1β-specific mRNA (a). Infected macrophages were harvested and lysed at 5 h p.i. and 20 µg protein was assessed for the presence of IL1β by Western blot analysis (b). Experiments were repeated three times with similar results.

Fig. 5. Extent of caspase-1 activation in virus-infected macrophages. Macrophages were infected with wild-type or NS1 mutant viruses. At 5 h p.i., macrophages were harvested, lysed and 20 µg protein was assessed for the presence of caspase-1 by Western blot analysis (a). Infected macrophages were lysed at 5 h p.i. and 100 µl cell lysate was tested for caspase-1 activity by using a fluorometric caspase-1 kit (b). Similar results were obtained in three separate experiments.
000; Pirhonen et al., 2001; Takizawa et al., 1996, 1999; Wurzer et al., 2003; Zhirnov et al., 1999). Since the PR8/delNS1 mutant virus could not counteract the virus-induced activation of PKR (Bergmann et al., 2000), we tested the possible link between PKR and the enhanced caspase-1 activity, early onset of apoptosis and high release of IL1β induced by the PR8/NS1del40–80 virus. We found that treatment of infected cells with PKR inhibitor (2-aminopurine) was as efficient as treatment with caspase-1 inhibitor in repression of IL1β release and delay in the onset of apoptosis in PR8/NS1del40–80 virus-infected macrophages (Fig. 7). Both inhibitors were able to reduce PR8/NS1del40–80 virus-induced apoptosis to the level of that in non-infected cells (Fig. 8). Moreover, treatment of macrophages with 2-aminopurine resulted in replication of the PR8/NS1del40–80 or PR8/delNS1 viruses in macrophages (data not shown). These results indicated a possible involvement of PKR in the caspase-1-mediated proteolytic cleavage of pro-IL1β.

DISCUSSION

Influenza A virus infection of monocytes and macrophages may have a serious impact on the establishment of inflammation and virus-specific immune responses, as these cells are strong producers of pro-inflammatory cytokines (Julkunen et al., 2001). Here, we have provided evidence that optimal function of the entire NS1 protein is essential for productive replication of influenza A virus in human

![Diagram of caspase inhibitors and cytokine release](http://vir.sgmjournals.org)

**Fig. 6.** Caspase-1 (Z-WEHD-FMK), caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK) inhibitors block IL1β and IL18 release from PR8/NS1del40–80-infected macrophages. Macrophages were infected with wild-type or NS1 mutant viruses in the presence or absence of the above caspase inhibitors. At 18 h p.i., cell-culture supernatants were harvested and assessed by ELISA for the presence of IL1β and IL18.

**Fig. 7.** Effect of PKR inhibitor [2-aminopurine (2-AP)] on IL1β release by PR8/NS1del40–80-infected macrophages. Macrophages were infected with wild-type or NS1 mutant viruses in the presence or absence of various concentrations of 2-AP. At 18 h p.i., cell-culture supernatants were harvested and assessed by ELISA for the presence of IL1β. Comparable data were obtained in three independent experiments.
macrophages. For example, the PR8/NS1–125 mutant virus could replicate efficiently in epithelial cells and mouse lungs (Egorov et al., 1998), but this virus appeared to be completely attenuated in human macrophages. This phenomenon could be partially explained by the inability of influenza A virus NS1 mutants to antagonize the production of type I IFNs (Garcı́a-Sastre et al., 1998). Here, we demonstrated that the NS1 protein is involved in the inhibition of not only type I IFNs, but also other pro-inflammatory cytokines, such as TNF-α, IL6, CCL3 (MIP-1α), IL1β and IL18. Moreover, we found that the regulation of cytokine production mediated by NS1 in human macrophages might involve several molecular mechanisms, where the different functional domains of the NS1 protein may play an important role.

The PR8/NS1–125 virus, encoding NS1 protein with an intact N terminus (125 aa), but possessing a large C-terminal deletion, was not able to prevent the production of IFN-β, TNF-α, IL6 and CCL3 (MIP-1α). Nevertheless, the PR8/NS1–125 mutant was as efficient as the PR8 wt virus at antagonizing the production of IL1β and IL18. It is known that the C-terminal domains of the NS1 protein interfere with cellular mRNA 3′-end processing machinery, thus inhibiting the maturation and nuclear export of cellular mRNAs (Krug et al., 2003). Our data indicated that the NS1 protein might also employ a similar mechanism in order to control the production of IFN-β, TNF-α, IL6 and CCL3 (MIP-1α), although further experiments are necessary to elucidate the precise mechanisms of their regulation. It should be mentioned that the inability of the PR8/NS1–125 virus to antagonize IFN-β, TNF-α, IL6 and CCL3 (MIP-1α) could not be attributed to the insufficient dimerization of its 125 aa NS1 protein, as it was able to bind dsRNA in vitro as efficiently as the full-sized NS1 protein (data not shown). Moreover, the PR8/NS1–125 mutant virus was capable of controlling virus-induced apoptosis of infected macrophages to the same extent as the PR8 wt virus.

On the other hand, viruses lacking the entire NS1 gene or expressing NS1 protein with an impaired RBD and dimerization domain induced 10–50 times more biologically active IL1β and three to five times more biologically active IL18 than the wild-type or PR8/NS1–125 viruses. The PR8/NS1del40–80 mutant virus, encoding an almost-intact C-terminal ED, displayed a phenotype very similar to that of the PR8/delNS1 mutant virus. Therefore, we believe that the non-dimerized NS1 protein encoded by the PR8/NS1del40–80 virus was also deficient in functions mediated by its C-terminal domains. It was noteworthy that the production of IFN-β, TNF-α, IL6 and CCL3 (MIP-1α) by this group of mutant viruses was diminished when compared with the PR8/NS1–125 virus, which was mainly due to the fast onset of apoptosis of the PR8/NS1del40–80 or PR8/delNS1 virus-infected macrophages.

Thus, our data imply that the NS1 protein can downregulate cytokine production by utilizing both of its functional domains. In our experimental set-up, the N-terminal part of the NS1 protein appeared to be crucial for the inhibition of IL1β and IL18 production, whereas the C-terminal part was important for the regulation of IFN-β, TNF-α, IL6 and CCL3 (MIP-1α) production in influenza A virus-infected human macrophages.

Previous reports have shown that the C-terminal domain of the NS1 protein mediates inhibition of IFN-β pre-mRNA maturation in infected cells. In our experimental set-up, we found that the C-terminal part of the NS1 protein of PR8 wt virus could inhibit the accumulation of IFN-β-specific mRNA (data not shown), but did not have any effect on the accumulation of IL1β-specific mRNA. A similar enhancement of pre-existing levels of IL1β-specific mRNA was detected in macrophages infected with any of the investigated viruses, including the PR8 wt virus.

Pro-IL1β and pro-IL18 are synthesized as biologically inactive precursors that must be cleaved by caspase-1 in order to become active (Ghayur et al., 1997; Gu et al., 1997; Li et al., 1995). Therefore, these two cytokines might be also regulated at the post-translational level. We could detect the mature form of IL1β protein only in macrophages infected with NS1 mutant viruses encoding non-functional NS1 proteins (PR8/delNS1 and PR8/NS1del40–80). The treatment of infected cells with caspase-1 inhibitor downregulated the release of the mature form of IL1β by 90%, confirming the key role of this enzyme in the post-translational processing of pro-IL1β. Surprisingly, the release of IL1β was also significantly repressed by other
caspase inhibitors (caspase-9, -8 and -3). The caspase-9-specific inhibitor, in particular, reduced the production of IL1β as efficiently as the caspase-1 inhibitor. We assume that caspase-9, -8 and -3 might also be involved in the regulation of IL1β and IL18 production by having a direct or indirect impact on the activation of caspase-1 (Cohen, 1997; Srinivasula et al., 1996).

It was previously shown that pro-caspase-1 is expressed constitutively in primary human macrophages and that its expression is not enhanced upon viral infection (Pirhonen et al., 1999, 2001). We also observed equal amounts of pro-caspase-1 in virus-infected and uninfected macrophages. Nevertheless, the enhanced activity of caspase-1 was detected only in macrophages infected with the PR8/delNS1 and PR8/NS1del40–80 viruses. Our findings imply that the NS1 protein restricts the production of the mature forms of IL1β and IL18 by preventing the activation of caspase-1. However, it is difficult to elucidate the precise mechanism of this interference at the molecular level, as the exact mechanism by which caspase-1 is activated is not yet known in detail.

In our experiments, we also observed that enhanced activity of caspase-1 in macrophages infected with the PR8/delNS1 or PR8/NS1del40–80 viruses correlated with an early onset of apoptosis in these cells. It was previously reported that the PR8/delNS1 virus rapidly induced apoptosis in IFN-competent cells, but not in IFN-deficient cells (Zhirkov et al., 2002). Thus, the NS1 protein, by inhibiting some of the IFN-induced factors, can prevent virus-induced apoptosis. In this context, PKR is considered to be one of the major factors responsible for influenza A virus-induced apoptosis (Takizawa et al., 1996). It was demonstrated that the NS1 protein could prevent the activation of PKR in PR8/delNS1 virus-infected cells (Bergmann et al., 2000). Influenza A virus-induced apoptosis in HeLa cells has been shown to be PKR-dependent and accompanied by the activation of caspase-8 and -3, but not caspase-1 (Takizawa et al., 1995, 1996, 1999). The involvement of caspase-1 in PR8/delNS140–80 virus-induced apoptosis was shown in our experiments by the repression of macrophage death following the addition of caspase-1-specific inhibitor to infected macrophages. Moreover, treatment of PR8/delNS140–80-infected cells with a PKR inhibitor (2-aminopyrine) resulted in complete inhibition of apoptosis and IL1β release. Based on these findings, we concluded that the mechanism by which NS1 protein inhibits caspase-1 activation in macrophages might include prevention of PKR activation. However, the link between PKR or other Ser/Thr kinases and caspase-1 activation remains to be elucidated.

The cytokines IL1β and IL18 belong to the class of major regulators of the antiviral immune response (Nakanishi et al., 2001). It was shown that influenza A virus-infected IL1β/IL18 knockout mice displayed a higher mortality rate than control mice, thus indicating the importance of IL1β and IL18 for host antiviral defence (Kozak et al., 1995; Liu et al., 2004). Enhanced induction of IL1β and IL18 by the PR8/delNS1 and PR8/delNS140–80 viruses might stimulate a robust immune response, due to the immuno-potentiating properties of IL1β and IL18 (Bradney et al., 2002; Staats et al., 2001). Recently, we found that these two viruses could elicit a significant Th1-type immune response that was sufficient to protect mice against wild-type virus challenge. Interestingly, mice immunized with the PR8/NS1del40–80 virus developed the strongest release of type I IFNs and IL1β in the mouse respiratory tract (unpublished data). We believe that the results of this study may contribute towards the development of a new generation of safe and immunogenic live influenza A virus vaccines based on NS1 mutant viruses encoding targeted modifications within the NS1 protein.

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REFERENCES


