Active NF-κB signalling is a prerequisite for influenza virus infection

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Influenza virus still poses a major threat to human health. Despite widespread vaccination programmes and the development of drugs targeting essential viral proteins, the extremely high mutation rate of influenza virus still leads to the emergence of new pathogenic virus strains. Therefore, it has been suggested that cellular cofactors that are essential for influenza virus infection might be better targets for antiviral therapy. It has previously been reported that influenza virus efficiently infects Epstein–Barr virus-immortalized B cells, whereas Burkitt’s lymphoma cells are virtually resistant to infection. Using this cellular system, it has been shown here that an active NF-κB signalling pathway is a general prerequisite for influenza virus infection of human cells. Cells with low NF-κB activity were resistant to influenza virus infection, but became susceptible upon activation of NF-κB. In addition, blocking of NF-κB activation severely impaired influenza virus infection of otherwise highly susceptible cells, including the human lung carcinoma cell lines A549 and U1752 and primary human cells. On the other hand, infection with vaccinia virus was not dependent on an active NF-κB signalling pathway, demonstrating the specificity of this pathway for influenza virus infection. These results might be of major importance for both the development of new antiviral therapies and the understanding of influenza virus biology.

INTRODUCTION

Influenza virus is a significant cause of morbidity and mortality and seasonal epidemics are responsible for approximately 40 000 deaths and 100 000 hospitalizations per year in the USA alone (Thompson et al., 2003). Pandemics such as that in 1918 have been responsible for the death of millions of people worldwide. Influenza viruses are impossible to eradicate as there is a large reservoir of influenza virus subtypes in wild aquatic birds that can contribute genes to new pathogenic virus variants (Shortridge et al., 2000). Although it was previously thought that these influenza virus strains were not pathogenic for humans, these viruses have been found to cause fatal outbreaks, such as in 1997 in Hong Kong, and might even have the potential for pandemic spread (Hatta & Kawaoka, 2002; Palese et al., 2002; Scholtissek et al., 2002; Seo et al., 2002). The continuous emergence of new virus variants poses a major problem for the design and development of antiviral therapies and underlines the constant threat to human health from this virus (Fleming, 2001; Gubareva et al., 2002; Bridges et al., 2003). Therefore, a better knowledge of cellular cofactors essential for virus infection might lead to the development of new antiviral therapies (Scholtissek & Muller, 1991; Ludwig et al., 2003). The importance of these cofactors for the outcome of influenza virus infection is demonstrated by the fact that, despite ubiquitous expression of cellular receptors for influenza virus, not all cells become infected efficiently (Nimmerjahn et al., 2003).
Whereas signalling events triggered after influenza virus infection are beginning to be defined (Ludwig et al., 2003; Root et al., 2000; Garcia-Sastre, 2001; Pleschka et al., 2001; Geiss et al., 2002; Wurzer et al., 2003), virtually nothing is known about cellular factors that predetermine the efficiency of virus infection. It has been suggested that certain protein kinase C isoforms are important for virus release from endosomes (Sieczkarski et al., 2003); however, it is not known whether this pathway is of general importance for influenza virus infection of human cells.

In this report, we have shown that influenza virus infection of human cells is essentially dependent on an active NF-κB signalling pathway. Cells with low NF-κB activity were virtually resistant to infection with influenza virus, indicating that the reported activation of the NF-κB signalling pathway by influenza virus infection is not sufficient to allow infection (Flory et al., 2000). This block of influenza virus infection, however, could be overcome by activation of the NF-κB signalling pathway. We furthermore showed that influenza virus infection of highly susceptible cells, e.g. lung epithelial cells, is severely impaired by inhibition of NF-κB signalling.

**METHODS**

**Cell lines and viruses.** Burkitt’s lymphoma (BL) lines and Epstein–Barr virus (EBV)-immortalized lymphoblastoid cell lines (LCLs) were cultured as described (Kempkes et al., 1996; Zimber-Strobl et al., 1996; Gires et al., 1997; Dudziak et al., 2003). 293T, MDCK, A549 and U1752 (kindly provided by Dr M. Bacher) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. Primary B cells were isolated from peripheral blood mononuclear cells by positive selection with magnetic beads coupled to CD19 antibodies (Miltenyi Biotech) and cultured for 4 days in RPMI containing 10% human autologous serum, 2 mM L-glutamine, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. EREB2/5, A1, p493/6 and 1194/3 cells were infected with the EBV P3HR1 strain, which has EBNA2 deleted (Kempkes et al., 1996; Zimber-Strobl et al., 1996; Bornkamm & Hammerschmidt, 2001; Polack et al., 1996). By stable transfection of these cells with a construct containing an oestrogen receptor–EBNA2 fusion protein, EBV latent gene expression can be induced by the addition of oestrogen. p493/6 cells are additionally transfected with a c-myc gene under the control of a tetracyclin-regulated promoter. Therefore, these cells can either proliferate under a c-myc (Burkitt-like) or EBNA2-controlled EBV programme. 1194/3 cells additionally express latent membrane protein 1 (LMP1) from an SV40 promoter. Influenza virus strains A/FPV/Bratislava (H7N7; FPV) and A/WSN/33 (H1N1; WSN) were propagated in Madin–Darby canine kidney (MDCK) cells. The introduction of heterologous genes into influenza virus particles was performed as described previously (Azeeh et al., 2001; Nimmerjahn et al., 2003). Unless otherwise stated, influenza virus was used at an m.o.i. of 1. Modified vaccinia virus Ankara, containing GFP (Vac–GFP), was kindly provided by Drs G. Sutter and C. Staib and used at the indicated m.o.i. values.

**Antibodies and reagents.** The inhibitors BAY11-7085, BAY11-7082 or Ly294002 at 2-5 μM for 8–10 h. BL33 and BL41 cells stably transfected with the NGFR–2134 LMP1 mutant were incubated with the inhibitors (2-5 μM) during the last two cross-links. Subconfluent A549 cells, U1752 cells and primary human fibroblasts were pre-treated for 4–6 h with 10 μM of the inhibitors. After washing, cells were infected with recombinant influenza or vaccinia virus and analysed for GFP expression 8–10 h later by FACS analysis.

**Induction of LMP1 signalling.** Cells (2 × 10⁶) were incubated with culture supernatant from the mouse hybridoma HB8737 at a dilution of 1:20 for 10 min at room temperature. After washing, cells were incubated with a goat anti-mouse F(ab)₂ fragment (Dianova) at 2 μg ml⁻¹ for 8–10 h. Following two more cross-links at 3 h intervals, cells were washed and infected with influenza virus containing GFP (FPV–GFP) or Vac–GFP at the indicated m.o.i. values. In control experiments, cells were either left untreated or only incubated with the anti-NGFR antibody without consecutive cross-linking.

**Inhibition of NF-κB activation.** LCLs were pre-treated with the inhibitor BAY11-7085, BAY11-7082 or Ly294002 at 2-5 μM for 8–10 h. BL33 and BL41 cells stably transfected with the NGFR–2134 LMP1 mutant were incubated with the inhibitors (2-5 μM) during the last two cross-links. Subconfluent A549 cells, U1752 cells and primary human fibroblasts were pre-treated for 4–6 h with 10 μM of the inhibitors. After washing, cells were infected with recombinant influenza or vaccinia virus and analysed for GFP expression 8–10 h later by FACS analysis.

**Microarray analysis.** The DNA microarray methods have been described in detail elsewhere (Alizadeh et al., 2000). Fluorescently labelled cDNA probes were generated from mRNA (Fast Track kit; Invitrogen), using the Cy5 dye to label cDNA from the indicated cell lines and the Cy3 dye to label cDNA from a reference pool of mRNA prepared from nine lymphoma cell lines. Lymphochip DNA microarrays containing 18 500 human cDNAs were prepared and used as described previously (Alizadeh et al., 2000). Initial microarray data selection was based on fluorescence signal intensity, with the requirement of 50 relative fluorescent units (r.f.u.) above background in both the Cy3 and Cy5 channels or 500 r.f.u. above background in either channel alone. DNA microarray analysis of gene expression was done essentially as described previously (Alizadeh et al., 2000). For the red/green diagram, datasets of different cell lines for a specific target gene were averaged. This mean value is shown in black, whereas deviations from this value are shown as different intensities of green (negative deviation) or red (positive deviation).
Virus attachment studies. Influenza virus attachment to target cells was analysed by incubating target cells with influenza virus (m.o.i. of 1) on ice for 1 h. After removal of excess influenza virus by washing the cells with ice-cold PBS three times, attached influenza virus was detected by staining with an anti-haemagglutinin antibody (anti-H7) followed by a PE-labelled secondary antibody and subsequent FACS analysis.

Titration of influenza virus. Influenza virus titres were determined by the classical MDCK plaque assay as described previously (Wagner et al., 2000). Briefly, confluent MDCK cell monolayers were infected with 10-fold dilutions of influenza virus in a total volume of 1 ml PBS/0.2% BSA for 1 h in 6 cm dishes. After washing, cells were covered with an overlay of DMEM cell culture medium containing 0.5% purified agar. Cells were incubated at 37°C under 5% CO2 and plaque formation was analysed 3 days post-infection.

RESULTS AND DISCUSSION

Expression of EBV latent genes confers susceptibility to infection with influenza virus

By using recombinant influenza viruses containing GFP (FPV–GFP and WSN–GFP) to detect virus infection, we observed that LCLs were efficiently infected with influenza virus while several BL cells and primary B cells isolated from healthy donors were not (Nimmerjahn et al., 2003, and data not shown). This indicated that cellular cofactors predetermine the efficiency of infection. One obvious difference between the highly susceptible and poorly susceptible group was the presence of EBV in the first group. To test whether the EBV status had an impact on the outcome of influenza virus infection, we used the EBV-negative BL cell line BL41, or BL41 cells infected with the wild-type EBV strain B95-8 (BL41-B95-8), or the EBNA2-deleted EBV strain P3HR1 (BL41-P3HR1). Because EBNA2, the master regulator of EBV latent gene expression, is deleted in the P3HR1 strain, the only latent gene expressed in BL41-P3HR1 cells is EBNA1. Strikingly, the cells became susceptible to infection with influenza viruses (FPV and WSN strains) only when wild-type EBV was present (Fig. 1a, b). Furthermore, the p493/6 cell line, which carries the P3HR1 EBV strain and an oestrogen-inducible EBNA2 protein (Pajic et al., 2000), could only be infected efficiently in the presence of EBV latent gene expression (Fig. 1c). Importantly, infection with vaccinia virus did not show such a preference (Fig. 1d).

To exclude the possibility that the observed pattern of susceptibility to influenza virus infection was due to differential receptor expression and therefore impaired virus attachment, we performed FACS analysis with lectins recognizing 2,3- and 2,6-branched sialic acid residues on the cell surface. All of the cells expressed the viral receptors, and influenza virus attachment to target cells was even slightly higher in the group that was only weakly susceptible to influenza virus infection (Fig. 2a, and data not shown).

After infection of B cells, EBV establishes a latency state in which only nine viral proteins are expressed (Bornkamm & Hammerschmidt, 2001). To identify EBV latent proteins important for rendering the cells susceptible to influenza virus infection, we made use of two other cell lines, EREB2/5 and 1194/3, which contain an oestrogen-inducible EBNA2 protein on a P3HR1 EBV background (Kempkes et al., 1996; Zimber-Strobl et al., 1996). After oestrogen deprivation, all EBNA2-dependent latent EBV genes were downregulated in both cell lines. In the 1194/3 cell line, however, EBV LMP1 expression was sustained due to control of LMP1 by a heterologous EBNA2-independent promoter (Fig. 2c). Influenza virus infection of EREB2/5 cells was crucially dependent on EBNA2 expression, whereas 1194/3 cells still could be infected efficiently (Fig. 2b). Moreover, LMP1 expression correlated well with the susceptibility to influenza virus infection in the BL41 and p493/6 cell lines (Fig. 1e and f). Thus, EBNA2 conferred susceptibility to influenza virus infection indirectly through the induction of its downstream target LMP1.

LMP1 or CD40 signalling renders B cells highly susceptible to infection with influenza virus

LMP1 signalling is mediated mainly by two signalling domains in the C terminus of LMP1, called the C-terminal transactivation regions (CTAR) 1 and 2 (Fig. 3a). To test the role of LMP1 signalling on influenza virus infection, we created several inducible forms of LMP1 and LMP1 mutants defective in the signalling domains by fusing the C termini of these LMP1 variants to the extracellular and transmembrane domains of the NGFR (Fig. 3a). It has been shown that an NGFR–LMP1 fusion protein can functionally replace wild-type LMP1 (Gires et al., 1997). BL33 and BL41 cells expressing the NGFR–LMP1 fusion protein became susceptible to influenza virus infection after induction of LMP1 signalling by cross-linking the NGFR (Fig. 3b, and data not shown). To test whether EBNA2 itself, which is also known to interfere with cellular signalling pathways, could further increase influenza virus infection efficiency, we made use of a cell line that carries an oestrogen-inducible EBNA2 on an EBV-negative background. This, however, did not lead to an additional increase in susceptibility to influenza virus infection, confirming that LMP1 signalling was mediating this effect (Fig. 3b, and data not shown). As LMP1 mimics CD40 signalling in B cells (Gires et al., 1997; Eliopoulos & Rickinson, 1998), CD40 signalling would be expected to have a similar effect on influenza virus infection. Indeed, after culturing different EBV-negative cell lines and primary B cells on 239T cells stably transfected with CD40L, these cells became highly susceptible to subsequent influenza virus infection (Fig. 4a). Again, CD40 signalling did not affect the infection efficiency of vaccinia virus as shown for the p493/6 cell line (Fig. 4b), demonstrating the importance of this signalling pathway, especially for influenza virus.

The most important signalling pathways triggered by LMP1 include the TRAFF/TRADD pathways leading to the activation of several downstream molecules such as...
NF-κB, AP1 and Jun (Lam & Sugden, 2003). To define further which signalling pathways are important for influenza virus infection, we made use of the inducible LMP1 mutants (Fig. 3a–c). Approximately 60–80 % of the LMP1-induced NF-κB activation is mediated by the CTAR2 domain (Huen et al., 1995; Mitchell & Sugden, 1995). Cross-linking the NGFR–2134 mutant, which had only the intact CTAR2 domain, resulted in the highest susceptibility to a subsequent influenza virus infection. If only a functional CTAR1 domain was present (NGFR–2078), there was a lower but still significant increase in infection efficiency, whereas inactivation of both signalling domains (NGFR–2131) abrogated this effect (Fig. 3b, c). These results suggested that LMP1-induced NF-κB activation is necessary for efficient influenza virus infection. Moreover, CD40 cross-linking has been described as activating NF-κB (Berberich et al., 1994).

As influenza virus can attach to BL cells (Fig. 2a), we reasoned that LMP1-mediated signalling might rescue influenza virus infection if triggered after virus attachment to target cells. As shown in Fig. 3(d), this is indeed the case, but the infection rate rapidly declined over time indicating that, in the absence of LMP1 or CD40 signalling, influenza virus bound to target cells is rapidly inactivated, probably by endocytosis and subsequent degradation in the endosomal/lysosomal compartment.

**Activation of NF-κB is responsible for the level of susceptibility to influenza virus infection**

To assess differences in NF-κB expression between cells in the highly susceptible and poorly susceptible groups, microarray analysis was performed. As shown in Fig. 5(a), molecules of the NF-κB family and NF-κB target genes were expressed at significantly higher levels in cells of the highly susceptible group. This correlated well with the observed increase in influenza virus infection of EREB2/5 cells after activation of EBNA2 (Fig. 5b). To investigate whether blocking NF-κB activation would interfere with influenza virus infection, we tested chemical inhibitors of NF-κB activation under several experimental conditions.
(Figs 5 and 6). Taken together, these results indicated that inhibition of NF-κB activation blocked influenza virus infection of otherwise susceptible cell lines. The observed increase in susceptibility to influenza virus infection in BL cells carrying the inducible NGFR–2134 mutant was abrogated if the cross-link was performed in the presence of the inhibitors BAY11-7085 or BAY11-7082 (Fig. 5c, and data not shown). Infection of EBV-immortalized cell lines was severely impaired if cells were pre-treated with these inhibitors (Fig. 5d), but not with LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3K). The observed level of inhibition was comparable with blocking influenza virus infection of EBV-positive and EBV-negative cell lines and LMP1-dependent susceptibility to infection. (a) Expression of 2,3-branched sialic acid residues on the surface of cells that do [p493/6(myc+EB2) and BL41-B95-8] or do not [p493/6(myc) and BL41] express EBV latent genes was analysed by FACS analysis with biotin-conjugated MAL (upper panel) with subsequent detection with PE-conjugated streptavidin (grey histograms). As a control, cells were incubated with streptavidin–PE alone (white histograms). Influenza virus attachment (FPV strain) was analysed by FACS analysis of cells that had been incubated with influenza virus at 4 °C for 1 h and subsequently stained with an anti-haemagglutinin (H7) antibody (lower panel) followed by detection with a PE-labelled secondary antibody (grey histograms). Cells that had not been incubated with influenza virus served as controls (white histograms). One of three independent experiments is shown. (b) EREB2/5 and 1194/3 cells were grown in the presence (with oestrogen) or absence (without oestrogen) of active EBNA2 and infected with FPV–GFP. Cells were infected in duplicate wells and the number of influenza virus-infected cells was quantified by FACS analysis. One of two representative experiments is shown. (c) Cells were grown in the presence or absence of oestrogen as in (b). LMP1 expression was determined by Western blot analysis. The equivalent of 5 × 10^5 cells was loaded per lane and one of two independent experiments is shown.
Fig. 3. The role of LMP1 signalling in influenza virus infection. (a) Schematic overview of the inducible wild-type and mutant LMP1 variants fused to the transmembrane and extracellular domain of the NGFR. CTAR1 and CTAR2 and amino acid motifs important for signalling (given as the one-letter code) are indicated. (b) FACS analysis of BL33 cells transfected with NGFR–LMP1 or the respective LMP1 mutant fusion proteins. Cells were infected with FPV–GFP either without (no cross-linking) or after previous cross-linking of the NGFR–LMP1 fusion protein with an NGFR-specific monoclonal antibody followed by a secondary antibody. One of three independent experiments is shown. (c) BL33 and BL41 cell lines stably transfected with the indicated NGFR–LMP1 mutants were infected with influenza virus (FPV–GFP) either with or without previous induction of LMP1 signalling by antibody cross-linking of the NGFR. Cells were infected in duplicate wells and one of three experiments is shown. (d) BL33::NGFR–2131 and BL33::NGFR–2134 cells were infected with FPV–GFP either 4 h after, or 4 or 24 h before, induction of signalling by cross-linking the NGFR fusion proteins. The number of GFP-positive cells was quantified by FACS analysis. One of two independent experiments is shown.
binding to its cellular receptor by pre-incubating the cells with MAL (not shown). Inhibition of NF-κB activation did not interfere with vaccinia virus infection, ruling out non-specific cytotoxic effects of the inhibitors (Fig. 5e). Moreover, influenza virus infection of primary human fibroblasts and the human lung carcinoma cells U1752 and A549, which are considered as the gold standard for influenza virus infection, were also dramatically reduced by inhibition of NF-κB activation (Fig. 6a, and data not shown). Importantly, receptor expression and virus attachment to cells was not impaired by inhibition of NF-κB activation (Fig. 6b, and data not shown). To exclude the possibility that the observed effects were due to the use of recombinant influenza viruses, we repeated the experiment with wild-type influenza virus and quantified the release of progeny virus particles after pre-treating the cells with NF-κB inhibitors. As before, influenza virus propagation was strongly impaired in the presence of NF-κB inhibitors (Fig. 6c).

These results suggested that an active NF-κB signalling pathway is a general prerequisite for influenza virus infection of human cells. One has to keep in mind, however, that despite the use of two independent chemical inhibitors of NF-κB activation and control compounds we cannot completely exclude the possibility that these inhibitors also influenced other as yet unknown pathways important for influenza virus infection and replication.

Nevertheless, our data indicate that, besides activation of haemagglutinin by cellular or bacterial proteases (Tashiro et al., 1987), the level of NF-κB activity might be a second determinant of influenza virus tropism in vivo. At first sight our results seem to contradict earlier findings that NF-κB activation is a hallmark of inflammatory and antiviral responses mediated especially by type I interferons that block virus replication. However, many other transcription factors and signalling pathways besides NF-κB (e.g. the Jak–Stat and Toll-like receptor signalling pathways) are specifically activated during these antiviral and inflammatory responses and are important to obtain an efficient cellular response (Hertzog et al., 2003). In contrast to this, our study most likely deals with qualitatively and quantitatively different levels of NF-κB activation. In the case of the B cell lines, this is due to LMP1 expression or the isolated triggering of CD40 signalling, whereas many factors can lead to a basal level of NF-κB activation in lung epithelial cells, e.g. dust particles, cell density and temperature shifts (Liden et al., 2003; Inoue et al., 2003). Therefore, inhibition of NF-κB activation may be effective in preventing or treating influenza virus infection. Interestingly, it has recently been shown that many epithelial cells including lung epithelial cells express CD40, especially under inflammatory conditions (Young et al., 1998; Kaufman et al., 2001), implicating that this pathway is of great importance for making these cells highly susceptible to influenza virus infection. Although still speculative, this finding might highlight the role of bacterial co-infections, which sustain inflammatory processes in the lung. Furthermore, our results suggest that NF-κB signalling might modulate specific steps in the endocytosis pathway, necessary for efficient influenza virus infection. We are currently trying
to identify NF-κB target genes that might be involved in rendering the cells susceptible to infection with influenza virus.

In addition, one might think of using inhibitors of NF-κB activation as a therapeutic agent to block the spread of influenza virus after infection of epithelial tissues in the respiratory tract. Whereas these inhibitors have been used systemically for the treatment of pathogenic inflammatory processes in vivo (Pierce et al., 1997), a more local application of these inhibitors to the respiratory epithelia might be indicated in the case of influenza virus infection. Indeed, besides the identification of specific NF-κB target genes, one of the next steps will be animal studies to define the potential of these inhibitors to block influenza virus infection in vivo.

**Fig. 5.** The role of LMP1–induced NF-κB activation in influenza virus infection. (a) The relative expression level of NF-κB and NF-κB target genes was compared between EBV-positive and EBV-negative cell lines by microarray analysis. The expression levels are depicted according to the colour scale at the bottom. (b) EREB2/5 cells cultured in the absence (5 days after oestrogen removal) or presence (for the indicated time points) of oestrogen were infected with FPV–GFP. One of two independent experiments is shown. (c) Signalling of the NGFR–2134 LMP1 mutant was induced by cross-linking in the presence or absence of inhibitors of NF-κB (BAY7085) or PI3K (Ly294002) in stably transfected BL33 and BL41 cell lines followed by infection with FPV–GFP. (d) EREB2/5 cells either were left untreated (w/o) or were pre-treated with BAY11–7085 (BAY7085) or Ly294002 followed by infection with influenza virus recombinant for GFP (FPV-strain). (e) EREB2/5 cells were left untreated or were pre-treated with the indicated inhibitors as in (d) and subsequently infected with Vac–GFP. All virus infections were carried out in duplicate wells and the number of GFP-positive cells was quantified by FACS analysis. One of two representative experiments is shown. M.f.i., mean fluorescence intensity.
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