Checks and balances between human cytomegalovirus replication and indoleamine-2,3-dioxygenase

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Despite a rigorous blockade of interferon-γ (IFN-γ) signalling in infected fibroblasts as a mechanism of immune evasion by human cytomegalovirus (HCMV), IFN-γ-induced indoleamine-2,3-dioxygenase (IDO) has been proposed to represent the major antiviral restriction factor limiting HCMV replication in epithelial cells. Here we show that HCMV efficiently blocks transcription of IFN-γ-induced IDO mRNA both in infected fibroblasts and epithelial cells even in the presence of a preexisting IFN-induced antiviral state. This interference results in severe suppression of IDO bioactivity in HCMV-infected cells and restoration of vigorous HCMV replication. Depletion of IDO expression nonetheless substantially alleviated the antiviral impact of IFN-γ treatment in both cell types. These findings highlight the effectiveness of this IFN-γ-induced effector gene in restricting HCMV productivity, but also the impact of viral counter-measures.

INTRODUCTION

Interferon-γ (IFN-γ) directly induces antimicrobial and antiviral transcriptional programmes leading to effector mechanisms preventing pathogen multiplication (Schroder et al., 2004). IFN-γ initiates the Jak-STAT signalling pathway (Darnell Jr, 1997; Stark et al., 1998) resulting in transcription of IFN-γ dependent genes. Indoleamine-2,3-dioxygenase (IDO) is strongly induced by IFN-γ. It catalyses the conversion of tryptophan to N-formylkynurenin, resulting in tryptophan depletion, thereby inhibiting the growth of bacteria (MacKenzie et al., 1998) and parasites (Däubener et al., 2001). Antiviral properties of IDO against several viruses, including measles (Obojes et al., 2005), herpes simplex types 1 and 2 (Adams et al., 2004a,b) and vaccinia virus (Terajima & Leporati, 2005), have been reported. Additionally, IDO was proposed to represent the prime effector restricting human cytomegalovirus (HCMV) growth in retinal pigment epithelium (RPE) cells when induced by IFN-γ (Bodaghi et al., 1999a). Beyond the well characterized enzymatic properties of IDO there is growing evidence that IDO exerts immune regulatory functions independently of its catalytic activity (Pallotta et al., 2011; Orabona et al., 2012).

Cytomegaloviruses (CMVs) belong to the herpesvirus β-subgroup and persist for life in infected hosts. As they replicate under continuous pressure of the immune system, CMVs have evolved effective mechanisms that counteract IFN-mediated antiviral defence (Trilling et al., 2012; Zimmermann & Hengel, 2006). HCMV employs several independent mechanisms to interfere with IFN-γ signal transduction in infected cells. In productively infected fibroblasts, HCMV blocks signal transducer and activator of transcription 1 (STAT1) tyrosine phosphorylation as early as 10 h p.i. and enhances cellular tyrosine phosphatase (SHP2) activity (Baron & Davignon, 2008). Subsequently, HCMV decreases Jak1 levels by a proteasome dependent mechanism (Miller et al., 1998) and prevents IFN-γ induced gene expression including MHC class II molecules (Miller et al., 1998) and transcription of immunoproteasome subunits (Khan et al., 2004). Hence, a selective IFN-γ-dependent induction of IDO appears hardly reconcilable with the strict control of IFN-γ signalling by HCMV, particularly when taking into account that the IFN-γ-dependent iNOS pathway was reported to be blocked under the same conditions (Bodaghi et al., 1999a). However, the commensurability of these studies is limited by the fact that
different HCMV permissive cell types and different HCMV strains were analysed. Moreover, a recent report indicated that HCMV infection itself might induce IDO expression through an IFN-γ-like transcriptional response mediated by the viral immediate early 1/pp72 protein (Knoblach et al., 2011). These conflicting data prompted us to reinvestigate whether and to what extent IDO induction escapes from HCMV control in epithelial cells, fibroblasts or both types of cells.

RESULTS

HCMV controls IFN-dependent IDO induction in infected fibroblasts

IDO has been reported to be induced upon IFN-γ treatment of HCMV-infected cells (Bodaghi et al., 1999a). Hence, we infected MRC-5 fibroblasts with HCMV AD169 and added 500 U ml⁻¹ IFN-γ simultaneously to induce IDO expression. High levels of IDO protein were readily detected in uninfected fibroblasts (Fig. 1a). However, no IDO protein was seen in HCMV-infected cells at any time post-infection indicating that HCMV blocked IDO induction (Fig. 1a). To determine whether a complete block of IDO induction was a regular event during HCMV infection we also investigated the endotheliotropic HCMV strain TB40/E and the clinical isolate UL1271 (Atalay et al., 2002). Progression of HCMV replication was followed by detection of HCMV non-structural proteins IE1/pp72 and IE2/pp86 as well as the matrix protein UL83/pp65 in lysates of infected cells. Both strains completely abolished IDO induction in infected cells.

To analyse the fate of IDO when already induced by IFN-γ added before HCMV infection, we monitored IDO protein expression in fibroblasts after 48 h preincubation with 500 U ml⁻¹ IFN-γ. Thereafter, IFN-γ-conditioned cells were either mock infected or infected with HCMV AD169 (5 p.f.u. per cell) and further exposed to IFN-γ for the remaining observation period. Expression of IDO protein was continuously increasing in uninfected cells (Fig. 1b). In HCMV-infected IFN-pretreated cells no increase of IDO protein expression was observed. In contrast, IDO expression declined over time in HCMV-infected cells (Fig. 1b). Again, similar results were obtained in IFN-pretreated cells infected with TB40/E and HCMV UL1271 (Fig. 1b). In agreement with earlier studies (Hengel et al., 1995), the extensive IFN-γ preincubiation had only a rather limited effect on HCMV gene expression. The immediate early gene product pp72 was expressed with the same temporal kinetics but at moderately reduced levels (compare respective lanes in Fig. 1a and Fig. 1b). Likewise, IFN-γ preincubation had only modest effects on pp65 abundance in infected cells (compare respective lanes in Fig. 1a and Fig. 1b).

In summary, the data demonstrated that HCMV efficiently prevents IDO protein synthesis in infected cells. Most importantly, the HCMV control of IDO induction was also effective in cells pretreated with IFN-γ and thereby exhibiting an IFN-induced antiviral state.

HCMV infection blocks IDO protein induction but not enzyme activity

To investigate whether HCMV is able to directly inhibit IDO enzyme function we monitored IDO catalytic activity by kynurenine production in HCMV-infected or mock-infected MRC-5 fibroblasts treated with IFN-γ (500 U ml⁻¹). In uninfected cells, IDO activity was rapidly detected within 8 h, reaching a maximum level 48 h after treatment with IFN-γ (Fig. 2a). As expected, infection with HCMV AD169 or TB40/E almost completely abolished IDO activity in infected cells (Fig. 2a). In IFN-γ pretreated cells, IDO activity was clearly detectable at the time point of infection and became further increased over time in mock-infected cells. In contrast, IDO activity continuously decreased upon HCMV infection with both AD169 and TB40/E strains (Fig. 2b). IDO activity largely correlated with the amount of IDO protein determined in both uninfected and HCMV-infected cells. Hence, HCMV infection had no obvious impact on the catalytic activity of preexisting IDO protein, indicating that HCMV controls IDO induction but does not actively inhibit or deplete preexisting IDO protein from infected cells.

HCMV controls IDO expression at the level of gene transcription

Next, we analysed IDO gene transcription by quantitative RT-PCR of IDO mRNA. IFN-γ treatment of MRC5 cells resulted in a more than 10000-fold induction of IDO mRNA within 24 h (Fig. 3a). In contrast, only a 20-fold increase of IDO mRNA transcripts was transiently observed in infected cells, peaking 24 h p.i. (Fig. 3a). As shown above, this transient mRNA induction was not sufficient to induce detectable amounts of IDO protein (Fig. 1a).

In IFN-γ pretreated cells the abundance of IDO mRNA was moderately enhanced by further IFN-γ incubation of uninfected cells (Fig. 3b). In contrast, HCMV infection resulted in a rapid and constant decline of IDO mRNA transcription, reaching a 5000-fold lower level after 48 h of infection (Fig. 3b), indicating that HCMV was able to efficiently counteract IDO mRNA induction even in IFN-γ pretreated cells. The decrease of mRNA production correlates closely with the decrease of IDO protein concentrations observed before (Fig. 1b).

To analyse whether the known inhibition of Jak-STAT signalling by HCMV was sufficient to explain the reduction of IDO mRNA transcription we used the Jak1-inhibitor pyridone-6 (Sarkis et al., 2006). To this end, we determined the decline of IDO transcription induced by a complete chemical block of Jak-STAT signalling and compared these kinetics to the effect of HCMV infection. HCMV infection even exceeded the effect obtained by Jak1 inhibition (Fig. 3c). To ensure that the concentration of pyridone-6 was
sufficient to block the Jak-STAT-mediated biological effect we analysed HCMV replication in the presence or absence of IFN-γ and pyridone-6. While HCMV replication was reduced by 2 log_{10} in the presence of 500 U ml^{-1} IFN-γ, HCMV growth restriction was completely reversed by the chemical interruption of Jak-STAT signalling using 0.2 mM pyridone-6 (Fig. 3d). Of note, the data also show that the modest alterations of HCMV protein expression documented in Fig. 1 are translated into a substantial titre reduction by IFN-γ.

In summary, the data demonstrated that HCMV prevents IDO transcription rigorously even in spite of a preexisting IFN-γ induced antiviral state. The observation that HCMV caused a faster decay of IDO mRNA than pharmaceutical Jak1 inhibition suggests that HCMV – in addition to the well-known block of Jak-STAT signalling – might directly affect transcription or stability of IDO mRNA.

**Cell-type dependent IDO induction is due to differences in HCMV gene expression**

The rigorous control of IDO induction observed in fibroblasts clearly challenged the notion that IDO represents the principal, if not exclusive, effector function of IFN-γ against HCMV as concluded from studies performed in epithelial cells (Bodaghi *et al.*, 1999a). Therefore, we extended our analysis to RPE cells. Analysis of HCMV AD169-infected RPE cells exposed to IFN-γ from the time point of infection revealed that IDO activity was indeed fully induced at 24 h p.i. (Fig. 4a), contrasting with the findings made with fibroblasts (Fig. 2a). However, despite the initial generation of

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**Fig. 1.** HCMV controls IDO induction in infected fibroblasts. (a) HCMV blocks IDO protein expression. MRC-5 cells were either mock infected or infected with HCMV AD169, HCMV TB40/E and the clinical HCMV-isolate UL1271 (5 p.f.u. cell^{-1} for all). IFN-γ (500 U ml^{-1}) was added at the time point of infection. Equivalent amounts of cell lysates prepared at the indicated time point post-infection (p.i.) were separated by SDS-PAGE before immunoblotting using antibodies against IDO, HCMV IE1-pp72/ pp86, HCMV-pp65 and β-actin. Each blot was representative of at least three independent experiments. (b) HCMV limits IDO protein expression in IFN-treated cells. MRC-5 cells were treated with IFN-γ (500 U ml^{-1}). After a 48 h preincubation period with IFN-γ cells were either mock infected or infected with HCMV AD169, HCMV TB40/E and the clinical HCMV-isolate UL1271 (5 p.f.u. cell^{-1} for all). IFN-γ (500 U ml^{-1}) was added again from the time point of infection. Cell lysates were prepared at the indicated time point p.i. Equivalent amounts of lysate were separated by SDS-PAGE and immunoblotted as in (a). Each blot was representative of at least three independent experiments. Corresponding experiments in (a) and (b) were run in parallel and detected from the same membrane to allow comparative analysis.
IDO the capability of HCMV to downregulate IDO bioactivity was readily recovered and maintained when HCMV infection proceeded (Fig. 4a).

In order to clarify the differential kinetics of IDO induction we compared HCMV gene expression in fibroblasts and epithelial cells. Whereas IE1/pp72 protein was detectable as early as 4 h p.i. in MRC-5 cells, it took almost 24 h in RPE. Likewise, reemergence of pp65 in lysates of RPE-infected cells was delayed until 48 h p.i. after the disappearance of initial pp65 protein derived from incoming HCMV particles. Hence, initial IDO induction in IFN-γ treated cells correlated with an obvious delay of HCMV gene expression even in the absence of IFN-γ when compared to MRC-5 fibroblasts (Fig. 4b). In the presence of IFN-γ, IE1 and pp65 were expressed with the same kinetics but to lower amounts compared to untreated RPE cells (Fig. 4b). The data indicated that HCMV gene expression in RPE is retarded with regard to IE and E gene products (Fig. 4b), explaining the initial IFN responsiveness of RPE cells due to the prolonged but transient absence of HCMV IFN antagonists after infection.

To verify these results and to rule out any bias by unequal infection rates of RPE cells versus fibroblasts, we assessed the responsiveness of individual HCMV-infected cells to IFN-γ by flow cytometry, taking advantage of the EGFP-expressing HCMV recombinants AD169-GFP (Borst & Messerle, 2000) and TB40-GFP, respectively. In both mutants the known HCMV-encoded inhibitors of MHC class II (Tomazin et al., 1999; Hegde & Johnson, 2003; Hegde et al., 2002) were deleted. Hence, HLA-DR was monitored as a known IFN-γ inducible marker without constitutive expression on the surface of MRC-5 and RPE cells (Fig. 4c, panels i, v, ix). HCMV-infected MRC-5 cells completely blocked IFN-dependent induction of HLA-DR throughout the replication cycle (Fig. 4c, panels ii–iv). In contrast, the majority of infected RPE cells exhibited HLA-DR surface expression when treated from the time point of infection (Fig. 4c, panel vi), indicating an almost intact responsiveness to IFN-γ. However, by 24 h p.i. almost 60 % of the infected RPE cells had lost their IFN responsiveness (Fig. 4c, panel vii). IFN-γ addition 48 h p.i. did not cause an increase of HLA-DR surface expression in the majority of infected RPE cells (Fig. 4c, panel viii), indicating that HCMV had established a complete block of IFN-γ signalling in infected RPE cells. In TB40-infected RPE cells the retardation of HCMV replication appeared even more pronounced. However, the eventual recovery of HCMV IFN antagonism during virus replication was also observed in this setting (Fig. 4c, panels x–xii). In conclusion, HCMV efficiently blocked IFN-γ signalling in both cell types, but with strikingly different kinetics.

**Contribution of IDO to IFN-γ mediated control of HCMV replication**

To determine the impact of differential induction of HCMV IFN antagonistic gene products on replication in MRC-5 versus RPE cells we analysed the IFN-γ mediated growth restriction under the same conditions used for HCMV protein analysis. In cells treated with IFN-γ, from the time point of infection no significant growth restriction...
was achieved by IFN-γ in MRC-5 whereas a substantial titre reduction (45-fold, $P<0.05$) was seen in RPE cells (Fig. 5a). After 48 h of preincubation of cells with IFN-γ, comparable titre reductions (110-fold and 160-fold, respectively) were seen. Hence, IDO induction and HCMV titre reduction appeared to correlate directly.

To investigate the contribution of IDO to the IFN-γ mediated effect, we performed HCMV growth analysis after 48 h of preincubation of cells with IFN-γ supplemented or not with 200 μg ml$^{-1}$ L-tryptophan. IFN-γ mediated a substantial reduction (400-fold, $P<0.05$) of HCMV replication in both MRC-5 and RPE cells (Fig. 5a, b). Complementation with L-tryptophan resulted in a partial rescue of HCMV replication. We observed a statistically significant increase of AD169 replication (10-fold in MRC-5, 20-fold in RPE cells, $P<0.05$) by tryptophan complementation (Fig. 5a). At the same time, IFN-γ still mediated a significant residual HCMV titre reduction in the presence of L-tryptophan (40-fold in MRC-5, 20-fold in RPE cells, $P<0.05$). It should be noted that a complete rescue of HCMV replication in IFN-γ treated cells was achieved by blocking Jak-STAT signalling via the Jak1 inhibitor pyridone-6 (Fig. 5c, d).
(a) 

IFN-γ incubation

0–24 24–48 48–72 h.p.i.

- No IFN-γ
- Uninfected + 200 U IFN-γ ml⁻¹
- HCMV-infected + 200 U IFN-γ ml⁻¹

IDO activity (µg kynurenine h⁻¹)

(b) 

HCMV TB40/E

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No IFN-γ incubation  No IFN-γ incubation  IFN-γ incubation from infection  48 h preincubation with IFN-γ

(c) 

IFN-γ incubation: none

0–72 24–72 48–72 h.p.i.

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IFN-γ-induced gene expression (HLA-DR)

IFN-γ-induced expression (µg kynurenine h⁻¹)
using the Jak inhibitor pyridone 6 (Fig. 3d). Essentially the same result was obtained when tryptophan complementation assays were performed with the endotheliotropic TB40/E strain (Fig. 5c).

To corroborate the results we employed an IDO siRNA to selectively remove IDO from IFN-γ-conditioned and HCMV-infected cells. IDO siRNA efficiently prevented IFN-γ-induced IDO protein synthesis 2 and 5 days post-transfection (Fig. 6a). Being aware that even minute amounts of IDO present after IDO knock-down might still provide a biological function, we checked whether the IDO knock-down was sufficient to inhibit an established highly sensitive tryptophan-dependent function (Schroten et al., 2001). To this end, we performed an IDO knock-down and subsequently added 100 c.f.u. of Staphylococcus aureus to cultures. While S. aureus growth was completely suppressed by IFN-γ treatment in control cells, the IDO knock-down completely rescued S. aureus from IFN-γ-mediated growth restriction (Fig. 6b). IDO siRNA proved to be equally effective in RPE cells (data not shown).

As expected, IDO siRNA treatment had no effect on HCMV replication in untreated fibroblasts and RPE cells. HCMV progeny determined in supernatants of IFN-γ pretreated, infected cells revealed a 400-fold reduction (P<0.05) of HCMV replication in MRC-5 and an 80-fold reduction (P<0.01) in RPE cells compared to untreated controls (Fig. 6c). HCMV replication in cells transfected with IDO siRNA was still reduced 40-fold and eightfold (P<0.05) in MRC-5 and RPE cells, respectively. Thus a substantial, but incomplete rescue from IFN-γ inhibition in both, MRC-5 and RPE cells (10-fold in both, P<0.05, Fig. 6c) was observed. These data corroborate the limited contribution of IDO to IFN-γ action observed by tryptophan complementation (Fig. 5a). However, in tryptophan complementation assays the IDO activity present in infected cells might even consume high amounts of tryptophan whereas minute amounts of IDO not detectable by Western blot might be sufficient to deplete tryptophan during siRNA experiments. Therefore, we combined both approaches and performed HCMV growth analysis on cells treated with siRNAs and supplemented with different amounts of tryptophan (Fig. 6d). With low tryptophan concentration (Fig. 6d, left panel) a substantial rescue was obtained (eightfold, P<0.05) reproducing siRNA-mediated rescue using HCMV TB40/E. High tryptophan substantially rescued HCMV replication (sevenfold, P<0.05). Importantly, no additive rescue was obtained by simultaneous siRNA treatment (Fig. 6d, right panel). Additionally, we monitored the kynurenine converted from tryptophan in the supernatants of infected cells at the end of the incubation period. Whereas high concentrations of kynurenine were detectable in supernatants from CTR-siRNA treated cells, IDO siRNA diminished tryptophan conversion to very low levels of mock-treated cells without IFN-γ (Fig. 6e). Hence, IDO siRNA effectively prevented tryptophan depletion in the supernatants of HCMV-infected cells. Yet, IFN-γ still mediated a significant residual HCMV titre reduction in both settings (10- and 20-fold, respectively, P<0.05). Taken together, these data identify IDO to represent an important effector among the IFN-γ induced factors mediating inhibition of HCMV replication. However, additional effectors must contribute to achieve the full potency of IFN-γ.

**DISCUSSION**

In this study, we demonstrated that HCMV rigorously controls the IFN-γ-dependent induction of IDO at the level of IDO mRNA transcription. This finding is consistent with previous work demonstrating a tight control of HCMV over IFN signal transduction via the Jak-STAT pathway (Baron & Davignon, 2008; Le et al., 2008; Miller et al., 1998; Paulus et al., 2006). On the other hand, induction of IDO mRNA was demonstrated in HCMV-infected RPE cells and fibroblasts (Baron & Davignon, 2008; Bodaghi et al., 1999a). While IDO transcription in MRC5 fibroblasts was found to decline during the course of HCMV Towne replication (Baron & Davignon, 2008), data from tryptophan complementation experiments performed with RPE cells led to the reverse conclusion that HCMV AD169 cannot counteract the IFN-induced IDO

**Fig. 4.** Cell-type specific delay of IDO repression in HCMV-infected cells. (a) Retarded inhibition of IDO induction in HCMV-infected RPE cells. RPE cells were either mock infected or infected with HCMV TB40/E at an m.o.i. of 5. IFN-γ (200 U ml⁻¹) was added at the indicated time point post-infection (p.i.). IDO activity (µg kynurenine h⁻¹) was determined from lysates prepared 24 h after addition of IFN. Each value represents the mean ± so from three independent determinations. (b) Expression of immediate early (IE) and early (E) genes in HCMV-infected RPE is delayed. MRC-5 (left panel) or RPE (right panels) as indicated were infected with HCMV TB40/E (5 p.f.u. per cell). Cells were either left untreated or were treated with 500 U ml⁻¹ IFN-γ from the time point with or without a 48 h period of preincubation with IFN-γ as indicated. Cell lysates were prepared at the indicated time point p.i. Equivalent amounts of lysate were separated by SDS-PAGE and then immunoblotted using antibodies IE-pp72/pp86, E-pp65 and β-actin. Each blot was representative of at least three independent experiments. (c) HCMV controls induction of IFN-γ dependent gene expression. AD169-GFP and TB40-GFP were used to infect MRC-5 (m.o.i. of 0.4) and RPE (m.o.i. of 4), respectively. Note that infection of RPE requires a higher virus dose to obtain comparable numbers of infected GFP-expressing cells. IFN-γ (200 U ml⁻¹) treatment was performed for the indicated time frames p.i. Cells were harvested 72 h p.i. and surface stained for expression of HLA-DR. GFP expression was used as an infection marker. The numbers in panels (i)–(xi) indicate the proportion of HCMV-infected cells (%) exhibiting HLA-DR surface expression (right upper quadrant) or not (left upper quadrant).
the time of HCMV replication. Tryptophan complementation experiments were performed in Trp-free media. (c) MRC5 cells (left panel) and RPE cells (right panel) were infected with HCMV AD169 (0.05 p.f.u. per cell). Titres of infectious virus from the supernatant were determined 8 days p.i. using the treatment protocols described above.

Pathway controlling replication in those cells (Bodaghi et al., 1999a). The controversial literature available prompted us to reinvestigate the precise potency of IDO in HCMV replication control in both cell types using various HCMV strains and infection conditions. Three basic findings were made: (i) all strains of HCMV invariably prevent IFN-γ-driven induction of IDO gene transcription; (ii) HCMV is also able to shut down IDO transcription in IFN-γ-conditioned cells exhibiting an antiviral state, albeit the kinetics of downregulation differed clearly between fibroblasts and RPE cells; (iii) under these conditions IDO represents an important but still subordinate anticytomegaloviral effector among the very large IFN-γ transcriptome, as concluded from tryptophan complementation data according to IDO gene knock-down experiments.

RPE cells represent a convenient epithelial cell infection model known to exhibit an altered HCMV protein expression pattern (Detrick et al., 1996) and decelerated HCMV gene expression (Adair et al., 2006) compared to fibroblasts. This distinction can be explained by a separate entry pathway via endocytosis into RPE cells versus membrane fusion into fibroblasts (Bodaghi et al., 1999b).

Hence, although not demonstrated before, it was not surprising that the protracted HCMV replication cycle in RPE cells has important consequences for HCMV interferon antagonism. Therefore, the data resolve the apparent contradiction between our results and an earlier report (Bodaghi et al., 1999a) describing early IDO induction after IFN treatment at the time point of infection. This observation is easily explained by the delay of HCMV gene expression, including IFN antagonists, in epithelial cells such as those from retinal pigment. Notably, a congruent situation was recently found when investigating human placenta tissue (Lopez et al., 2011). HCMV was described as effectively repressing IDO induction in placenta tissue cultures, but the extent of repression strictly relied on tissue composition, e.g. early and term placenta. Our data also indicate that IDO, once induced by IFN-γ, remains stable and catalytically active in HCMV-infected cells with a half-life comparable with published values (Pallotta et al., 2011). While the catabolic function of IDO clearly contributes to the anticytomegaloviral effect of IFN-γ, tryptophan depletion alone was not sufficient to explain the antiviral potency of IFN-γ. Very recently, it was reported that HCMV itself could elicit an IFN-γ-like response via the IE1 protein which results in the selective induction of IFN-γ-dependent gene products including IDO (Knoblach et al., 2011). This might give HCMV the opportunity to profit from the immunosuppressive properties of IDO (Soliman...
Fig. 6. Role of IDO for the IFN-mediated restriction of HCMV growth. (a) IDO siRNA efficiently inhibits IDO induction by IFN-γ. MRC-5 cells were transfected with the indicated siRNA: control, CTR; IDO siRNA, IDO. After 3 h IDO expression was induced by addition of IFN-γ (500 U ml⁻¹). Cell lysates were prepared 2 and 5 days after transfection. Equivalent amounts of lysate were separated by SDS-PAGE and then immunoblotted using antibodies against IDO and β-actin as a loading control detected as
Fig. 6. (cont.) reblot from the same membrane. (b) IDO siRNA efficiently protects bacteria from IFN-γ-mediated growth restriction by IFN-γ. MRC-5 cells were either mock-transfected (No), transfected with IDO-specific siRNA (IDO) or a scrambled control siRNA (CTR), incubated with IFN-γ (500 U ml⁻¹) and inoculated with 100 c.f.u. of *Staphylococcus aureus*. Bacterial growth was determined by serial dilution titration of bacteria from the supernatant 16 h after inoculation. Each value represents the mean ± SD from three independent determinations. (c) IDO siRNA does not provide a complete rescue of HCMV from IFN-γ-mediated growth restriction. MRC-5 (left panel) or RPE (right panel) were transfected with siRNA as indicated. Infection of transfected cells was performed with HCMV AD169 (0.05 p.f.u. per cell) after 48 h of preincubation without IFN or with 500 U ml⁻¹ IFN-γ as indicated. Incubation was continued throughout the time of HCMV replication. Titres of infectious virus from the supernatant were determined 6 days p.i. Each value represents the mean ± SD from three independent determinations. (d) Combined tryptophan (Trp) complementation and IDO siRNA treatment completely rescues HCMV from IFN-γ-mediated growth restriction. MRC-5 cells were transfected with siRNA as indicated. Infection of transfected cells was performed with HCMV TB40/E (0.05 p.f.u. per cell) after 48 h of preincubation without IFN or with 500 U ml⁻¹ IFN-γ as indicated. Incubation was continued throughout the time of HCMV replication. Tryptophan concentration of DMEM (16 µg ml⁻¹) was used for low tryptophan determinations (left panel) or tryptophan was supplemented to 200 µg ml⁻¹ for high tryptophan values (right panel). Titres of infectious virus from the supernatant were determined 6 days p.i. Each value represents the mean ± SD from three independent determinations. (e) IDO siRNA prevents tryptophan depletion in transfected cells. Tryptophan consumption (µg tryptophan ml⁻¹) was determined from the supernatants of infected MRC-5 cells supplemented with 200 µg ml⁻¹ tryptophan and analysed in (d). Each value represents the mean ± SD from three independent determinations.

et al., 2010). However, our data show that the transient induction of IDO mRNA during infection (Fig. 1b) did not induce detectable amounts of IDO protein in infected cells. This was not unexpected since the reported HCMV-induced IDO induction was dependent on phosphorylated STAT1 (Knoblauch et al., 2011) becoming rapidly dephosphorylated during lytic infection (Baron & Davignon, 2008; Le et al., 2008). Additionally, efficient control of IDO induction appears necessary since IDO-dependent tryptophan depletion is one of the major mediators of IFN-γ-induced autophagy (Fougeray et al., 2012). Autophagy is promoted by HCMV infection in response to incoming virions (McFarlane et al., 2011) and might become severely enhanced without effective control of IDO induction by HCMV. Although HCMV itself counteracts autophagy induction by the TRS1 protein (Chau morcel et al., 2012), limitation of IDO-dependent tryptophan depletion might be a necessary prerequisite to escape immune activation induced by autophagic processes.

Our findings demonstrate that HCMV is able to largely block IFN-γ signalling in IFN-conditioned cells exhibiting the antiviral state. This interference is not restricted to optimally permissive fibroblasts but also readily established in epithelial cells exhibiting a more protracted induction of the HCMV replication cycle. Thus the viral control limits the time window available to induce IDO bioactivity and promotes viral growth. Given the broad antimicrobial potential of IDO, HCMV-mediated IDO suppression might compromise the immune control of a number of IDO-restricted pathogens (MacKenzie et al., 2007) which may concomitantly multiply in HCMV-infected cells (Gelderman et al., 1968). Indeed, very recently, we were able to demonstrate that HCMV infection severely interferes with IDO-mediated antimicrobial and antiparasitic control. Furthermore, HCMV infection abrogates the IDO-mediated immune-suppressive properties of human fibroblasts in co-culture with activated T cells (Heseler et al., 2013).

In the transplantation setting, IDO-mediated immunoregulatory functions limit autoreactive T-cell reactions, thereby exhibiting protective capacity from transplant rejection (Meisel et al., 2004; Munn et al., 1999). Disturbance of this delicate balance by HCMV-mediated alterations of IDO activity in inflamed tissue could trigger and promote severe transplant complications. Taken together, both beneficial and detrimental effects of IDO in the context of HCMV infection and disease must be carefully considered and delineated in future studies.

METHODS

Cells. Human MRC-5 fibroblasts (ATCC CCL-171, passages 3–15) and human retinal pigmented epithelium cells ARPE (ATCC CRL-2302) were grown in DMEM supplemented with 10% FCS, 100 units penicillin ml⁻¹, 100 mg streptomycin ml⁻¹ and 2 mM glutamine.

Viruses, infection conditions and virus titration. The HCMV strains used were AD169 (Hengel et al., 1995), AD169-GFP lacking the genes US2–US11 (Borst & Messerle, 2000), the endotheliotropic strain TB40/E (Sinzer et al., 1999) and the clinical isolate UL1271 (Atalay et al., 2002). For the construction of TB40-GFP, a PCR fragment was generated using the primers AZ-Agt-Kana1 (CACTGGTTCCACATGTACGGCTAGACTAGTCGGTAC) and AZ-Agt-Kana2 (CGATGCAAGTTGTGCAGTTGACGTAAATTCGAAAGTCGGTAC) and the plasmid pSFRTKn (Atalay et al., 2002) as template. The PCR fragment containing a kanamycin resistance gene substituted the gpt sequence of the parental TB40/E-BAC4 lacking the genes US 2–6 (Sinzer et al., 2008) by homologous recombination in E. coli. Kan was subsequently excised by FLP-mediated recombination (Wagner & Koszinowski, 2004) leaving an ftr-site within the gpt sequence. An ftr-site-flanked fragment encompassing the MCMV-derived major IE promoter/enhancer in front of the GFP gene was introduced into TB40-Agt to generate TB40-GFP. Purified HCMV stocks were prepared on MRC-5 cells as described previously (Le et al., 2008). Virus titres were determined by standard plaque assay. HCMV infection was enhanced by...
centrifugation at 800 g for 30 min. Susceptibility to IFNs was assayed by virus growth in the presence of recombinant human IFN-γ (PBL Biomedical Laboratories) after preincubation with IFN for 48 h prior to infection.

**siRNA transfection.** siRNAs (Dharmacon) were delivered in a final concentration of 25 nM using Superfect Transfection Reagent (Qiagen) according to the manufacturer’s instructions.

**Immunoblot analysis.** For immunoblotting, equal amounts of cell lysate were suspended in Laemmli sample buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. Immunoblot analyses were performed with the following antibodies: mouse monoclonal antibody (mAb) against IDO (upstate), HCMV separated by SDS-PAGE and transferred to nitrocellulose filters. Lysate were suspended in Laemmli sample buffer. Proteins were visualized using the ECL-Plus chemiluminescence system (Amersham).

**IDO activity assay.** IDO activity in HCMV-infected cells was quantified by the ascorbate/methylene blue activity assay as previously described (Takikawa et al., 1988).

**RNA analysis.** RNA was extracted from cells with the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. The extracted RNA was reverse transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR was performed using a LightCycler 480 Master Mix and specific primers to amplify the IDO gene. The reactions were performed in a 384-well plate format on a LightCycler 480 instrument (Roche Applied Science). The expression levels of IDO were normalized to the expression of 18S rRNA, and the fold change was calculated using the 2^ΔΔCt method.

**Flow cytometry.** HCMV DNA was detected by the EGFP-expressing AD169-GFP (a kind gift of M. Messerle, Hannover, Germany) whereas HLA-DR was monitored as a known IFN-γ inducible surface marker. FACS was performed with an antibody specific for HLA-DR (L243, Leinco) detected by an allophycocyanin conjugated goat anti-mouse IgG secondary antibody (BD). Fluorescence intensities were measured by a FACSCanto II (BD).

**Statistical analysis.** A two-tailed Student’s t-test was applied to determine statistically significant titre differences.

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