Stimulatory effects of human cytomegalovirus tegument protein pp71 lead to increased expression of CCL2 (monocyte chemotactic protein-1) during infection

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Received 30 October 2014
Accepted 19 February 2015

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Human cytomegalovirus (CMV) is the most common infectious cause of congenital birth defects in developed countries. Studies of infected amniotic fluid and placentae show CMV infection leads to a pro-inflammatory shift in cytokine profiles with implications for pathogenesis of foetal disease. ELISA, immunofluorescence and real-time-PCR assays were used to investigate CCL2 (monocyte chemotactic protein-1) and TNF-α changes following CMV infection of human fibroblasts, as well as following transient expression of CMV gene products in HeLa cells. Infection of human fibroblasts with CMV AD169 resulted in increased cytoplasmic and extracellular expression of CCL2 during early stages of infection, followed by marked downregulation of the chemokine at late times. Induction of CCL2 was not observed with CMV clinical strain Merlin, consistent with the postulated immune-evasion potential of this genetically intact WT strain. Comparison between live and UV-irradiated virus infections showed that changes in CCL2 levels were a direct response to active CMV replication. There were no significant changes in TNF-α expression during a parallel time-course of CMV infection. In transient transfection assays, overexpression of CMV tegument protein pp71 resulted in intracellular and extracellular upregulation of CCL2 protein. mRNA analysis showed that pp71-induced elevation in CCL2 was mediated through transcriptional upregulation. The data showed that CMV-induced upregulation of CCL2 during early stages of infection was mediated, at least in part, by stimulation of viral pp71, which may contribute to viral pathogenesis through enhanced virus dissemination.

INTRODUCTION

Human cytomegalovirus (CMV) establishes lifelong latency within the host following primary infection (Griffiths & Emery, 1997). It is also known as Human herpes virus 5 and is a member of the subfamily Betaherpesvirinae. Primary infection with CMV is usually asymptomatic in healthy individuals, although infection of immunocompromised patients, including pregnant women, can result in disease (Pass, 2001). Pathogenesis of disease in the infected foetus following maternofoetal transmission likely involves virus-induced upregulation of inflammatory mediators (Hamilton et al., 2012; Scott et al., 2012). CMV encodes proteins with immunomodulatory functions – some of which affect cytokine secretion and chemokine responsiveness, whilst others modulate NK- and T-cell functions, allowing the virus to evade host immune responses (Llano et al., 2003; Michelson, 2004; Penfold et al., 1999; Wilkinson et al., 2008). However, the effects of CMV on the immune response are likely to change with time, as well as with host and viral factors (Scalzo et al., 2007).

Previous studies have shown that CMV induces a cytokine profile with predominant pro-inflammatory effects, with
increased production of IL-1β, IL-6, IL-8, CCL2 (monocyte chemotactic protein-1) and TNF-α during different stages of infection (Chan et al., 2002; Cheeran et al., 2001; Chou et al., 2006; Yurochko & Huang, 1999). Pro-inflammatory cytokines are elevated in a number of clinical settings in immuno-compromised individuals, including in CMV-infected transplant recipients, where the development of CMV disease has been associated with pro-inflammatory immune responses (Humar et al., 1999; Tong et al., 2001). During pregnancy, the normal maternal immune response is a predominant T helper cell (Th2) bias, allowing maternal partial tolerance of paternal MHC antigens present in foetal cells (Marzi et al., 1996; Wegmann et al., 1993). This Th2 bias is reversed with birth of the baby (Buonocore et al., 1995) and during some intrauterine infections (Koga et al., 2009). Maternal CMV infection has been demonstrated to upregulate CCL2 and TNF-α in amniotic fluid from mothers of babies born with congenital CMV (Scott et al., 2012). The placenta from babies stillborn due to CMV infection also demonstrated intracellular upregulation of pro-inflammatory cytokines, suggesting the increased inflammation may have contributed to the adverse foetal outcome (Hamilton et al., 2012).

The immunomodulatory properties of some CMV gene products (UL111a, UL146, UL147) in mediating cytokine effects are known (Nachtwey & Spencer, 2008; Penfold et al., 1999), although the cytokine-inducing effect of other CMV gene products has only been demonstrated to a lesser extent. Previous studies have shown that CMV virion glycoprotein components gB and gH stimulate the IFN-responsive pathway, as well as transcriptional activators Sp1 and NF-κB, upon binding of virion to cellular receptors (Boyle et al., 1999; Yurochko et al., 1997). Transient expression of CMV immediate-early (IE) proteins IE1p72 and IE2p86 in trophoblast cells resulted in TNF-α secretion and trophoblast apoptosis, which was inhibited by TNF-α antibodies (Chan et al., 2002).

CMV IE (IE2p86), early (pUL27, pUL44, pUL50, pUL53, pUL54) and early-late (pUL69, pUL97, pp71) products represent gene products with different expression kinetics that are critical for efficient viral replication. These viral proteins exhibit regulatory and broad stimulatory effects on viral and cellular gene expression (IE2p86, pp71, pUL69) (Chau et al., 1999; Lang et al., 1995; Liu & Stinski, 1992; Sommer et al., 1994; Wara-Aswathi et al., 1999; Winkler et al., 1994), viral DNA replication (pUL44, pUL54) (Alvisi et al., 2009; Pari & Anders, 1993), nuclear egress of viral capsids (pUL50, pUL53) (Milbradt et al., 2007) and protein kinase activity (pUL97) (Marschall et al., 2003). These known functions are consistent with potential other involvement in stimulation of the host immune response during viral replication. In this study, the regulatory and intracellular properties of CCL2 and TNF-α were investigated in human cell lines during CMV infection, and during the transient expression of individual CMV proteins.

## RESULTS

**Active CMV infection leads to alteration in CCL2 levels during the course of infection**

The accumulated CCL2 protein levels were measured in the medium of CMV-infected and uninfected MRC-5 fibroblast cultures at various time points. CCL2 was continually secreted by uninfected MRC-5 cells, resulting in a significant accumulation of the chemokine in the culture medium over the time-course (Fig. 1a–d). Infection of MRC-5 fibroblasts with CMV laboratory strain AD169 resulted in an initial upregulation of CCL2 secretion at early times compared with uninfected controls (Fig. 1a–c). The induction of CCL2 was most prominent at 12 h post-infection (p.i.), with extracellular CCL2 levels approximately twofold higher than those secreted by mock-infected fibroblast cultures (Fig. 1a–c). However, the degree of CCL2 upregulation was independent of the m.o.i. The duration of CCL2 induction was inversely related to the m.o.i. used, with a more prolonged induction of CCL2 observed at a lower m.o.i. of 1. The initial upregulation was followed by marked inhibition of CCL2 secretion from CMV-infected fibroblasts at late times (48 and 96 h p.i.) (Fig. 1a–c).

In order to determine whether CCL2 was induced by a virion component or de novo gene expression, MRC-5 fibroblasts were also infected with UV-irradiated AD169 (UV-AD169). Infection with UV-AD169 resulted in extracellular CCL2 levels similar to those observed in mock-infected fibroblast cultures, irrespective of the different infection doses used (m.o.i. 1, 3 or 10). This showed that upregulation of CCL2 during early stages of infection was due to de novo gene expression during active viral replication. Additionally, extracellular CCL2 levels were also investigated in MRC-5 fibroblasts infected with CMV clinical strain Merlin. In contrast to AD169 infection, there was no significant elevation in CCL2 during the early stages of infection, although a marked inhibition of CCL2 secretion was observed in Merlin-infected fibroblast cultures at late times (48 and 96 h p.i.), similar to that seen with AD169 (Fig. 1d). The pro-inflammatory cytokine TNF-α was not detected in the culture medium of MRC-5 fibroblasts infected with AD169 or Merlin at all time points investigated (2, 6, 12, 24, 48 and 96 h p.i.) (data not shown).

Confocal imaging of cytokine expression and intracellular distribution patterns in fibroblasts showed a low-level constitutive expression of CCL2 throughout the nuclear and cytoplasmic compartments of mock-infected cells (Fig. 2). Similar levels of intracellular CCL2 were observed in fibroblasts infected with AD169 at early time points (6, 12 and 24 h p.i.). However, CCL2 was markedly increased in the cytoplasm of CMV-infected fibroblasts at 48 h p.i., with surrounding uninfected cells expressing CCL2 at background levels comparable to levels in mock-infected cells (Fig. 2). In contrast, TNF-α expression was undetectable in mock-infected fibroblasts and there was no increased intracellular TNF-α produced during the course of CMV infection (data not shown).
CMV tegument protein pp71 stimulates CCL2 mRNA and protein expression

Investigation of the effects of individual CMV gene products on CCL2 and TNF-α expression showed a moderate, but significant, upregulation or accumulation of intracellular CCL2 protein in transient transfection settings. This occurred to some extent upon overexpression of the CMV proteins IE2p86, pUL27 (protein with a role in maribavir susceptibility), pUL44 (processivity subunit of DNA polymerase), pUL54 (catalytic subunit of DNA polymerase), pUL50 (inner nuclear membrane protein with a role in nuclear egress of viral capsids), pUL69 (tegument phosphoprotein with regulatory role in RNA transport) and pp71 (tegument phosphoprotein with multiple regulatory roles including transcription and interference with innate immunity) (Fig. 3). The increased intracellular CCL2 was not observed with overexpression of pUL53 (nuclear matrix protein with a role in nuclear egress of viral capsids) and pUL97 (viral protein kinase). For some of these viral proteins, the induction of CCL2 was due to transcriptional upregulation, as CCL2 mRNA was significantly upregulated upon transient expression of pp71 (2.3-fold, \( P=0.008 \)) and pUL50 (2.1-fold, \( P<0.001 \)) compared with pcDNA plasmid control (Table 1). The ELISA detection of CCL2 in the culture medium of transfected cells revealed a significant upregulation of CCL2 upon transient expression of CMV tegument protein pp71, but there were no significant changes in extracellular CCL2 levels on overexpression of other CMV proteins (IE2p86, pUL27, pUL44, pUL54, pUL50, pUL53, pUL69, pUL97) (Table 1). This suggested that increased intracellular CCL2 detected during the transient expression of some CMV proteins (IE2p86, pUL27, pUL44, pUL54, pUL53, pUL69, pUL97) was likely to be a result of increased intracellular accumulation, rather than induction of CCL2. None of the CMV proteins investigated in this study (IE2p86, pUL27, pUL44, pUL54, pUL50, pUL53, pUL69, pUL97, pp71) suppressed CCL2 mRNA or protein expression in our expression assays (Table 1). None of these CMV proteins altered TNF-α mRNA or protein expression (data not shown).

DISCUSSION

The regulatory and intracellular properties of CCL2 and TNF-α were investigated in human cells during CMV infection or transient expression of CMV gene products. Infection of human fibroblasts with a purified laboratory strain of CMV (AD169) resulted in upregulation of CCL2 secretion during early stages of infection, followed by
In contrast to AD169, infection with CMV clinical strain Merlin did not induce CCL2 secretion, although the repression of CCL2 was evident at late times. This indicates that CMV strain variation affects induction of CCL2. These CCL2 changes can influence viral pathogenicity and virulence, as CCL2 is a potent chemotactic factor for the infiltration of monocytes, macrophages, lymphocytes and NK-cells to sites of infection, with a critical role in controlling the spread of infection (Allavena et al., 1994; Deshmamne et al., 2009). However, these naive, uninfected immune cells attracted to the site of CMV infection may become infected, thereby facilitating dissemination of the virus. Lack of CCL2 induction early post-infection by Merlin may represent a superior immune-evasion potential of this genetically intact WT strain compared with AD169. AD169 has large (15 kb) deletion in the UL (unique long)/b’ region, as well as numerous mutations elsewhere in the genome due to adaptation for growth in fibroblast culture (Cha et al., 1996; Murphy et al., 2003). Consistent with this, fibroblasts infected with clinical CMV strains have been shown to confer resistance to the action of NK-cells, whereas those infected with high-passaged laboratory strains are more susceptible to recognition by NK-cells (Cerboni et al., 2000). Hence, differences in NK recognition of CMV infection may be mediated, at least in part, by CMV strain-dependent differences in induction of CCL2.

Consistent with previous findings in CMV-infected human foreskin fibroblast (HFF) cultures (Michelson et al., 1994), infection of fibroblasts with AD169 or Merlin did not stimulate the production of intracellular or extracellular TNF-α. This lack of TNF-α production by infected fibroblasts suggests that TNF-α is not the mediator of CCL2 upregulation during CMV infection, despite previous suggestions that it may be the inducer of CCL2 in CMV-infected fibroblast cultures (Hirsch & Shenk, 1999).

Using ELISA, immunofluorescence and real-time (RT)-PCR assays, we demonstrated that pp71 is a moderate, but important, inducer of CCL2 mRNA and protein expression during transient transfection, and this likely occurs during early stages of CMV infection. CMV tegument protein pp71 (UL82) has been demonstrated to act as transactivator and strongly stimulate transcription from the major IE promoter through ATF (activating transcription factor) and AP-1 cis-acting sequences (Liu & Stinski, 1992). Furthermore, pp71 can stimulate additional viral (US11 early gene) and cellular transcription via the ATF and CREB (cAMP response element-binding protein) sites within the promoter (Chau et al., 1999; Liu & Stinski, 1992). The CCL2 promoter contains binding sites for the transcription factors NF-κB, AP-1 and Sp1 (Ueda et al., 1994, 1997). Furthermore, CMV-induced expression of CCL2 has been shown to occur in concert with modulation of transcriptional activators NF-κB and IFN regulatory factor-3 (Hamilton et al., 2013), suggesting that pp71 triggers transcriptional upregulation via activation of one or more of these sites in the CCL2 promoter. As infection with AD169, but not UV-AD169, induces CCL2 secretion, the upregulation of CCL2 is likely dependent on de novo expression of pp71 during infection.

downregulation of CCL2 at late times, consistent with previous findings (Bodaghi et al., 1998; Hamilton et al., 2013; Hirsch & Shenk, 1999). This alteration in CCL2 levels was a direct response to viral infection, as infection with UV-AD169 did not induce such changes, instead showing CCL2 levels similar to those detected in uninfected fibroblast cultures. Immunofluorescence and confocal imaging revealed a slight delay in upregulation of intracellular CCL2, occurring specifically in CMV-infected cells, suggesting induction of CCL2 was a local response to active CMV infection, not mediated by any secreted factors. These findings strongly agree with our previous report (Hamilton et al., 2013), although Hirsch & Shenk (1999) showed that the induction of CCL2 was due to factors present in the crude virus stock, such as TNF-α and IL-1β.
The marked inhibition of CCL2 expression during late-stage infection indicates the possibility that some CMV gene products suppress CCL2 mRNA transcription or block CCL2 secretion through direct binding of the chemokine. The CMV US28 gene encodes a functional receptor for CCL2 and other β-chemokines (Gao & Murphy, 1994), which has been demonstrated to act as a chemokine scavenger (Bodaghi et al., 1998). However, CCL2 repression during late-stage infection of fibroblasts occurs at the transcriptional level, without a contribution from pUS28 binding (Hamilton et al., 2013; Hirsch & Shenk, 1999). The virus-encoded inhibitor of CCL2 transcription is yet to be identified as none of the CMV proteins investigated in this study (IE2p86, pUL27, pUL44, pUL50, pUL54, pUL53, pUL69, pUL97, pp71) suppressed CCL2 expression.

**Table 1.** CCL2 mRNA and extracellular protein expression levels during transient expression of CMV gene products in HeLa cells

CCL2 mRNA results are presented as the expression ratio relative to pcDNA 3.1 vector transfection, with an expression ratio of 1.00 representing control-level expression. All values represent the mean with 95% confidence intervals of triplicate samples.

<table>
<thead>
<tr>
<th>Expression construct</th>
<th>CCL2 mRNA expression ratio</th>
<th>Extracellular CCL2 protein level (pg ml⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>pcDNA 3.1</td>
<td>1.00</td>
<td>396.5 (274.8-518.1)</td>
</tr>
<tr>
<td>IE2p86 (UL122)</td>
<td>0.97 (0.90–1.04)</td>
<td>372.0 (214.6–529.4)</td>
</tr>
<tr>
<td>pp71 (UL82)</td>
<td>2.33 (1.80–2.86)*</td>
<td>696.9 (613.0–780.8)*</td>
</tr>
<tr>
<td>pUL27</td>
<td>0.93 (0.77–1.09)</td>
<td>371.4 (276.7–446.1)</td>
</tr>
<tr>
<td>pUL44</td>
<td>0.84 (0.77–0.91)</td>
<td>317.8 (183.7–451.9)</td>
</tr>
<tr>
<td>pUL54</td>
<td>1.23 (0.98–1.48)</td>
<td>487.8 (273.7–701.9)</td>
</tr>
<tr>
<td>pUL50</td>
<td>2.13 (1.90–2.20)*</td>
<td>428.4 (237.6–619.3)</td>
</tr>
<tr>
<td>pUL69</td>
<td>0.92 (0.81–1.03)</td>
<td>369.9 (269.0–470.9)</td>
</tr>
<tr>
<td>pUL97</td>
<td>0.98 (0.70–1.26)</td>
<td>421.1 (183.5–658.7)</td>
</tr>
<tr>
<td>pUL53</td>
<td>1.03 (0.92–1.14)</td>
<td>382.5 (353.6–411.4)</td>
</tr>
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*Statistically significant difference (P<0.05) in CCL2 expression levels compared with pcDNA 3.1 transfection.
CCL2 mRNA or protein expression in transient transfection assays.

Our results demonstrate that CMV induces cytoplasmic and extracellular upregulation of CCL2 during early stages of infection, which is stimulated at least in part by de novo expression of viral tegument protein pp71. CMV-induced upregulation of CCL2, with resulting monocyte chemotraction, can lead to tissue damage associated with altered cytokine profiles and could also facilitate the dissemination of the virus. Moreover, elevated levels of CCL2 have been associated with adverse outcomes of pregnancy, including pre-eclampsia, preterm delivery and pregnancy loss (Chaiworapongsa et al., 2002; Esplin et al., 2005). Differences in the degree of CCL2 induction by clinical and laboratory CMV strains provide important insights into the diverse immunogenic properties of CMV strains, which may be the determinants for the pathogenic potential of the virus.

**METHODS**

**Cell culture.** Human cervical carcinoma cells (HeLa; ATCC) were cultivated in Dulbecco’s minimal essential medium (DMEM; Life Technologies) containing 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin/l-glutamine (PSG). Human embryonic lung fibroblasts (MRC-5) and HFFs were obtained from ATCC, and maintained in minimal essential medium (MEM; Life Technologies) supplemented with 10% (v/v) FBS and 1% (v/v) PSG. Retinal pigment epithelial (RPE-1) cells were provided by Barry Slobedan (University of Sydney, Sydney, Australia) and cultured in DMEM/F12 + GlutaMAX medium (Life Technologies) containing 10% (v/v) FBS and 1% (v/v) PSG.

**Preparation of virus stocks.** The bacterial artificial chromosome (BAC) recombinant of CMV clinical strain Merlin (clone pAL1120) containing the full complement of the CMV genome was provided by Richard Stanton (Cardiff University, Cardiff, UK) (Stanton et al., 2010). Merlin-BAC DNA was extracted from bacterial culture using a Nucleobond BAC 100 extraction kit (Macherey-Nagel) and then transfected into MRC-5 fibroblasts using Lipofectamine 2000 (Life Technologies). Reconstituted Merlin was further propagated in RPE-1 epithelial cells to minimize the risk of mutation. Laboratory CMV strain AD169 (ATCC) was propagated in MRC-5 fibroblasts.

Merlin and AD169 stocks were prepared by high-speed centrifugation (32 800 g, 16 °C, 60 min) of cell-free viruses in a 20% sucrose density gradient. The supernatant was collected from the bottom of the gradient and concentrated by ultracentrifugation in a 20% sucrose cushion at 70 000 g for 16 h. Finally, the concentrated virus was resuspended in MEM supplemented with 2% FBS and 1% PSG and stored at −80 °C.

**CMV infection assays.** MRC-5 fibroblasts grown to 90–100% confluence in 24-well plates were inoculated with AD169 or UV-AD169 at m.o.i. 1, 3 and 10 per cell. MRC-5 cells were also infected with Merlin or UV-Merlin at m.o.i. 1. Mock-infected cultures were included as controls. Virus-inoculated cultures were incubated at 37 °C with 5% CO2 for 2 h, and then supernatant was removed and replaced with fresh medium (MEM supplemented with 2% FBS and 1% PSG). Cells were reincubated at 37 °C with 5% CO2, until culture supernatants were collected at 2, 6, 12, 24, 48 and 96 h p.i., and stored at −80 °C. Three independent experiments were performed.

**CMV gene expression plasmids.** Expression constructs containing CMV genes (pcDNA-UL27-MYC, pcDNA-MYC-pp71, pcDNA-MYC-UL50, pcDNA-MYC-UL69, pcDNA-FLAG-IE2p86, pcDNA-UL44-FLAG, pcDNA-UL53-FLAG, pcDNA-UL54-FLAG, pcDNA-UL97-FLAG) were provided by Professor Manfred Marschall and Professor Thomas Stamminger (Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, Germany). Expression constructs were generated in a similar way (Marschall et al., 2001) by PCR amplification of the respective ORFs within the CMV AD169 genome using primers containing gene-specific sequences, FLAG- or MYC-tag sequences and restriction site-specific sequences. Amplification was followed by restriction digestion and ligation of DNA containing the CMV gene with N- or C-terminal FLAG or MYC sequences into pcDNA 3.1 vector (Life Technologies). These vectors containing CMV gene inserts were transformed in Escherichia coli DH10B (Life Technologies) by electroporation and colonies with the desired inserts were subjected to overnight culture in LB medium, followed by plasmid DNA extraction using a PureLink HiPure Plasmid Midiprep kit (Life Technologies).

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For immunofluorescence, MRC-5 fibroblasts cultured on coverslips in six-well plates were infected with AD169 at m.o.i. 1.0 as above, and analysed at 6, 12, 24 and 48 h p.i.

**Transfected transfection assays.** For immunofluorescence, HeLa cells grown on coverslips were transfected with 4 µg individual CMV gene constructs using Lipofectamine 2000 (Life Technologies) and analysed for cytokine expression levels at day 3 post-transfection.

For CCL2 mRNA and protein expression analysis, HeLa cells grown in 24-well culture plates were transfected with CMV gene constructs (2 µg DNA per well). Culture supernatants were harvested on day 3 post-transfection. Cells were also harvested and lysed in 600 µl lysis/binding buffer (RNAqueous kit; Ambion) and stored at −80 °C.

**CMV and TNF-α ELISA.** CCL2 and TNF-α proteins were measured in the culture supernatants collected from CMV infection assays using human CCL2 (MCP-1) ELISA (Life Technologies) and TNF-α EASIA (Life Technologies) kits, following the manufacturer’s instructions.

**Indirect immunofluorescence analysis.** For immunofluorescence analysis, coverslips were transferred to new six-well plates, and cells were fixed with 4% (w/v) paraformaldehyde and permeabilized in 0.2% (v/v) Triton X-100 (Progen), followed by non-specific binding block with horse serum for 30 min at 37 °C. The following primary antibodies were used for incubation at 37 °C for 90 min: (i) for infection assay, goat anti-CCL2 and rabbit anti-IE2p86 or mouse anti-TNF-α and rabbit anti-IE2p86; (ii) for transfection assay, goat anti-CCL2 and mouse anti-FLAG/MYC antibodies or mouse anti-TNF-α and rabbit anti-FLAG/MYC antibodies. Cells were washed with 1× PBS and incubated with appropriate secondary antibodies: (i) for infection assay, Alexa Fluor 488 donkey anti-goat and Alexa Fluor 555 donkey anti-rabbit antibodies or Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 goat anti-rabbit antibodies; (ii) for transfection assay, Alexa Fluor 488 donkey anti-goat and Alexa Fluor 555 donkey anti-mouse antibodies or Alexa Fluor 488 goat anti-mouse and Alexa Fluor 555 goat anti-rabbit antibodies (Life Technologies). Nuclei were counterstained with Vectashield mounting medium with DAPI (Vector Laboratories). Immunofluorescence imaging was done using a Leica TCS SP5 confocal microscope, and immunofluorescence was analysed at excitation wavelengths of 450–490 and 510–560 nm. Confocal images were processed with Adobe Photoshop (version 3). CCL2 and TNF-α expression levels were quantified as the mean intensity of staining calculated based on the total staining intensity of
positive pixels to total pixels in the image using the ImageJ program (http://imagej.nih.gov/ij/).

**Analysis of CCL2 mRNA expression levels.** Total RNA was extracted using a RNAqueous kit (Ambion) and RNA extracts were DNase-treated using a DNA-free DNase Treatment and Removal kit (Ambion). RNA integrity was analysed by a NanoDrop 1000 spectrophotometer. CCL2 mRNA levels were determined using RT-PCR with SYBR Green I One-Step RNA Amplification Master Mix (Roche) on a Roche LightCycler 2.0. PCR primers and cycling conditions were as described previously (Hamilton et al., 2012). Briefly, 25 ng total RNA template (2.5 μl) was added to a 17.5 μl reaction mixture containing 4 μl LightCycler RT-PCR Mix (Roche), 2 mM MgCl2 (Roche), 0.4 μM each primer (MCP1-3F/MCP1-3R) (Sigma) and 0.4 μl LightCycler RT-PCR Enzyme Mix (Roche). Melting curve analysis and visualization with gel electrophoresis were used for determination of PCR specificity. CCL2 mRNA expression levels were normalized against the geometric mean of EIF4A2 and SF3A1 reference genes, which were found to be stably expressed under culture and experimental conditions. CCL2 mRNA expression data were calculated as mRNA expression ratios relative to pcDNA 3.1 transfection control using the Pfaffl formula (Pfaffl, 2001).

**Statistical analysis.** CCL2 mRNA and protein expression ratios were expressed as the mean with 95% confidence intervals of triplicate samples. Student's t-test was applied to determine the significance of differences in CCL2 expression in response to CMV infection or transient expression of CMV gene products versus pcDNA control. P<0.05 was considered statistically significant. Analysis was performed using SPSS version 19.0 (SPSS).

**ACKNOWLEDGEMENTS**

This collaborative study was supported by funding from Group of Eight (Australia-Germany Joint Research Co-operation Scheme). The authors thank the staff at Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg for support during this study. This project was also supported by a scholarship from the Australian Centre for Perinatal Science.

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