Toll-like receptor 4 is involved in the cell cycle modulation and required for effective human cytomegalovirus infection in THP-1 macrophages

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ABSTRACT

Suitable host cell metabolic conditions are fundamental for the effective development of the human cytomegalovirus (HCMV) lytic cycle. Indeed, several studies have demonstrated the ability of this virus to interfere with cell cycle regulation, mainly by blocking proliferating cells in G1 or G1/S. In the present study, we demonstrate that HCMV deregulates the cell cycle of THP-1 macrophages (a cell line irreversibly arrested in G0) by pushing them into S and G2 phases. Moreover, we show that HCMV infection of THP-1 macrophages leads to Toll-like receptor 4 (TLR4) activation. Since various studies have indicated TLR4 to be involved in promoting cell proliferation, here we investigate the possible role of TLR4 in the observed HCMV-induced cell cycle perturbation. Our data strongly support TLR4 as a mediator of HCMV-triggered cell cycle activation in THP-1 macrophages favouring, in turn, the development of an efficient viral lytic cycle.

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Introduction

Human cytomegalovirus (HCMV), the prototype member of the β-herpesviruses (Mocarski and Courcelle, 2001), is a ubiquitous and widespread agent that infects the majority of the population during early childhood and then establishes a lifelong latency. It is the main viral cause of birth defects following congenital infection and one of the most important opportunistic pathogens leading to severe and even fatal diseases in “at-risk” categories of individuals, such as those with a deficient immune system due to iatrogenic (e.g. transplant patients) or acquired (e.g. HIV-infected subjects, patients with cancer) causes (Alford and Brit, 1996; Bosch et al., 2011; Cobbs et al., 2002; Pass, 2001; Ramsay et al., 1991; Razonable and Paya, 2003). The multi-organ involvement frequently observed in severe cases of HCMV diseases, in particular those connected with the symptomatic reactivation of the endogenous virus, is mainly due to its ability to infect a variety of cell types, including different immune system effectors, such as myeloid progenitors, dendritic cells, monocytes and macrophages (Rölle and Olweus, 2009; Slobedman et al., 2004; Södeberg-Naucler et al., 2001). In particular, monocytes are important reservoirs of latent virus, while macrophages are permissive to HCMV lytic infection and mediate its dissemination into host tissues (Smith et al., 2004).

Another striking characteristic of HCMV, accounting for the success of this agent as a pathogen, is its prominent capacity to counteract host defence mechanisms, mainly by interfering with intracellular signalling and inducing the transcription of cellular genes involved in the activation of innate immunity, the inhibition of apoptosis and the cell cycle regulation (Billstrom Schroeder et al., 2002; Castillo and Kowalik, 2004; Compton et al., 2003; Hertel and Mocarski, 2004; Salsman et al., 2012; Wolf et al., 2012). With regard to the latter, it is known that HCMV can block the progression of the cell cycle at G1 phase or during G1/S transition in proliferating cells (Arcangeletti et al., 2011; Bain and Sinclair, 2007; Dittmer and Mocarski, 1997; Fortunato et al., 2002; Hertel and Mocarski, 2004; Wiebusch et al., 2003). In contrast, confluent fibroblast monolayers and human testicular embryonic carcinoma cells (i.e. transiently or stably quiescent cell types, respectively) have been described to re-enter the cell cycle upon HCMV infection (Bresnahan et al., 1996; Jault et al., 1995; Sinclair et al., 2000; Song and Stinski, 2005). Many in vitro studies investigating the possible mechanism(s) through which HCMV might be able to perturb the cell cycle have focussed on the possible role of viral immediate-early proteins IEp72 and IEp86; these proteins are thought to interact with important cellular activators of specific transcription factors crucial for cell entry into S phase (Bain and Sinclair, 2007; Hagemeier et al., 1994; Poma et al., 1996). Nevertheless, the above-mentioned ability of HCMV to efficiently infect different kinds of
cells deserves particular attention, as it suggests that the virus might be able to co-opt alternative and/or additional mechanisms to deregulate the cell cycle and promote its replication.

Paradoxically, a number of cellular functions exploited by HCMV for an efficient infection are also connected with the activation of innate immunity, often triggered through the stimulation of Toll-like receptors (TLR). The latter constitute the main class of immune-sensor molecules capable of recognizing a broad range of pathogens, and an increasing body of literature highlights the involvement of specific TLR during the infection of different DNA and RNA viruses (Boheme and Compton, 2004; Boehme et al., 2006; Cai et al., 2012; Cervera et al., 2007; Ducloix et al., 2005; Gaur et al., 2011; Harwani et al., 2007; Kurt-Jones et al., 2004; Lögren et al., 2010; Xagorari and Chlichlia, 2008; Xia et al., 2008). Interestingly, other studies provide evidence indicating novel functions of specific TLR in the regulation of the cell cycle (Hasan et al., 2005, 2007).

Several HCMV gene products exhibiting extensive immuno-modulatory effects have been identified, which result in the evasion of host cell defences or the exploitation of immune effectors for the improvement of viral infection, reactivation, and dissemination (Mocarski, 2002a, 2002b). For instance, HCMV-encoded IL-10 may profoundly attenuate host immunity, facilitating the establishment and maintenance of a persistent infection (Chang and Barry, 2010). Some important examples of immune effectors co-opted by HCMV to its own benefit include monocyte-derived dendritic cells, rendered functionally impaired by HCMV infection (Rölle and Olweus, 2009), and inflammatory cytokines, found to be activated in specific cell models via the HCMV-induced stimulation of TLR2, which in turn increase the severity of HCMV-induced disease (Compton et al., 2003). Moreover, it has been described that the reactivation of murine cytomegalovirus from latency is dependent on the activation of TLR4 (Xagorari and Chlichlia, 2008).

Considering the relevance of macrophages as a key innate immune effector targeted by HCMV in vivo and the fact that this cell type expresses the highest number of TLR subsets (Hornung et al., 2002; Zarembek and Godowski, 2002), the THP-1 macrophage cell model used in this study provides an ideal opportunity to analyse the effect of HCMV infection on the cell cycle of these resting cells and to assess whether specific TLR are in some way involved in HCMV infection. Here, we show that HCMV disrupts the cell cycle homeostasis of THP-1 macrophages and provide experimental data supporting an important role of TLR4 signalling in mediating the HCMV-induced cell cycle perturbation and effective HCMV infection.

**Results**

**Cell cycle progression in HCMV-infected THP-1 macrophages**

The present study makes use of the THP-1 human monocytic cell line that differentiates into macrophages following TPA treatment. THP-1 macrophages therefore provide a suitable cell model, mimicking the HCMV natural target, for studying the mechanisms through which HCMV controls host cell metabolism to its own benefit. Here, we focus on cell cycle regulation and the possible involvement of specific subsets of TLR during the HCMV infectious cycle.

In a preliminary series of control experiments (data not shown), we confirmed that THP-1 monocytes behave similarly to the in vivo cells in that they are non-permissive to HCMV infection, only becoming permissive following their differentiation into macrophages; using flow cytometry analysis and macrophage differentiation markers we also confirmed that THP-1 cells differentiate correctly.

We next analysed whether the virus was able to rescue THP-1 macrophages (irreversibly withdrawn from the cell cycle) from G0; indeed, over the course of a three-day-infection, using flow cytometry analysis to study the DNA distribution in the cell cycle phases of uninfected and HCMV-infected THP-1 macrophages, we observed that HCMV is able to cause a progressive re-entry of these cells into the cell cycle, with an increased number of cells in S phase at 48 h post-infection (p.i.) and in G2/M transition at 72 h p.i. (Fig. 1A, white bars) compared to the mock infected cells (Fig. 1A, black bars). Surprisingly, cell counts did not reveal any significant increases in the cell population at later time points (data not shown).

In order to verify that this effect was dependent on the presence of an active virus, we concomitantly analysed the DNA distribution in THP-1 macrophages infected with UV-inactivated virus (Fig. 1A, grey bars). In this case, the results show a DNA distribution similar to that of the uninfected cells (Fig. 1A, grey vs black bars). Mock infected undifferentiated THP-1 cells were also included in this analysis (Fig. 1, pale grey bars) in order to compare these proliferating cells with the G0-arrested THP1 macrophages. The full permissivity of the latter to the HCMV lytic cycle was also confirmed by analysing the viral transcript patterns (immediate-early, early, early-late and late) at the considered times post-infection, which were typical of a lytic program (Fig. 1B).

To confirm the results obtained from cytofluorimetry, which demonstrated a virus-induced re-entry into the cell cycle with increased S and G2/M phases, we next analysed the transcript expression levels of cyclins E and A at the same time points as those shown in Fig. 1 in order to evaluate their pattern during (active and UV-inactivated) HCMV infection, as well as in mock infected differentiated and undifferentiated THP-1 cells (Fig. 2). Cyclin E is known to be required for the G1/S transition to occur, and cyclin A is necessary for and mainly expressed in late S and G2 and for the activation of mitosis-promoting factors (Hwang and Clurman, 2005; Pagano et al., 1992; Vermeulen et al., 2003). As shown in Fig. 2, the expression levels of cyclin E and A are significantly higher in active-virus-infected THP-1 macrophages and, as expected, in mock infected undifferentiated THP-1 cells compared to inactive-virus-infected and mock infected THP-1 macrophages, thus confirming a true re-entering into the cell cycle of HCMV-infected THP-1 macrophages.

**TLR transcript levels in mock infected, active HCMV- and UV-inactivated HCMV-infected THP-1 macrophages**

Considering the ability of HCMV to activate specific effectors of innate immunity in different cell models, in particular TLR (Boheme et al., 2006; Xagorari and Chlichlia, 2008), we next investigated whether TLR were co-opted in HCMV-infected THP-1 macrophages. To this end, we analysed the pattern of the TLR transcripts known to be expressed in macrophages at 24, 48, and 72 h in active or UV inactivated virus-infected or mock infected THP-1 macrophages (Fig. 3). The results show that TLR3, TLR4, and TLR5 transcript levels increased during active HCMV infection, reaching a peak at 48–72 h p.i.; on the other hand, no significant expression differences were observed for TLR8 (Fig. 3). TLR2 and TLR9 (data not shown) compared to the mock infected or UV inactivated HCMV-infected cells. Interestingly, in the latter experimental condition, only the TLR4 transcript level is upregulated, supporting the hypothesis of a HCMV-induced TLR4 stimulation occurring earlier than viral replication.

**Effects of TLR3, TLR4 and TLR5 activation on the cell cycle in uninfected THP-1 macrophages**

Studies have demonstrated that TLR3, TLR4, and TLR5 are involved not only in the classical role of innate immunity “sentinels”, but also...
in the modulation of cell proliferation (Hasan et al., 2005, 2007). Considering this, together with the finding that the expression of these specific TLR is up-regulated in infected THP-1 macrophages (see Fig. 3), we next investigated whether the activation of the above-mentioned TLR was able to push uninfected THP-1 macrophages (irreversibly blocked in G0) into the cell cycle.

To this end, a cytofluorimetric analysis of the DNA distribution of uninfected THP-1 macrophages was performed at 24, 48, and 72 h following their treatment with TLR3, TLR4, or TLR5 agonists: polyinosinic:polycytidylic acid (Poly I:C), lipopolysaccharide (LPS) or flagellin (FLA), respectively (Fig. 4). The activation of these TLR was checked by monitoring the transcription levels of specific cytokines known to be increased following agonist treatment; specifically, the transcript pattern of interleukin 8 (IL-8) was analysed by RT-PCR to confirm TLR3 activation, and interferon β (IFN β) transcript was checked to verify TLR4 and TLR5 stimulation.

Interestingly, only the activation of TLR4 (Fig. 4, column "TLR4 [LPS]") led THP-1 macrophages to re-enter the cell cycle. It is also noteworthy that the DNA distribution in the different phases of the cell cycle exhibited temporal kinetics similar to those observed in HCMV-infected cells: following agonist treatment, an increased quantity of cells could be observed in S phase and G2/M transition at 48 and 72 h, respectively. On the other hand, TLR3 and TLR5 stimulation did not alter the DNA distribution, which remained typical of resting cells.

Based on the aforementioned results, the next series of experiments focussed on TLR4 and its possible involvement in HCMV infection in THP-1 macrophages. Effects of the TLR4 signalling pathway inhibition on cell cycle progression in uninfected THP-1 macrophages

In order to confirm that the observed LPS-induced cell cycle activation could be attributed to TLR4 stimulation, two specific inhibitors, CLI and NeutrAb, were used to block TLR4 signalling. These antagonists act in different ways: CLI specifically blocks signalling mediated by the TLR4 intracellular domain, inhibiting the production of nitric oxide and pro-inflammatory cytokines,
whilst NeutrAb is a TLR4 neutralising antibody recognising the external portion of the receptor, thus blocking TLR4 receptor signalling by masking its binding site.

Uninfected THP-1 macrophages were treated with LPS alone, to stimulate TLR4 or, alternatively, with LPS and one of the two TLR4 inhibitors, as detailed in the "Materials and methods" section; cell DNA distribution was analysed by flow cytometry at 0, 24 and 48 h (Fig. 5). Stimulation of TLR4 with LPS (Fig. 5, white bars) typically induced cell entry into S phase after 48 h, confirming our previous results (see Fig. 4). Conversely, the concomitant presence of either of the TLR4 inhibitors (Fig. 5, grey bar: CLI; black bar: NeutrAb) clearly prevented THP-1 macrophages from entering the cell cycle. Changes in IFN-β (TLR4 activation marker) transcript levels were also monitored by RT-PCR in the three experimental conditions (not shown).

**IFN-β transcript level pattern in TLR4-activated mock infected or HCMV-infected THP-1 macrophages**

The IFN-β transcript expression pattern was monitored by RT-PCR in mock infected and HCMV-infected THP-1 macrophages (Fig. 6). Analysis was performed at 0, 24 and 48 h after TLR4 activation in mock infected (Fig. 6 “LPS”) or active HCMV-infected (Fig. 6 “HCMV”) THP-1 macrophages; in both cases, cells were untreated (Fig. 6 “LPS” and “HCMV”) or treated with CLI (Fig. 6 “LPS+CLI” and “HCMV+CLI”, respectively) or NeutrAb (Fig. 6 “HCMV+NeutrAb”).
“LPS + NeutrAb” and “HCMV + NeutrAb”, respectively). The most relevant data show that IFN-β activation does occur following HCMV infection in untreated differentiated THP-1 cells, and that this activation is blocked in the presence of TLR4 inhibitors; thus, IFN-β activation is most likely a downstream effect of TLR4 stimulation.

**Effects of TLR4 signalling pathway inhibition on HCMV replication**

To investigate whether TLR4 inhibition by CLI or NeutrAb could have a negative impact upon the infection of THP-1 macrophages by HCMV (possibly connected with the lack of cell cycle activation, as observed in untreated cells; see Fig. 5), the same inhibitors were used during HCMV infection to analyse their effects on viral immediate-early gene expression and viral yield (Fig. 7). IE1 transcript pattern was assessed at 24 and 48 h p.i. in untreated cells (Fig. 7A a), or in the presence of CLI (Fig. 7A b) or NeutrAb (Fig. 7A c); the percentage of cells expressing the viral major IE proteins was analysed in the same experimental conditions at 24, 48 and 72 h p.i. (Fig. 7B, bar graphs). Finally, the viral yield from THP-1 macrophages was also assessed at 5, 6 and 7 days post-infection in the absence or presence of TLR4 inhibitors (Fig. 7C, bar graphs), as detailed in the “Materials and methods” section. The results show a significant reduction in HCMV IE1 transcript levels, IE proteins and viral yield following treatment of THP-1 macrophages with TLR4 antagonists compared to the untreated controls (Fig. 7A–C, compare control vs treated cells).

In order to exclude the possibility that the observed effect could be attributed to a decreased virus adsorption/entry in the presence of TLR4 inhibitors, virus uptake was investigated (Supplementary Figure 1) by analysing HCMV DNA (IE sequence) by PCR at the beginning (“0 h”) and the end (“2 h”) of the adsorption time in the absence (“HCMV”) and presence of each TLR4 inhibitor (“HCMV + CLI”; “HCMV + NeutrAb”). No appreciable differences in HCMV DNA are observed between the infection control and treated cells, thus demonstrating that a differential virus uptake is not responsible for the inhibition of IE (transcript and protein) expression and for the viral yield decrease observed in Fig. 7 upon treatment with TLR4 inhibitors.

Although cell viability was previously confirmed to be unaffected by the presence of the TLR4 antagonists (as described in the “Materials and methods” section), to exclude the possibility that inhibition of HCMV infection in THP-1 macrophages resulted from antagonist-induced side effects, the same experimental protocol was repeated in MRC5 fibroblasts that are highly permissive to HCMV infection and express neither TLR4 mRNA nor protein (Friboulet et al., 2010; Matsumoto et al., 2002; Rudd et al., 2005). As shown (Fig. 7D–F), neither of the antagonists affected the IE1 transcript (Fig. 7D) nor protein levels (Fig. 7E) nor viral yields (Fig. 7F) in MRC5 infected cells.

**Effects of TLR4 signalling pathway inhibition on HCMV-induced re-entry of THP-1 macrophages into the cell cycle**

In the next series of experiments, differentiated THP-1 cells were infected with HCMV for 24 (not shown), 48 and 72 h in the absence (Fig. 8, column “HCMV”) or presence of one of the two selected TLR4 inhibitors (Fig. 8, columns “HCMV + CLI” and “HCMV + NeutrAb”). Flow cytometry (both dot plot and histogram representations are shown) was applied to analyse DNA distribution patterns in uninfected (black dots and histograms) and HCMV infected (green dots and histograms) cells. Since the main effects of infection on the cell cycle were most evident at 48 and 72 h, only the data obtained at these later times are shown.

The treatment of HCMV infected THP-1 macrophages with TLR4 antagonists resulted in a significantly reduced concentration of infected cells (expressing viral IE proteins) at both 48 and 72 h p.i. (Fig. 8, columns “HCMV + CLI” and “HCMV + NeutrAb”, IE+ percentages in the dot plot graphics) compared to that observed in HCMV infected cells in the absence of antagonists (Fig. 8, column “HCMV”, IE+ percentages). As can be deduced from the relative mean percentage values for G0/G1, S and G2/M shown in the top right-hand corner of each histogram, it is once again evident that HCMV drives quiescent THP-1 cells into the S and G2 phases of the cell cycle (Fig. 8, column “HCMV”, green histograms and green dots), since a DNA distribution pattern
typical of actively proliferating cells is observed (in particular, 34.1% and 34% of cells are in S phase in green graphics at 48 and 72 h p.i., respectively), corroborating the results presented in Fig. 1A. On the other hand, in THP-1 macrophages infected with HCMV and treated with CLI or NeutrAb, cell cycle activation is significantly reduced, as observed in the corresponding graphics and confirmed by the S phase percentages (10.1% and 13.1% in the presence of CLI and 13.1% and 7.9% in the presence of NeutrAb at 48 and 72 h, respectively).

Discussion

Viruses have evolved a variety of mechanisms to efficiently infect their hosts, which mainly involve the interference with host cell regulation systems and signalling pathways (Bruggeman, 2007; Gale et al., 2000; Mirandola et al., 2006). The strategies employed by HCMV are numerous and can vary drastically depending on whether it resides latent within a host cell or has entered its lytic cycle (Adler and Sinzger, 2009; Arrode and Devrinche, 2003; Lunardi et al., 2005; Pass, 2001). During latent infection, although the HCMV genome remains stably present, it is mostly unexpressed and its impact on the host cell metabolism is quite restricted (Slobedman et al., 2004). In the case of lytic infection, the virus competes for and exploits different functions of the host cell in order to develop efficient infection and produce viral progeny (Hertel and Mocarski, 2004).

The goal of the present study was to extend our knowledge of the cellular mechanisms co-opted by HCMV that enable it to carry out productive infection and create the ideal conditions for its replication in a resting cell, such as a macrophage model. The THP-1 macrophages, mimicking one of the major strategic targets exploited by HCMV in vivo, represent an ideal paradigm in which to study HCMV infection. Indeed, the differentiation of peripheral blood monocytes into macrophages provides the latent virus with a cell environment within which it is able to sustain a lytic infection, favouring its dissemination into host tissues (Mendelson et al., 1996; Taylor-Wiedeman et al., 1994). In the monocyte-macrophage system, a tight correlation between cellular differentiation and HCMV reactivation from latency is likely to exist; specifically, the post-mitotic stage that initiates the maturation of monocytes into macrophages is thought to stimulate the viral gene expression that leads to productive infection (Sinclair and Sissons, 2006).
Mimicking the natural situation, THP-1 macrophages are fully permissive to HCMV infection; in fact, as shown here, the viral transcript expression pattern is representative of all the phases of HCMV lytic cycle and the viral progeny is also produced, thus confirming previous data from our group (Ioudinkova et al., 2006).

When considering the possible strategies adopted by HCMV to productively infect these cells, it is important to take into account the following three important points. First of all, as in the in vivo situation, THP-1 macrophages are irreversibly withdrawn from the cell cycle. One might therefore expect that the virus would not find an adequate supply of deoxyribonucleotides and cofactors (abundant in late G1 and G1/S of cycling cells) necessary for the efficient replication of its genome; nevertheless, it has been demonstrated that HCMV can efficiently infect macrophages in vivo (Castillo and Kowalik, 2004). The second important point deals with the ability of this virus to manipulate the cell cycle, keeping in mind that in cycling cells HCMV is able to block the cell cycle, mainly in late G1 phase or G1/S transition, by concomitantly abolishing cellular DNA replication (Arcangeletti et al., 2011; Bain and Sinclair, 2007; Dittmer and Mocarski, 1997; Fortunato et al., 2002; Hertel and Mocarski, 2004). The third important point concerns HCMV infection of quiescent cells, such as the THP-1 macrophages used in this study. A previous study (Sinclair et al., 2000) has described the ability of HCMV to push G0 cells into early S phase in a quiescent human embryo teratocarcinoma cell line (NT2D1).

To this regard, it was of crucial importance to ascertain whether this viral agent had developed the capacity to circumvent the lack of S phase components in a strategically important resting cell in vivo, such as the macrophage.

To this end, the first aim of this study was to check whether HCMV could alter the THP-1 cell cycle to its own advantage. Our results demonstrate that HCMV is indeed able to induce these cells to re-enter the cell cycle, with a gradual increase in the
amount of DNA in S and G2/M phases, although this did not lead to an increase in the number of cells. In order to expand these results, we analysed the transcript pattern levels of cyclin E (required for G1/S transition) and A (necessary for the late S and G2 and for the activation of mitosis-promoting factors) (Hwang and Clurman, 2005; Pagano et al., 1992; Vermeulen et al., 2003), and found that they are significantly increased in active-virus-infected THP-1 macrophages.

These data support the notion of a virus-induced cell cycle activation that, in contrast to that observed in the NT2D1 model (Sinclair et al., 2000) leading to the early S phase activation, goes through the late S and G2 phases.

Of the possible mechanisms that HCMV might exploit to activate the cell cycle in THP-1 macrophages, the present study focussed on the potential involvement of the TLR family on the basis of a series of notions already presented in the literature. First of all, macrophages are among the most important effectors of the innate immune response against pathogens and they constitute one of the cell types expressing the highest number of TLR subtypes (Hornung et al., 2002; Zarember and Godowski, 2002). Second, not only does HCMV possess different strategies to evade the host immune defences, but it is also able to activate and exploit host immune responses (particularly the inflammatory process) to increase its virulence (Boheme and Compton, 2004; Compton et al., 2003; Kovacs et al., 2007; Mocarski, 2002a, 2002b; Varani and Landini, 2011). Third, and most importantly, specific TLR receptors have been described not only for their classical function in the immune response, but also for having a role in cell cycle regulation, with particular emphasis placed upon TLR3, TLR4 and TLR5 (Hasan et al., 2005, 2007).

In the present study, we show that active HCMV-infected THP-1 macrophages present a significant increase in the transcription levels of the genes encoding TLR3, TLR4 and TLR5 proteins compared to uninfected controls. These results lie in accordance with the observations made by others investigating different cell models and/or viruses (Iversen et al., 2009; Jiang et al., 2008; West and Damania, 2008). The presence of cross-talk between different TLR could explain how the expression of more than one TLR subtype may be influenced at the same time (Palazzo et al., 2008), as observed in the present work.

An important finding of this study is that in uninfected THP-1 macrophages only TLR4 activation is connected with re-entry into the cell cycle. Furthermore, inhibition of the TLR4-mediated signalling pathway drastically inhibited the efficiency of HCMV infection in this in vitro model. Interestingly, it has been shown that TLR4 activation leads to pulmonary reactivation of murine cytomegalovirus in mice and that inhibition of TLR4 signalling by a monoclonal antibody was able to block this reactivation (Xagorari and Chlichlia, 2008). Moreover, our data demonstrate that TLR4 inhibition significantly reduces the virus-induced activation of the cell cycle in HCMV-infected THP-1 macrophages, leading to the hypothesis that an interaction between HCMV and TLR4 exists and, considering the cell membrane location of this receptor, that it could be part of a receptorial or co-receptorial complex exploited by HCMV in THP-1 macrophages. In support of this hypothesis, here we demonstrate that an increase in the TLR4 transcription level (but not TLR3 and TLR5) also occurs with the UV-inactivated HCMV, in accordance with data obtained by Boehme et al. (2006), studying TLR1/TLR2 heterodimer in a HCMV-infected fibroblast model.

Nevertheless, in this macrophage-like model we also demonstrate that the expression of the IFN $\beta$ transcript is significantly increased during THP-1 macrophage infection with active HCMV (with levels and a time course comparable to those observed in TLR4-activated uninfected cells) and that the presence of TLR4 specific inhibitors interfere with this expression, thus supporting...
the notion that the stimulation of this cytokine might be a downstream effect of TLR4 activation.

Taking into account data showing that once stimulated, this receptor may become internalised (Husebye et al., 2006), and that the internalisation of TLR4 into the endosomal network is required to regulate type I IFN expression (Chiang et al., 2011; Zanoni et al., 2011), the present set of data let us hypothesise that, not only could TLR4 form part of a receptorial or co-receptorial complex for HCMV in THP-1 macrophages, but that it might also be involved in later stages of infection, such as the internalisation process and in events connected with cell cycle activation. This is also coherent with our finding that only active HCMV leads to the activation of the cell cycle in THP-1 macrophages.

Concerning the possible connection between cell cycle deregulation and IFN production, the latter has been described to influence cell cycle progression by prolonging the S phase and G2/M transition, whilst inhibiting cell proliferation (Katayama et al., 2007). Accordingly, here we observe a similar effect of HCMV infection upon the cell cycle in THP-1 macrophages, with a gradual increase in the quantity of DNA residing in S and G2/M phases, in the absence of concomitant cell proliferation.

Work in our laboratory is currently in progress to elucidate the role of TLR4 internalisation and interferon β production in the observed cell cycle modulation induced by HCMV in THP-1 differentiated cells.

In conclusion, using a human macrophage model closely related to the HCMV natural target in vivo, the present study provides the first evidence demonstrating virus-induced activation of the cell cycle which, in turn, requires TLR4 activation. These results are relevant as they are able to account for the efficient productive infection of these important cell targets and explain how they may be exploited by HCMV to favour its tissue dissemination.

 Whilst many questions remain open, our finding that a significant reduction in HCMV lytic replication occurs in this human macrophage model, following TLR4 inhibition, unveils novel routes for identifying innovative targets for prophylactic and/or therapeutic approaches to counteract HCMV replication.

Materials and methods

Cell culture

Cultures of the monocytic cell line THP-1 (“Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia-Romagna”) were maintained in suspension in RPMI 1640 medium, supplemented with 1% l-glutamine, 1% sodium pyruvate, 50 μM β-mercaptoethanol, 10% foetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cell differentiation into macrophages was induced by adding 80 nM 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma-Aldrich) to the medium for 48 h to obtain fully adherent cells.

Monolayer cultures of MRC5 human embryo lung fibroblasts (American Type Culture Collection, ATCC; CCL-171) were grown in Earle's modified Minimum Essential Medium (E-MEM), supplemented with 1% l-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 10% FBS and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Cell culture medium and supplements were obtained from PAA (The Cell Culture Company).

Virus strain, titration and cell infection

HCMV reference Towne strain (ATCC VR-977) was propagated in MRC5 cells; viral infectious titre was determined as previously described (Arcangeletti et al., 2003). THP-1 monocytes were grown and differentiated in 6-well plates at the concentration of 3 x 10^6 cells/well. After differentiation, THP-1 cells were washed in E-MEM without FBS, then infected with HCMV Towne at a multiplicity of infection (m.o.i.) of 2 plaque forming units (PFU)/cell. Virus adsorption was performed by centrifugation at 700 x g for 45 min then incubation at 37 °C for 75 min. Subsequently, the virus inoculum was removed and replaced with 10% RPMI. The infected cells were incubated at 37 °C for the indicated times. HCMV infection of MRC5 fibroblasts was carried out at an m.o.i. of 0.2.

Viral yield analysis was performed as already described (Loudovikova et al., 2006), by using a 10-fold lower m.o.i. for the infection of THP-1 macrophages (m.o.i. of 0.2) and MRC5 fibroblasts (m.o.i. of 0.02), in the presence or the absence of TLR4 antagonists. Briefly, at the indicated days p.i., THP-1 and MRC5 cells supernatants were collected, centrifuged at low speed (7,500 x g) for 10 min then used to infect MRC5 fibroblasts. HCMV yield titration was evaluated using the immunofluorescence technique (as described in the “Indirect immunofluorescence” section).

Virus inactivation

For the UV inactivation of HCMV Towne, an aliquot of virus was diluted into 1 ml of E-MEM without FBS (to obtain an m.o.i. of 2 for the infection of 3 x 10^6 THP-1 macrophages) and transferred to a 3 cm Petri dish. The viral suspension was placed on ice and UV-irradiated for 2 h at a distance of 5 cm from the UV lamp. The UV-inactivated virus did not replicate or produce any detectable levels of IE gene products (data not shown).

Treatment with specific agonists of the TLR3, TLR4, and TLR5 signalling pathways

Differentiated THP-1 cells, seeded in 6-well plates at the concentration of 3 x 10^6 cells/well, were treated for 6 h with one of the following: a synthetic dsRNA analogue (Poly I:C high molecular weight, InvivoGen) acting as a TLR3 agonist, at the final concentration of 1 μg/ml; lipopolysaccharide (Ultra Pure E. coli K12 LPS, InvivoGen) a TLR4 agonist, at the final concentration of 1 μg/ml; or flagellin (Salmonella typhimurium FLA, InvivoGen), a TLR5 agonist, at the final concentration of 500 ng/ml. Following treatments, TLR agonists were withdrawn and cell monolayers were HCMV-infected or mock-infected for the established times. Cell viability was checked using trypan blue (Sigma-Aldrich) and chromatin staining by DAPI (described in the “Antibodies and fluorescent dyes” section).

Treatment with the TLR4 antagonist CLI

Uninfected THP-1 macrophages were pre-treated for 1 h with 1 μg/ml CLI (InvivoGen), a cycloexene derivative known to antagonise TLR4 activation, and then treated with lipopolysaccharide (LPS) together with 10 μg/ml CLI for 6 h. After the latter incubation time, the TLR4 antagonist was maintained in the cell culture medium at the concentration of 100 ng/ml for the established times. The same protocol was applied to HCMV infected cells. Cell viability was assessed as stated above.

Treatment with a neutralising polyclonal antibody directed against TLR4 (NeutrAb)

Uninfected THP-1 macrophages were pre-treated with a neutralising polyclonal antibody directed against TLR4 (NeutrAb, InvivoGen) for 1 h at the concentration of 10 μg/ml. Following pre-treatment, 5 μg/ml NeutrAb was kept in the cell culture medium for the duration of LPS treatment, then maintained in the cell culture medium at the concentration of 2.5 μg/ml for the
established times. The same protocol was applied to HCMV infected cells. Cell viability was assessed as stated above.

**RNA isolation and RT-PCR assay**

Total RNA was extracted from cells using the NucleoSpin® RNA II kit according to the manufacturer’s instructions (Macherey-Nagel). Protein and DNA contaminations were checked in RNA extracts using an Eppendorf biophotometer (absorbance at 260 and 280 nm and extinction coefficient, respectively). Furthermore, to confirm the absence of DNA contamination, PCR for viral (IE1) or cellular (gyceraldehyde-3-phosphate dehydrogenase [GAPDH]) genes were done on not-retrotranscribed RNA extracts.

Template RNAs were then reverse transcribed using the ReverTaid first strand cDNA synthesis kit (Fermentas). The obtained cDNA was subjected to PCR amplification using the following: specific primers for viral transcripts IE1 (303 bp), DNA pol (237 bp), pp65 (213 bp) and pp150 (206 bp) (louidinkova et al., 2006); specific primers for Cyclin E (F: 5’-GTCCTGGCTGAATGTATACATGC-3’; R: 5’-CCCTATTTTGTTCAGACAACATGGC-3’), Cyclin A (F: 5’-GTCACACATACATGGACATG-3’; R: 5’-AAGTTTTCTCTCAGCATTGAC-3’, 300 bp), TLR3 (F: 5’-AGGAATCCCTTGCCCTTGTG-3’; R: 5’-CAAGACCTCCCAATCTGCA-3’, 172 bp), TLR4 (F: 5’-AACTCTCTGTGGCATTAGG-3’; R: 5’-CAGACCAACAGCCTCTGTAG-3’, 212 bp), TLR5 (F: 5’-GCTTTTGATCCCCAGGACT-3’; R: 5’-GAACTTTGT-212 bp), and TLR8 (F: 5’-TCAAAAATCCCGACTTGAAA-3’; R: 5’-CCTTTTGTATCCCCAGGACT-3’, 181 bp); specific primers for interferon β (303 bp) (Opitz et al., 2006) and interleukin 8 (260 bp) (Lihn et al., 2003). The specificity of every couple of primers was controlled before use by means of amplification reactions with a mock sample (water) and agarose gel electrophoresis. A GAPDH fragment of 242 bp (louidinkova et al., 2006) was amplified as a sample loading control calibration.

**Antibodies and fluorescent dyes**

For conventional immunofluorescence, a purified monoclonal antibody (Mab clone E13; working dilution 1:30) specific for the common epitope encoded by exon 2 of the immediate early (IE) major products (72 and 86 kDa proteins) (Argene) was used as primary antibody. A monoclonal anti-rat IgG1-K antibody (AbD Serotec) was used as negative control for flow cytometry analysis. The immunoreactions were revealed by Alexa-Fluor fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (H + L) (Invitrogen; working dilution 1:500) to detect viral antigens. The fluorescent compound with high affinity for DNA 4’,6-diamin-2-phenylindole chloride hydrate (DAPI-385 nm, Sigma-Aldrich) was also used as chromatin marker. Working dilutions were prepared in 0.2% bovine serum albumin (BSA) in phosphate buffered saline (PBS: 7 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4, 137 mM NaCl, 2.7 mM KCl). Propidium iodide (PI; 10 μg/ml) was used to stain DNA for cytotoxicity analyses. All chemicals were from Sigma-Aldrich.

**Indirect immunofluorescence**

Differentiated THP-1 cells were gently fixed with methanol at −20 °C for 10 min the detection of HCMV IE proteins, and then stained and analysed as previously described (Arcangeletti et al., 2003). To establish the number of IE-positive cell nuclei, 10 fields per dish were counted and positive cells expressed as mean percentages of the total number of cells per field (evaluated by DAPI counterstaining of nuclei).

For THP-1- and MRC5-derived viral yield titration, the number of IE-positive fibroblasts was counted at 24 h.p.i. and expressed as percentage value of the total number of cells per dish (evaluated by DAPI counterstaining of nuclei).

**Indirect immunofluorescence for flow cytometry analysis**

At the established times, THP-1 macrophages were trypsinised, harvested and centrifuged at 800 × g for 10 min at room temperature. The cells were treated as previously described (Arcangeletti et al., 2011). Briefly, the supernatant was discarded and the pellet resuspended in 0.6 ml PBS and 1.4 ml ethanol 100% (ice-cold ethanol 70%) added in a drop-wise manner, then fixed/permeabilised overnight at −20 °C. Cells were subsequently centrifuged at 800 × g for 10 min at room temperature, the supernatant discarded and the pellet resuspended in 0.5 ml PBS. Cells were pelleted again and immunostained for 30 min at room temperature in the dark using a primary antibody directed against HCMV immediate early major products. Cells were then rinsed in wash buffer (0.5% BSA-PBS) and centrifuged; the pellet was resuspended in the appropriate dilution of Alexa-Fluor FITC-conjugated goat anti-mouse IgG secondary antibody and incubated for 30 min at room temperature in the dark. Cells were then rinsed in wash buffer, pelleted and resuspended in PBS containing PI (10 μg/ml) and RNase (100 μg/ml) and incubated for 30 min at room temperature. Control cells were stained using a monoclonal anti-rat IgG1-K.

**Flow cytometry**

THP-1 cells, prepared as described above, were analysed using the EPICS® XL-MCL cytometer (Beckman Coulter). DNA content and viral parameters in the different cell cycle phases were examined using Expo32 Software (Beckman Coulter). Peaks corresponding to G0/G1, S and G2/M were obtained excluding doublets and cell debris by FL3lin/RATIO and FS/FL3log dot plot analysis, respectively (Gobbi et al., 2009).

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**Appendix A. Supporting information**

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.01.021.

**References**


