Mouse cytomegalovirus inhibits beta interferon (IFN-β) gene expression and controls activation pathways of the IFN-β enhanceosome

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We have investigated beta interferon (IFN-β) and IFN-α4 gene expression and activation of related transcription factors in mouse cytomegalovirus (MCMV)-infected fibroblasts. mRNA analysis demonstrated an initial phase of IFN gene induction upon MCMV infection, which was followed by a sustained MCMV-mediated simultaneous downregulation of IFN-β and IFN-α4 gene expression. The induction of IFN transcription resulted from the activation of the components of the IFN-β enhanceosome, i.e. IFN regulatory factor (IRF) 3, nuclear factor (NF)-κB, activating transcription factor (ATF)-2 and c-Jun. Activation of the transcription factors occurred rapidly and in a sequential order upon infection, but only lasted a while. As a consequence, IFN-α4/β gene expression became undetectable 6 h post-infection and throughout the MCMV replication cycle. This effect is based on an active interference since restimulation of IFN gene induction by further external stimuli (e.g. Sendai virus infection) was completely abolished. This inhibition required MCMV gene expression and was not observed in cells infected with UV-inactivated MCMV virions. The efficiency of inhibition is achieved by a concerted blockade of IκBα degradation and a lack of nuclear accumulation of IRF3 and ATF-2/c-Jun. Using an MCMV mutant lacking pM27, a signal transducer and activator of transcription (STAT) 2-specific inhibitor of Jak/STAT signalling, we found that the initial phase of IFN induction and the subsequent inhibition does not depend on the positive-IFN feedback loop. Our findings indicate that the MCMV-mediated downregulation of IFN transcription in fibroblasts relies on a large arsenal of inhibitory mechanisms targeting each pathway that contributes to the multiprotein enhanceosome complex.

INTRODUCTION

Upon virus infection the type I interferon (IFN-α/β) response is one of the earliest innate host defence mechanisms, and many viruses have found means to avoid IFN gene expression (Hengel et al., 2005; Haller et al., 2006). Production of IFN-α/β is induced by the recognition of pathogen-associated molecular patterns (PAMP) (Medzhitov & Janeway, 2002) and initiates transcriptional upregulation of IFN-stimulated genes (ISGs) to establish an antiviral state. IFN-α/β is synthesized by virus-infected cells bind to the IFN-α/β receptor complex, termed IFNAR, and activates the Jak/STAT signal transduction cascade, leading to the formation of the IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds to IFN-stimulated response elements (ISRE), located in the promoter region of IFN-inducible target genes mediating IFN effector functions (Darnell et al., 1994).

Initiation of IFN transcription and its subsequent autocrine and paracrine amplification is a prerequisite for efficient IFN effector responses. IFN-β gene transcription is controlled by a higher order transcription enhancer complex, known as enhanceosome (Maniatis, 1986; Maniatis et al., 1998). The multi-component complex includes three distinct transcription factors binding cooperatively to the IFN-β promoter and the architectural high mobility group protein HMG-I(Y). Viral infection triggers activation of the latent transcription factors IRF3 and nuclear factor (NF)-κB that initiate, synergistically with ATF-2/c-Jun, IFN-β gene expression by recruitment of the coactivators p300 and CREB-binding protein (CBP). The IFN-β promoter comprises several positive-regulatory domains (PRDs) (Goodbourn & Maniatis, 1988). PRD I and PRD III are related sequence elements that are recognized by members of the IFN regulatory factor (IRF) family, IRF3 and IRF7, respectively (Wathelet et al., 1998). Virus infection stimulates phosphorylation, dimerization and nuclear translocation of IRF3, which binds to PRD I and PRD III (Lin et al., 1998). PRD II constitutes the binding site for the NF-κB p65/p50 complex. In unstimulated cells NF-κB is found in a complex with an inhibitory protein, predominantly IκBα. NF-κB activation requires phosphorylation, ubiquitination and subsequent proteasomal degradation of IκBα to release p65/p50 (Karin & Ben...
neriah, 2000). the third component of the IFN-β enhancesosome is the heterodimeric transcription factor ATF-2/c-Jun. Activated ATF-2/c-Jun binds to PRD IV of the IFN-β promoter (maniatis et al., 1998). To attain sustained IFN gene expression a positive-feedback regulation is established in fibroblasts through de novo expression of IRF7 induced by IFN-β (sato et al., 1998). in turn IRF7 acts in cooperation with IRF3 to maintain IFN responses (marie et al., 1998).

Viral antagonists of IFN-β gene induction, IFN receptor signal transduction and the action of antiviral effector proteins have been identified (Katze et al., 2002; Hengel et al., 2005). For cytomegaloviruses (CMV), a number of genes were reported to code for inhibitors counteracting IFN responses. the mouse cytomegalovirus (MCMV) M27 protein downregulates STAT2 to disrupt IFN-α/β and IFN-γ signal transduction, resulting in a dramatic attenuation of viral replication in vivo (Khan et al., 2004; Zimmernann et al., 2005). The human CMV (HCMV) immediate-early 1 (ie1)-encoded protein pp72 was described as a STAT-interacting protein that diminishes ISGF3-dependent transcription (Paulus et al., 2006). HCMV TRS/IRS1 and MCMV m142/m143 bind to dsRNA, resulting in a reduced activation of specific antiviral proteins, e.g. protein kinase R and RNaseL (child et al., 1998). Moreover, the HCMV IE2 protein pp86 was identified as blocking IFN-β gene induction by inhibiting NF-κB DNA binding (Taylor & Bresnahan, 2006a).

to gain information as to whether MCMV copes with IFN induction, we analysed IFN-α and IFN-γ transcription and related activation pathways in infected fibroblasts. herein, we describe the ability of MCMV to interfere with distinct molecular events along the IRF3, NF-κB and ATF-2/c-Jun activation pathways. our findings document that MCMV uses effective inhibitory mechanisms to down-regulate IFN transcription.

METHODS

Cells and viruses. Primary mouse embryonic fibroblasts (MEF) were prepared as described previously (Brune et al., 1999). MEF were cultured in complete medium [Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 200 μg penicillin and streptomycin ml−1] and used for experiments within three passages. For culture of NIH3T3 fibroblasts (ATCC CRL1658), FCS was replaced by newborn calf serum. The MCMV used in this study was the bacterial artificial chromosome (BAC)-derived recombinant MW97.01 with wild-type (wt) properties in vitro and in vivo (Wagner et al., 1999). Before use, MCMV stocks were tested for mycoplasma contamination by PCR as described previously (van Kuppeveld et al., 1994). MCMV was propagated and titrated on MEF (polic et al., 1998). MCMV infection was enhanced by centrifugation at 800 g for 30 min. UV-inactivation of MCMV was performed for 20 min at 265 nm. Sendai virus (SeV) infection was performed by low volume incubation with 100 HA U ml−1 for 30 min at room temperature.

Expression analysis by semi-quantitative RT-PCR. Total RNA was extracted from MCMV-infected NIH3T3 cells using the RNeasy Mini kit (Qiagen). Total RNA was digested with DNase I to eliminate possible DNA contamination. Semi-quantitative RT-PCR analysis was performed by using the OneStep RT-PCR kit (Qiagen) with different dilutions of total RNA as template. Gene-specific primers (Supplementary Table S1 available in JGV Online) were used to quantify relative amounts of target transcripts. Target transcripts were normalized to GAPDH transcripts. Amplification from two log10 dilutions was routinely performed to ensure equal levels of the GAPDH mRNA standard. Non-GAPDH RT-PCR products were amplified from those dilutions of total RNA, which were determined as limiting for the detection of the specific transcript.

Northern blot analysis of specific transcripts. Total RNA was subjected to electrophoresis and transferred to nylon membranes. Probes were prepared by PCR with gene-specific primers and digoxigenin-labelled dUTP (Roche) for detection of specific transcripts (Table S1). Hybridization and detection were performed as described by roche manuals.

Western blot analysis of viral and cellular proteins. Cells were lysed according to established protocols (meyer et al., 2002). Equal amounts of whole cell, nuclear or cytosolic lysates were separated by SDS-PAGE or native PAGE and transferred to nitrocellulose membranes. Immunoblot analysis was performed using specific antibodies detecting MCMV IE1 (CROMA101), IRF3 (Zymed), phospho-IκBα (Ser32; Cell Signalling), IκBα (C-21; Santa Cruz), phospho-ATF-2 (Thr71; Cell Signalling), ATF-2 (C-19; Santa Cruz), phospho-c-Jun (Ser63; Santa Cruz), c-Jun (Cell signalling), GAPDH (Hy Test Ltd), Lamin A/C (Cell Signalling) and β-actin (Sigma). Proteins were visualized using the enhanced chemiluminescence-plus system (Amersham).

Electrophoretic mobility shift assay (EMSA). Cells were infected and washed before being lysed and analysed as described previously (Zimmermann et al., 2005). Nuclear lysates were incubated with 1 ng 32P-labelled probe (x-basis: 5’-AGTTAGGGACCTTTCCCAGGC-3’; Santa Cruz). Binding activity was visualized by autoradiography.

Immunofluorescence microscopy. Subconfluent NIH3T3 fibroblasts were grown on coverslips and infected with MCMV. At different time points post-infection (p.i.) cells were fixed with 3% paraformaldehyde for 20 min before being permeabilized with 0.02% saponin/PBS for 30 min. Cells were subsequently incubated with primary antibodies detecting MCMV IE1/pp89 (CROMA101) and IRF3 (Zymed) for 1 h at room temperature, washed with 0.002% saponin/PBS and incubated for 1 h with secondary antibodies conjugated with Cy2 and Cy3, respectively (jackson ImmunoResearch). Cells were counterstained with DAPI. Coverslips were sealed on slides and cells were visualized using Nikon TE2000 microscope and LUCIA 4.60.

RESULTS

MCMV shuts down IFN-α/β gene expression after an initial phase of induction

When analysing an MCMV mutant lacking M27, a gene encoding an inhibitor of STAT2-mediated IFN signalling, an
increased sensitivity of replication to exogenously added type I IFN was observed, whereas ΔM27-MCMV replication was wt-like in the presence of endogenously produced IFN (Zimmermann et al., 2005). Based on this observation we hypothesized that MCMV induces only very low amounts of endogenous type I IFN in fibroblast cultures and may be able to avoid IFN gene induction. Type I IFN activities detected in the supernatant of infected MEF yielded approximately 10–20 U ml⁻¹, whereas MEF infected with UV-inactivated MCMV produced 30–50 U ml⁻¹ (data not shown). Next, we monitored gene transcription of IFN-β and IFN-α4 (type I IFNs directly induced upon infection) during the MCMV replication cycle in infected fibroblasts by semi-quantitative RT-PCR analysis. IFN-β and IFN-α4 mRNA were detected in infected fibroblasts 2–5 h p.i. (Fig. 1a), but the expression of both IFN-β and IFN-α4 was rapidly diminished by 6 h p.i. Downregulation of IFN gene expression was dependent on the infectious dose. Infection with a lower m.o.i. resulted in a more delayed IFN-β and IFN-α4 downregulation compared with a higher m.o.i. (Fig. 1a). The observed shut down of IFN-α/β production required MCMV gene expression since UV-inactivated MCMV was not able to reduce type I IFN levels with the same kinetics (Fig. 1b). Restriction of MCMV gene expression by the late phase inhibitor phosphonoacetic acid had no effect (data not shown). In conclusion these findings suggest a time and dose-dependent interference of MCMV IE/early gene products with the IFN-β and IFN-α4 gene induction.

**MCMV actively disrupts IFN-β induction**

IFN-β gene expression in fibroblasts is limited to a short period of time after MCMV infection. Two explanations are conceivable. One possibility is a lack of induction, i.e. the MCMV stimuli, leading to IFN-β induction, fall below a critical threshold at later time points of MCMV infection. Alternatively, the loss of IFN-β transcription could be due to active inhibition by MCMV. To distinguish between these possibilities, we performed superinfection experiments using MCMV to ensure that the second stimulus bears the same PAMP. Fibroblasts were first infected with MCMV, then 4 h later the cells were superinfected with MCMV. After 3 h (i.e. 7 h after the primary infection), IFN-β gene expression was analysed. In contrast to primary MCMV infection, 3 h p.i. IFN-β transcription remained undetectable upon MCMV superinfection (Fig. 2b), suggesting active inhibition rather than lack of IFN stimulation. To ensure efficient gene expression of superinfecting virions, a ΔM84-MCMV deletion mutant was used as primary virus. M84 is expressed with early kinetics and M84 transcripts are detectable early after infection (Fig. 2b). As shown in Fig. 2(c), the lack of IFN-β transcription coincided with intact M84 transcription of superinfected wt-MCMV, indicating active inhibition of IFN production by MCMV gene expression. We used SeV, a potent inducer of IFN-β production (Wathelet et al., 1998), as an external stimulus to test whether the ability of MCMV could also prevent SeV-induced IFN-β transcription. For determination of the appropriate time points for coinfection experiments with MCMV, we measured IFN-β gene expression at different time points after SeV infection. Initiation of IFN-β transcription upon SeV infection was delayed (6 h p.i., Supplementary Fig. S1a available in JGV Online) compared with induction by MCMV infection (2 h p.i.). Coinfection with MCMV abolished activation of the IFN-β promoter by SeV (Fig. 2d, e). The effectiveness of IFN-β inhibition depended on the time point and order of MCMV and SeV infection. The earlier MCMV coinfection
was allowed the stronger was the inhibition of SeV-induced IFN-β gene expression (Fig. 2d). Coinfection with UV-inactivated MCMV did not diminish IFN-β induction upon SeV infection (Fig. 2e), indicating that MCMV gene expression is required to mediate the inhibitory effect. IFN-β gene expression seemed to be even more pronounced under this condition. As a proof for SeV replication in MCMV-coinfected cells, SeV nucleoprotein (SeV NP) transcripts were determined (Fig. 2d, e; Supplementary Fig. S1b available in JGV Online). Taken together these findings support the notion that MCMV codes for suppressive factors of the IFN induction pathway.

**MCMV downregulates IRF3 phosphorylation, dimerization and nuclear accumulation after an initial phase of activation**

To get insight into the pathways leading to the initial IFN-β gene induction we tested the activation of the transcription factors forming the IFN-β enhanceosome (Maniatis et al., 1998). First we investigated the activation of IRF3 by detection of phosphorylated and dimerized IRF3. Due to the lack of available phospho-specific antibodies recognizing mouse IRF3, we displayed phosphorylation of IRF3 by the altered migration property of phosphorylated proteins in SDS gels. The dimerization was analysed by native PAGE. Our data revealed that MCMV infection leads to phosphorylation and dimerization of IRF3, which only lasted transiently. A decrease of phosphorylated and dimerized IRF3 occurred with proceeding time of infection (Fig. 3a). Next, subcellular distribution of IRF3 was compared in non-infected and MCMV-infected cells using immunofluorescence (IF) microscopy (Fig. 3b). IF analysis revealed a translocation of IRF3 to the nucleus at 3 h p.i., which was not observed at 7 h p.i., in line with the absence of IRF3 phosphorylation and dimerization at this phase of MCMV replication (Fig. 3a). This effect parallels the decrease of IFN-β and IFN-α4 expression in MCMV-infected cells and was accompanied by
a transient expression of an IRF3 target gene, ISG56 (data not shown). The rapidity and efficiency of the MCMV-mediated inhibition accentuates the important role of IRF3 in antiviral response.

NF-κB signalling is controlled at multiple steps of the activating pathways

The NF-κB transcription factor controls the expression of diverse host genes mediating cell survival and encoding proinflammatory and immune response proteins, including IFN-β (Kucharczak et al., 2003). In non-activated cells NF-κB is bound by IκBα, preventing nuclear translocation by masking the nuclear localization site of NF-κB. Activation of NF-κB is the consequence of phosphorylation and subsequent proteasomal degradation of IκBα. Thus, the cytoplasmic protein level of IκBα indicates the activation status of NF-κB. We analysed IκBα protein levels in MCMV-infected cells and found degradation of IκBα at 3 h p.i., indicating NF-κB activation, but this status only lasted briefly and IκBα levels were restored within 6 h p.i. (Fig. 4a) and maintained throughout MCMV replication (data not shown). Next, we tested whether tumour necrosis factor (TNF-α)-α, which is a potent activator of NF-κB (Pfeffer, 2003), was able to stimulate NF-κB in MCMV-infected fibroblasts. In uninfected cells, treatment with TNF-α for 30 min resulted in a substantial reduction of IκBα protein levels, while in cells infected with MCMV for 7 h the IκBα amount remained unaltered (Fig. 4b), pointing towards an effective MCMV-mediated block of the TNF-α-mediated signalling cascade already at early time points of infection. To confirm that the cellular IκBα protein levels are in fact a true marker for the NF-κB activation status, we analysed IκBα phosphorylation and DNA binding of activated NF-κB complexes in MCMV-infected cells. Immunoblot analysis with a phospho-IκBα-specific antibody demonstrated the expected coherence between IκBα phosphorylation and degradation (Fig. 4c). EMSA analysis using a κB-consensus site as probe revealed increased levels of DNA-binding complexes peaking at 4 h p.i., which disappeared by 6 h p.i. and later time points (Fig. 4c). The specificity of the probe was controlled by using lysates from TNF-α-treated cells and addition of competitive unlabelled oligonucleotides (Fig. 4c). To gain insight into the mechanisms used by MCMV to interfere with the NF-κB pathway, we tested the stability of IκBα protein in MCMV-infected cells. Using cycloheximide (CHX) blocking protein biosynthesis the intrinsic half-life of IκBα was found to be approximately 50 min, whereas the IκBα half-life was prolonged for more than 6 h in cells infected with MCMV (Fig. 4d). The observed ability of MCMV to extend the stability and to increase steady-state levels of IκBα depends on viral gene product(s) that are expressed after the initial phase of NF-κB activation, since IκBα half-life is not altered before 4 h p.i. (data not shown). From these findings we conclude that activation of NF-κB is under tight temporal control of MCMV, which is kept at multiple checkpoints of the pathway.
MCMV interferes with ATF-2/c-Jun activation

The heterodimeric transcription factor ATF-2/c-Jun is also part of the IFN-β enhanceosome. When analysing the phosphorylation and activation status of ATF-2/c-Jun upon MCMV infection, we noted an initial phase of activation followed by a continuous decrease of phosphorylated ATF-2 (Fig. 5a). However, the kinetics of activation and subsequent downregulation were less rapid when compared with the deactivation of IRF3 and NF-κB signalling (see Figs 3 and 4). At 24 h p.i., MCMV also prevented the restimulation of ATF-2 phosphorylation induced by external activation, e.g. UV-treatment (Fig. 5b). Analysis of nucleoplasmic lysates showed a peak of activated ATF-2 at 4 h p.i. and declining thereafter, compatible with a progression of viral inhibition. Interestingly, the reduction of phosphorylated ATF-2 occurred mainly in the nucleoplasmic extracts, while cytoplasmic levels were maintained. This finding suggests that phosphorylated ATF-2 protein is retained in the cytoplasm or rapidly dephosphorylated in the nucleus at early time points after infection (Fig. 5c). The purity of the lysates was controlled by measuring the cytoplasmic and nucleoplasmic markers GAPDH and Lamin A/C (Fig. 5c). UV-treatment of MCMV-infected cells induced only marginal phosphorylation of ATF-2 (Fig. 5b). The subcellular localization of this activated ATF-2 was predominantly found in the cytoplasm (data not shown), confirming that MCMV interferes independently with both phosphorylation and nuclear translocation of ATF-2/c-Jun.

The initial phase of IFN-β induction is prolonged in MCMV-infected macrophages

To analyse the ability of MCMV to interfere with type I IFN induction in further permissive cell types, IC-21 macrophages were analysed. We found that the phase of IFN-β transcription was prolonged when compared with infected fibroblasts (Fig. 6a, b). Nonetheless, levels
of IFN-β transcripts were continuously decreased over time and complete downmodulation of IFN-β gene expression was achieved at 38 h p.i. and sustained during the MCMV replication cycle, which is more protracted as compared with fibroblasts (data not shown). Similar kinetics were observed with IFN-α4 (data not shown). Interestingly, the efficiency of downregulation of IFN-β transcription was inversely correlated with the progression of MCMV gene expression represented by the regulation of MCMV ie1 transcription (shown by Northern blot analysis, Fig. 6) and genome replication (data not shown). We conclude that MCMV-mediated downregulation of type I IFN transcription takes place in different productively infected cells, albeit with different kinetics.

DISCUSSION

It is now over 50 years ago that type I IFN was discovered when Isaacs and Lindenmann observed that chicken fibroblasts exposed to influenza virus started to produce a cytokine interfering with influenza virus replication (Isaacs & Lindenmann, 1957). Here, we have analysed type I IFN induction in mouse fibroblasts by replicating and UV-inactivated MCMV. We show that MCMV sustains a long lasting inhibition of IFN-α/β gene expression after an initial phase of induction. IFN gene transcription results from a rapid activation of IRF3, NF-κB and ATF-2/c-Jun transcription factors upon infection. However, transcription of IFN-α4 and IFN-β occurs only transiently due to the MCMV-encoded capacity to downmodulate the IRF3, NF-κB and ATF-2/c-Jun signalling cascades. The unresponsiveness of MCMV-infected cells exposed to external stimuli including SeV, TNF-α treatment or UV-irradiation confirmed that MCMV actively blocks all of the signalling cascades implicated in IFN-α/β production.

MCMV infection activates IFN-α/β inducing signalling pathways

MCMV triggers all IFN-α/β inducing signalling cascades early upon infection during a short period of time before activation of transcription factors becomes downregulated and IFN-α/β production is abolished. The distinct activation kinetics indicate that ATF-2/c-Jun stimulation occurs unrelated to IRF3 and NF-κB activated pathways. Our data reveal that the activation of ATF-2/c-Jun occurs...
for a longer period of time after MCMV infection when compared with IRF3 and NF-κB. Given the proviral capabilities of ATF-2/c-Jun, as observed in the context of influenza virus infection (Ludwig et al., 2001), it is conceivable that ATF-2/c-Jun transcription is also exploited by MCMV to enhance replication efficiency. Compatible with this idea, earlier reports confirmed c-Jun to be involved in regulation of CMV ie transcription (Lee et al., 2004; Wang & Sonenshein, 2005). Nonetheless, the cellular components responsible for IFN-α/β induction by replicating MCMV lying upstream of the transcription factors are not clear. Several studies have identified Toll-like receptor (TLR) pathways activated upon MCMV infection of mice or dendritic cells (DCs), leading to production of type I IFN (Krug et al., 2004; Tabeta et al., 2004) although TLR-independent, MyD88-dependent and -independent perception of MCMV occurs as well (Delale et al., 2005). The TLR3/Trif and TLR9/MyD88 pathways are activated by distinct microbial components and reported as sensors of infection limiting MCMV replication in vivo (Tabeta et al., 2004). While TLR3 is broadly expressed including in fibroblasts and thus likely to be relevant in our experimental setting, TLR9 expression appears restricted to a few cell types including DCs (Perry et al., 2005). Both TLRs interact with the endoplasmic reticulum protein UNC93B via their transmembrane regions before reaching endosomal compartments (Brinkmann et al., 2007). Remarkably, a single point mutation in UNC93B disrupting this interaction abolishes TLR signalling and leads to a lack of proinflammatory cytokine production in MCMV-infected mice, which includes IFN-β (Tabeta et al., 2004, 2006). The UNC93B mutation is associated with strongly enhanced MCMV replication and even fatal disease, highlighting the essential role of UNC93B- and TLR3/9-dependent signalling for MCMV immune control. To date, the molecular signals delivered by MCMV that are detected by host pattern recognition receptors are not yet defined. The existence of TLR-independent receptors for intracellular DNA has been hypothesized (Ishii & Akira, 2006; Ishii et al., 2006; Stetson & Medzhitov, 2006); this would represent obvious sensors for MCMV. The recently identified cytosolic DNA sensor DAI (Takaoka et al., 2007) could be involved in the TLR-independent recognition of MCMV. For HCMV, several components including gB and gH (Yurochko et al., 1995, 1997), the TNF-receptor homologue UL144 (Poole et al., 2006) and virion-associated activated casein kinase (Nogalski et al., 2007) were reported to induce an extended activation of NF-κB and IRF3 (Yurochko et al., 1997; Boehme et al., 2004). HCMV stimulates NF-κB activity binding to HCMV promoter elements (Sambucetti et al., 1989; Sun et al., 2001), and complete repression of NF-κB signalling was found only in the late phase of HCMV replication (Jarvis et al., 2006). In contrast, the MCMV-mediated inhibition of TNF-α-induced IkBα degradation occurs early after infection (Fig. 4) and becomes manifested in the presence of late phase inhibitors like phosphonoacidic acid (data not shown). Taken together, our findings are compatible with the notion that HCMV infection generates higher threshold levels of NF-κB activity compared with MCMV where a sustained repression of NF-κB signalling dominates after a short phase of induction.

**MCMV-mediated inhibition of IRF3, NF-κB and ATF-2/c-Jun-dependent signalling**

Sustained repression of IFN-α/β transcription results from the concerted inhibition of IRF3, NF-κB and ATF-2/c-Jun transcription factors. Coinfection experiments with SeV revealed that not only MCMV-induced IFN-β expression but RG-I helicase-dependent induction (by RNA viruses; Kato et al., 2006) is inhibited. This blockade requires MCMV gene expression as UV-inactivated virions were not able to mediate this inhibition and was likely caused by multiple and independent MCMV factors. This assump-

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**Fig. 6.** The initial phase of IFN-β induction is prolonged in macrophages. NIH3T3 fibroblasts (a) and IC-21 macrophages (b) were mock-infected or infected with MCMV (NIH3T3: m.o.i. 5; IC-21: m.o.i. 15). At the indicated time points p.i., cells were harvested for RNA extraction. IFN-β expression was analysed by semi-quantitative RT-PCR using specific primers. MCMV ie1 transcripts were determined by Northern blot analysis with a genespecific DIG-labelled probe.
tion is supported by the observation that the down-regulation of IRF3, NF-κB and ATF-2/c-Jun followed pathway-specific kinetics. In remarkable contrast to the reported mechanisms of NF-κB inhibition by HCMV (Montag et al., 2006; Jarvis et al., 2006; Taylor & Bresnahan, 2006a), we found that MCMV infection influences the intrinsic half-life of IkBα (Fig. 4) and downregulates the basal NF-κB activity. The HCMV tegument protein pp65 (pUL83) was reported to counteract innate antiviral defence, including NF-κB activity (Browne & Shenk, 2003) and IRF3 activation (Abate et al., 2004). In addition, an indirect effect on inhibition of IFN-β expression by deletion of the UL83-ORF was described previously (Taylor & Bresnahan, 2006b). Our data exclude the MCMV gene homologues of HCMV UL83, i.e. MCMV M83 and M84, to be major inhibitors of IFN-β production (see Supplementary Material available in JGV Online). Deletion of M83 or M84 from the MCMV genome did not affect the downregulation of IRF3 nor impair the recovery of IkBα protein levels, indicating that the MCMV homologues may serve non-conserved functions. Moreover, induction and repression of IFN-β transcription in MCMV-infected cells occur independently of the MCMV gene M27 (see Supplementary Material) encoding a selective inhibitor of STAT2-mediated IFN-α/β receptor signalling (Zimmermann et al., 2005). Therefore, autocrine type I IFN receptor signalling does not contribute to the initial phase of IFN-α/β induction nor does it influence repression of IFN-α/β transcription by MCMV.

DCs respond to MCMV with a strong type I IFN production (Krug et al., 2004; Tabela et al., 2004; Delale et al., 2005; Andoniou et al., 2005). At a first glance, our findings seem to be incompatible with those studies lacking any indication of MCMV-mediated suppression of IFN production. We found that inhibition of IFN-α/β transcription requires MCMV gene expression (Fig. 1). A reason for the obvious discrepancy in IFN-α/β gene induction between cell types might be the limited MCMV gene expression in DCs, especially mature DCs, which are very poor in replicating genomes when compared with fibroblasts (Mathys et al., 2003). Interestingly, the window of active IFN-β transcription was prolonged in IC-21 macrophages when compared with fibroblasts (Fig. 6), suggesting that the extent of inhibition may differ between cell types and could be low in cells with enhanced signalling and cytokine secretion capabilities like DCs, which are known as potent producers of type I IFN in MCMV infection (Krug et al., 2004). Exerting proinflammatory effects type I IFNs contribute to cytokine-mediated symptoms of virus disease (Vilcek, 1984). Given the usually asymptomatic course of primary and reactivated CMV replication in immunocompetents, limiting the amount of type I IFN secretion in productively infected tissues may indeed contribute to host health and establish a suitable condition for host–virus coexistence. Albeit type I IFNs are relatively poor inhibitors of MCMV replication on their own, they can reach sufficient antiviral priming of neighbouring cells even at low concentrations in combination with synergistic cytokines like lymphotoxins and IFN-γ (Benedict et al., 2001; Zimmermann et al., 2005).

**Potential consequences of IFN-α/β repression and CMV accommodation to an IFN producing environment**

How to reconcile the contrasting findings of IFN-α/β production in fibroblasts and DCs? It appears that MCMV creates ‘virus factories’ in certain cell types (e.g. fibroblasts), i.e. an intracellular milieu optimized for genome replication and morphogenesis (Novoa et al., 2005). In these cells IFN-α/β induction is strictly avoided, while in other cell types prototypically represented by plasmacytoid DCs (pDC) IFN induction is tolerated or even desired. By means of a rigorous interference with type I IFN production in selected tissues a high yield of progeny virus could be reached, sufficient for horizontal spread within the infected host. The CMV interference with IFN production is complemented by further independent strategies counteracting the efficacy of IFNs. Through establishing a state of IFN receptor unresponsiveness by disrupting Jak/STAT signalling and antiviral gene expression (Heise et al., 1998; Presti et al., 2001; Zimmermann et al., 2005) MCMV builds protected ‘virus factories’ enabling efficient viral replication even in the presence of significant concentrations of IFNs. On the other hand MCMV triggering of IFN producing capacities in pDCs could be essential for the augmenting of natural killer and cytotoxic T lymphocyte responses attaining a balanced virus–host relationship. In such a scenario robust type I IFN production in some cells and blocked IFN production in other cells appear to be a barter deal between MCMV and the host establishing détente in the adversities of antiviral defence. With the availability of an IFN-β reporter mouse model this concept will become amenable to experimental verification.

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