Human cytomegalovirus directly modulates expression of chemokine CCL2 (MCP-1) during viral replication

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Human cytomegalovirus (CMV) infects monocytes and other haematopoietic progenitor cells which then act as reservoirs for latency and virus dissemination. The chemokine CCL2 (monocyte chemotactic protein-1 or MCP-1) exhibits potent chemotactic activity for monocytes and is a likely target for CMV-induced immunomodulation. In this study, we demonstrate CMV modulates CCL2 expression in MRC-5 fibroblasts with multiplicity-dependent kinetics, where CCL2 is upregulated during early stage infection, followed by CCL2 inhibition at late stage infection. This CMV-induced CCL2 modulation was dependent upon virus replication, as UV-inactivated virus did not elicit any changes in CCL2 levels. Dual immunofluorescence staining showed CMV strains AD169, purified AD169, Merlin, FIX WT (FLAG-US28/WT) and pUS28-deficient FIX (FIX-ΔUS28) all induced upregulation of CCL2 primarily within infected cells. Focal upregulation of CCL2 within FIX-ΔUS28-infected cells demonstrated intracellular CCL2 accumulation was independent of CCL2 sequestration by the CMV-encoded chemokine receptor US28. Infection with purified virus confirmed CMV-induced CCL2 upregulation was not due to any CCL2-inducing factors contained within non-purified virus stocks. The CMV-induced CCL2 expression kinetics occurred concurrently with modulation of the CCL2 transcriptional activators NF-κB, interferon regulatory factor 3 and cytokine IFN-γ, independent of virus strain, and with the establishment of viral replication compartments within infected cell nuclei. This is the first report to our knowledge to demonstrate CMV modulation of CCL2 expression within infected cells during viral replication. This immune modulation may facilitate virus dissemination, establishment of latency and pathogenesis of CMV-induced host disease.

INTRODUCTION

Human cytomegalovirus (CMV) is a ubiquitous infection, with seropositivity rates in the US adult population ranging between 40 and 90% (Staras et al., 2006). The manifestations of CMV disease are diverse, and clinical disease is closely linked to immune status of the infected host (Compton, 2004). CMV infection is usually asymptomatic in individuals with normal immune function, whilst infection can have serious consequences for the immunocompromised.

CMV has immunomodulatory properties and immune-evading strategies that alter the immune response to infection, properties which may be implicated in virus dissemination and disease pathogenesis. Monocytes are important immune effector cells in the innate and adaptive immune response, and are targeted by CMV as cell reservoirs for latency and dissemination. The chemokine CCL2 (monocyte chemotactic protein-1 or MCP-1) is a potent chemotactic protein for monocytes and other leukocytes to sites of infection and inflammation (Göser et al., 2005). CMV infection has been associated with increased CCL2 expression in renal transplant recipients (van de Berg et al., 2010), amniotic fluid following congenital infection (Scott et al., 2012), CMV-infected placenta from stillborn babies (Hamilton et al., 2012) and cell culture monolayers (Bodaghi et al., 1998; Cheenan et al., 2001; Hirsch & Shenk, 1999), including latently infected cells during in vitro culture (Stern & Slobedman, 2008). Furthermore, aberrant expression of CCL2 has been associated with a number of adverse clinical outcomes.
independent of CMV infection, including pregnancy loss (Chaiworapongsa et al., 2002; Esplin et al., 2005; Katabuchi et al., 2003).

The transcriptional factors NF-κB and interferon regulatory factor 3 (IRF-3) are key regulators of the cellular immune response to infection. Previous studies have shown CMV modulates the activity of these transcriptional activators (Abate et al., 2004; Jarvis et al., 2006; Paladino et al., 2006; Yurochko et al., 1995); however, the exact mechanisms and kinetics are not well defined. CCL2 expression at the transcriptional level is controlled by a network of transcriptional regulators of the promoter, including NF-κB, IκBζ, AP-1, Sp-1 and C/EBP, with the dominant regulator being NF-κB (Hildebrand et al., 2013). Activation of IRF-3 could also indirectly upregulate CCL2 transcription by activating a variety of interferon-stimulated genes (ISGs). This might include IFN-β, which can induce nuclear translocation of the CCL2 transcription factors STAT-1 (Platanias, 2005) and NF-κB (Yang et al., 2000) via autocrine/paracrine signalling.

Our recent investigations into CMV-induced cytokine changes within the placenta (Hamilton et al., 2012) and amniotic fluid of CMV-infected pregnant women (Scott et al., 2012) led us to further investigate the effects of CMV on CCL2 expression kinetics during in vitro infection of cell cultures. This study demonstrated CMV directly modulates CCL2 expression within infected cells during viral replication, which may have important implications for the pathogenesis of CMV disease.

RESULTS

CMV productive infection causes upregulation of CCL2 mRNA and protein expression during early stages of infection followed by CCL2 inhibition during late stage infection

The effects of CMV infection on CCL2 mRNA and protein expression kinetics in fibroblast cell cultures were determined using relative real-time PCR and ELISA, respectively. Infection of MRC-5 fibroblast cells with CMV strain AD169 resulted in distinct expression kinetics of CCL2 mRNA and protein in a multiplicity-dependent manner, where CCL2 was upregulated during early stage infection followed by a decline from peak levels during late stage infection (Fig. 1a). At the higher m.o.i. of 2 p.f.u. per cell, CCL2 mRNA was increased 10-fold relative to mock-infected cells at 1 day post-infection (p.i.). However, by 4 days p.i. CCL2 mRNA expression was decreased 12-fold, and decreased 20-fold by 7 days p.i. At lower m.o.i. values, a similar, yet delayed expression kinetic profile was observed with initial increases of up to fivefold in CCL2 mRNA transcripts by 4 days p.i. followed by a decline of expression up to 30-fold by 7 days p.i. The cellular reference genes EIF4A2 and SF3A1 remained stably expressed over the time course and over the varying m.o.i. values (data not shown). Expression kinetics of CCL2 at the transcriptional level were reflected at the translational level (Fig. 1b). Secreted CCL2 protein levels in cell culture supernatant were concordant with the mRNA data, and highly correlated using a two-tailed Spearman’s correlation coefficient test (P<0.0001).

The changes in CCL2 expression were then assessed for dependence upon viral replication by inoculating MRC-5 cells with UV-inactivated virus at comparable m.o.i. values. Complete UV-inactivation of virus replication in this study was confirmed using immunofluorescence and real-time PCR analysis of CMV antigen expression and CMV DNA replication, respectively. Cells infected with AD169 showed nuclear staining for CMV immediate early (IE)/early (E) protein whereas no staining for CMV IE/E protein was observed in UV-inactivated AD169 (UV-AD169)-inoculated cells (see Fig. S1a, available in JGV Online). Detection of CMV pp65 E/late tegument protein in cells inoculated with AD169 was observed in nuclei with some cells also showing diffuse staining in cytoplasm, while UV-AD169-inoculated cells showed limited punctate staining in cell cytoplasm with no nuclear or diffuse staining in cell cytoplasm observed (see Fig. S1a). Staining for CMV antigen was not observed in any mock-infected cells. These results demonstrate complete inactivation of virus replication capability was achieved whilst retaining virus cell binding and entry capability. Supplementary real-time PCR analysis of CMV DNA accumulation in cell culture supernatant over the 7 day time course confirmed successful inactivation of UV-AD169 viral replication (see Fig. S1b).

For analysis of CCL2 mRNA expression kinetics in response to UV-AD169, MRC-5 cells were inoculated, and mRNA expression analysed, using identical protocols to that used with replication-competent virus. Fibroblast cells inoculated with UV-AD169 at m.o.i. values ranging from 0.02 to 2 p.f.u. per cell did not result in any substantial changes in CCL2 mRNA expression at 1, 4 or 7 days p.i. relative to mock-infected cells (Fig. 1c). An inflammatory response to UV-AD169 virus inoculation was confirmed by measuring CXCL10 (IFN-γ-induced protein 10 or IP-10) mRNA expression, a marker of an intracellular antiviral response (Paladino et al., 2006). CXCL10 mRNA expression was upregulated in a multiplicity-dependent manner 1 day p.i., and then decreased over time (Fig. 1d). These results demonstrated UV-AD169 virus stocks retained immunostimulatory properties following UV irradiation, despite inoculum failing to induce increases in CCL2 mRNA transcription, suggesting CMV-induced modulation of CCL2 expression was driven by viral replication. UV-AD169 induction of CXCL10, independent of CCL2 modulation, is consistent with the model advocated by Paladino et al. (2006), where low-level virus cell entry induces expression of a subset of ISGs prior to viral replication and in the absence of NF-κB-induced pro-inflammatory cytokine production.
CMV-induced upregulation of CCL2 expression is a direct response to CMV replication within infected cells

The localization and source of increased CCL2 protein expression in the cell culture monolayers was assessed using dual immunofluorescence to co-localize CMV antigen with CCL2 protein. Cells infected with unpurified AD169, purified AD169 and CMV WT strain Merlin at 4 days p.i. (0.2 p.f.u. per cell) showed intense focal staining for CCL2 exclusively within infected cells, and not in uninfected cells (Fig. 2a). The CMV-induced focal staining of CCL2 (including occasional intracellular redistribution) was also observed when CMV antigen detection was omitted from the immunocytochemical analyses, demonstrating focal CCL2 expression was not due to non-specific antibody interactions (see Fig. S2). UV-AD169 inoculation of fibroblast cell cultures did not produce focal staining for CCL2 in any cells of the monolayer, but showed diffuse low-level expression of CCL2 comparable to the localization observed in mock-infected cell cultures (Fig. 2a). No difference in CCL2 expression could be observed between UV-AD169 and mock-infected cell cultures. These results suggest the elevated expression of CCL2 observed was a direct response to CMV replication within infected cells of the monolayer independent of CMV strain or CCL2-inducing factors contained within unpurified virus stocks.

CMV encodes the chemokine receptor US28, which is a functional receptor for a number of chemokines including CCL2, RANTES, macrophage inflammatory protein (MIP)-1α, MIP-1β (Bodaghi et al., 1998). This receptor binds and sequesters chemokines from the extracellular environment into the CMV-infected cell cytoplasm. CMV strains FIX WT and pUS28-deficient FIX (FIX-ΔUS28) were utilized to determine if the observed co-localization of CMV antigen and CCL2 protein was a result of CMV US28 sequestration of CCL2 from the extracellular environment into the infected cells. Cells infected with both FIX WT and FIX-ΔUS28 (0.2 p.f.u. per cell) showed identical co-localization patterns of CMV IE/E and CCL2 proteins to those observed in AD169- and Merlin-infected monolayers at 4 days p.i., confirming the focal staining for CCL2 expression was not due to US28 sequestration of CCL2 in infected cell cytoplasm (Fig. 2b).

CMV-induced modulation of CCL2 expression temporally localizes with modulation of the CCL2 transcriptional activators NF-κB, IRF-3 and IFN-β

The cellular mechanisms driving CMV-induced CCL2 modulation were investigated by examining expression and cellular localization of the CCL2 transcriptional activators NF-κB, IRF-3 and IFN-β. Utilizing triple immunofluorescence with the CMV IE/E antibody cocktail (detecting pIE72 and pUL44 antigen) allowed observation of temporal CMV replication kinetics simultaneously with modulation of CCL2 and the transcriptional activators of CCL2. While CMV immediate early IE72 protein is diffusely expressed throughout the nucleus during immediate early and early stages of replication, pUL44 is expressed only during early/late stages and is identified by formation of multiple discrete viral replication compartments (RCs) that enlarge and fuse during the replication cycle (Xiaofei et al., 2012). These RCs were readily distinguished from pIE72 detection by a higher intensity Alexa Fluor 594 fluorescence signal.

At 4 and 24 h p.i., CMV-infected MRC-5 cells (0.2 p.f.u. per cell) showed pIE72 expression within cell nuclei, no pUL44 expression, and limited diffuse expression of CCL2, NF-κB, IRF-3 and IFN-β in cell cytoplasm. At 48 h p.i., immature RC formation was observed as discrete compartments within infected nuclei that co-localized with activation of both NF-κB and IRF-3, and modest upregulation of CCL2 and IFN-β protein. During later stages of infection (96 h p.i.), mature RCs were established within infected nuclei that coincided with intense staining for NF-κB and IRF-3 within both cytoplasm and nucleus, and intense focal expression of CCL2 and IFN-β within cytoplasm. At late stage infection (168 h p.i.), pIE72 staining was limited within infected nuclei and pUL44 was predominantly detected. This coincided with a marked reduction in staining for NF-κB, IRF-3, IFN-β and CCL2. Fig. 3 shows representative images for the expression kinetics of CMV IE/E antigens, CCL2 protein and the CCL2 transcriptional activators in purified AD169-infected MRC-5 cells (0.2 p.f.u. per cell) during the course of infection. The temporal expression of NF-κB, IRF-3, IFN-β and CCL2 in response to CMV replication occurred exclusively within CMV-infected cells and no differences could be observed between any of the replication-competent CMV strains used in this study (Fig. 4). UV-AD169 and mock-infected cultures did not show any changes in NF-κB, IRF-3, IFN-β or CCL2 expression at 4, 24, 48, 96 or 168 h p.i. (data not shown).

DISCUSSION

In this study, CMV infection of MRC-5 fibroblast cell cultures resulted in distinct, multiplicity-dependent, CCL2 expression kinetics with upregulation of CCL2 during early stages of infection, followed by CCL2 inhibition during late stage infection. This CMV-induced CCL2 modulation was dependent upon virus replication, as UV-inactivated virus did not elicit any changes in CCL2 levels. Reproducible co-localization of CMV antigen and CCL2 protein showed CCL2 induction occurs primarily within CMV-infected cells with no or minimal indirect effect on uninfected cells observed. Simultaneous expression of CMV antigen and CCL2 protein in cells infected with purified virus demonstrated this result was independent of CCL2-inducing factors contained within crude virus stocks. US28-mediated CCL2 sequestration was also excluded as a cause for this focal expression due to identical staining...
patterns observed in cells infected with CMV strains AD169, Merlin, FIX WT and FIX-DUS28. These findings demonstrate for the first time to our knowledge that CMV directly modulates CCL2 expression within infected cells during viral replication. However, some previous studies have suggested the initial upregulation of CCL2 in CMV-infected fibroblast cultures may be an indirect effect caused by uninfected cells responding to CCL2-inducing factors such as TNF-α and IL-1β, either found in virus inoculum or secreted by CMV-infected cells (Hirsch & Shenk, 1999). The discrepancy between the findings in this study and the former study (Hirsch & Shenk, 1999) may be primarily due to differences arising from the use of different host cell subpopulations (MRC-5 versus human foreskin fibroblasts), virus stock concentrations (and inoculum-associated immunostimulatory proteins), and lack of immunocytochemical analyses in the former study for detection of tissue-fixed chemokine.

CCL2 upregulation in response to CMV infection was found to occur during early stages of viral replication and concurrent with the establishment of viral RCs within infected cell nuclei, but not observed during immediate early or late stages of infection. The CMV-induced CCL2 response occurred with simultaneous activation of the CCL2 transcriptional activators NF-κB, IRF-3 and cytokine IFN-β, which were then inhibited during late stage infection in conjunction with CCL2 downregulation. Previous studies have demonstrated that CMV-induced activation of IRF-3, IFN-β and other ISGs during immediate early stages of infection occurs through the DNA sensor DAI/ZBP1 (DeFilippis et al., 2010). However, in the current study, inhibiting expression of ZBP1 by treating cells with the small interfering RNA molecule ZPB1 no. 57 (DeFilippis et al., 2010) prior to CMV infection did not affect the CMV-induced activation or expression kinetics of NF-κB, IRF-3, IFN-β and CCL2. These data suggest additional pathways and mechanisms are involved (data not shown). Decreasing CCL2 levels during late stage infection was found to occur at the
transcriptional level, consistent with previous findings (Hirsch & Shenk, 1999). This was based on our observation of convergent CCL2 mRNA and protein expression kinetics data and stable reference gene mRNA expression over the time course and varying m.o.i. values. CCL2 transcriptional inhibition during late stage infection was observed with concurrent inactivation of NF-κB and IRF-3, suggesting CMV inhibits CCL2 transcription possibly via CMV-induced NF-κB inhibition (Jarvis et al., 2006) or, as our results suggest, via concerted inhibition of both NF-κB and IRF-3. CMV-induced CCL2 upregulation during early stages of infection is postulated to benefit the virus by transiently attracting a limited number of monocytes and other leukocytes to sites of infection which then allow virus dissemination, and act as reservoirs of latency. CCL2 increase could also slow viral clearance by impairing virus-specific CD8+ T-cell expansion and differentiation into effector cytotoxic T-lymphocytes as observed in the murine model (Daley-Bauer et al., 2012). Subsequent inhibition of CCL2 expression during later stages of infection may assist with concealing the infected cell from the infiltrating immune effector cells, and facilitate other immune evasion mechanisms such as downregulation of lytic viral gene expression, allowing the establishment of latency. This immune modulation may also contribute to pathogenesis of disease, leading to adverse outcomes of CMV infection in conditions such as pregnancy via immune dysregulation with infected organ (placental) dysfunction (Hamilton et al., 2011; Stern & Slobedman, 2008). Understanding the mechanisms by which CMV modulates the immune environment within infected hosts is vital to understanding the pathogenesis of CMV-induced disease. This study demonstrates CMV directly modulates the expression of the chemokine CCL2 within CMV-infected cells during the viral replication cycle, thereby potentially facilitating virus dissemination, establishment of latency and pathogenesis of disease.

**METHODS**

**Cell lines and preparation of virus stocks.** Human MRC-5 fetal lung fibroblasts (ECACC) were maintained in modified minimum essential medium (MEM; Invitrogen) supplemented with 10% FBS (Bovogen) and 100 U penicillin G ml⁻¹, 100 μg streptomycin ml⁻¹ and 29.2 μg L-glutamine ml⁻¹ (1× PSG; Invitrogen). Cell lines were Mycoplasma-free and maintained at 37°C with 5% CO₂. Bacterial artificial chromosome (BAC) recombinants of FIX strain (FLAG-US28/WT) and pUS28-deficient FIX (FIX-ΔUS28) derived from the CMV clinical isolate VR1814 (Stropes & Miller, 2008) were kindly provided by Gabriele Hahn (The Wistar Institute of Anatomy and Biology, PA, USA). Since FIX-BAC WT contains a large deletion in the unique short (Us) region, CMV Merlin-BAC (Stanton et al., 2010) was also utilized in this study. This latter virus contains the full complement of the CMV genome and was kindly provided by Richard Stanton (Cardiff University, Cardiff, UK). DNA was transfected and propagated in ARPE-19 cells (ECACC) in Dulbecco’s modified Eagle’s medium/F12+Gluta-MAX (Invitrogen) supplemented with 5% FBS and 1× PSG, with one passage in MRC-5 cells to increase viral titre. The CMV laboratory strain AD169 (ATCC) was propagated in human MRC-5 fetal lung fibroblasts maintained in MEM supplemented with 2% FBS and 1× PSG. Supernatants containing extracellular virus were stored at −80°C. The titre of virus stocks was determined by standard plaque assay in MRC-5 cells. UV-inactivated AD169 virus (UV-AD169) was obtained by irradiating 3 ml of virus stock with four doses of 0.12 J cm⁻² (total dose 0.48 J cm⁻²). This dosage has been previously reported to be sufficient to inactivate virus replication ability while retaining cell viability.

**Fig. 2.** CMV-induced upregulation of CCL2 expression is a direct response to CMV replication within infected cells. Localization of CMV IE/E and CCL2 protein in MRC-5 fibroblast cell cultures infected with (a) UV-AD169, AD169, purified AD169 (pAD169), Merlin, mock, and (b) FIX WT and FIX-ΔUS28 (0.2 p.f.u. per cell) at 4 days p.i.
binding and entry function (Child et al., 2002; Zhu et al., 1997). Purified AD169 virus was obtained by ultracentrifugation using a linear 20–50 % sucrose density gradient as previously described (Britt, 2010).

**MRC-5 culture assays.** MRC-5 cells seeded in 24-well plates and cultured to 90–100% confluence were inoculated with AD169 or UV-AD169 (in duplicate) for each time point of analysis, at an m.o.i. of 2, 0.2 and 0.02 p.f.u. per cell. Mock-infected cultures were also included for each plate in duplicate. Plates were centrifuged at 770 g for 30 min followed by 1 h incubation at 37 °C with 5% CO₂. Supernatant was removed and replaced with fresh medium. Cells were incubated at 37 °C with 5% CO₂ until cell harvest at day 1, 4 or 7 p.i. using 0.25 % trypsin (Invitrogen). The cell suspension was transferred to a 1.5 ml tube, centrifuged at 770 g for 5 min and the pellet collected for RNA extraction.

**RNA extraction and quantitative real-time PCR.** Total RNA was extracted and purified using RNAqueous and DNA-Free DNase Treatment and Removal Reagents kits (Ambion) following the manufacturer’s protocol. RNA quantity and purity were assessed using spectrophotometry (ND-1000 NanoDrop; Thermo Fisher). Quantitative real-time PCR was performed using a Roche 2.0 LightCycler with One-Step RNA Amplification SYBR Green I master mix (Roche) and 25 ng of total RNA. CCL2 and CXCL10 oligonucleotide primers were as follows: CCL2, 5′-ATTCCCCAGGGTGCTGC-3′ and 5′-ACTTGCTGTTGATTCAGTA-3′; CXCL10, 5′-TTCAAGGAGTACCTCTCTCTAG-3′ and 5′-CTGGATTCCAGACATTCTCTCTC-3′. The reference genes EIF4A2 and SF3A1 were selected for normalization from a panel of 12 reference genes (PrimerDesign) based on stability of expression in response to CMV infection using GeNorm Software (Vandesompele et al., 2002) (data not shown). Reactions were carried out under the following conditions: cDNA synthesis at 55 °C for 15 min, denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 15 s. Specificity of the PCR mRNA products was determined using melting curve analysis, gel electrophoresis and control PCR experiments excluding cDNA synthesis (data not shown). Cytokine mRNA was normalized against the geometric mean of reference gene mRNA, and expression ratios calculated relative to mock infection using the Pfaffl formula (Pfaffl, 2001). Amplification efficiency for each individual reaction was determined using LinReg software (Ruijter et al., 2009) and the (SavrgE) model was used for analysis as described (Karlen et al., 2007).

**CCL2 ELISA.** For cell culture supernatant protein analysis, 1 ml aliquots of cell culture supernatant were harvested prior to total RNA extraction and frozen at −80 °C. CCL2 protein was measured in culture supernatant harvested from cell culture assays at days 1, 4 and 7 p.i. using a CCL2 (MCP-1) ELISA kit (Invitrogen), following the manufacturer’s protocols.

**Immunofluorescence.** MRC-5 cells were seeded onto two-well chamber slides (BD Biosciences) in MEM supplemented with 2 % FBS.
plus 1 × PSG and inoculated with CMV strains AD169, UV-AD169, purified AD169, Merlin, FIX WT or FIX-US28 at an m.o.i. of 0.2 p.f.u. per cell. Mock-infected cultures were also established. After 2 h incubation at 37 °C with 5 % CO₂, inoculum was replaced with fresh medium. At 4 days p.i., cells were washed with PBS and fixed in 4 % paraformaldehyde for 7 min. Cells were washed twice in PBS and permeabilized in 0.2 % Triton X-100 in PBS for 20 min at 4 °C. Cells were then washed four times in PBS. Non-specific staining was blocked by incubation with 2 % BSA in PBS for 30 min. CCL2 was first detected using mouse monoclonal anti-human MCP-1 IgG₂b isotype (R&D Systems; 1: 20 dilution) for 60 min followed by 40 min incubation with Alexa Fluor 488 IgG₂b goat anti-mouse (Invitrogen; 1: 1000 dilution). CMV was then detected using mouse monoclonal anti-CMV immediate early (IE) (IE IE72 protein) and early (E) (delayed early DNA-binding protein p52 or pUL44) (IE/E) antibody IgG₁ and IgG₂a isotypes (clones DDG9 and CCH2; Dako; 1: 100 dilution) incubated for 60 min followed by 40 min incubation with Alexa Fluor 594 IgG goat anti-mouse secondary antibody (Invitrogen; 1: 1000 dilution). The use of different murine mAb isotypes allowed sequential staining for proteins of interest with limited background or cross-reactivity and staining for CCL2 protein prior to staining for CMV IE/E antigens ensured accurate CCL2 detection. The sections were washed extensively in two changes of wash buffer (Dako) after each antibody incubation step. Cells were then covered with ProLong

Fig. 4. CMV-induced upregulation of CCL2 expression and activation of NF-κB, IRF-3 and IFN-β is independent of CMV viral strain. Localization of CMV IE/E antigen and CCL2 protein with the CCL2 transcriptional activators NF-κB, IRF-3 and IFN-β in MRC-5 fibroblast cell cultures infected with UV-AD169, AD169, purified AD169 (pAD169), Merlin, FIX WT or FIX-US28 (0.2 p.f.u. per cell) or mock-infected at 4 days p.i.
Gold Antifade Reagent containing DAPI (Invitrogen) and mounted with a coverslip.

For triple immunofluorescence, CCL2 was first detected as described above. CMV IE/E antibody (1:100 dilution) was then mixed with either rabbit polyclonal anti-human NF-κB p105/p50 antibody (Abcam; 1:250 dilution), rabbit polyclonal anti-human IRF-3 antibody (Abcam; 1:200 dilution) or rabbit monoclonal anti-human IFN-β antibody (Abcam; 1:100 dilution) and added to cells for 60 min incubation. The secondary antibodies Alexa Fluor 594 IgG goat anti-mouse (Invitrogen; 1:1000 dilution) and Alexa Fluor 350 IgG donkey anti-rabbit (Invitrogen; 1:1000) were then mixed and added to cells for 40 min incubation. Again, the sections were washed extensively in two changes of wash buffer after each antibody incubation step. Cells were then covered with CC/Mount (Sigma) and mounted with a coverslip. For all experiments, mock-infected cells and cells incubated with universal mouse IgG isotype primary antibody (Dako) served as negative controls (data not shown). Imaging of all cells was carried out using a Nikon Eclipse E400 light microscope with a Y-Fli Epi Fluorescence attachment and a DS camera control unit DS-L2, DS camera head DS-Fi1 (Nikon).

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