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Cytomegalovirus Enhances Macrophage TLR Expression and MyD88-mediated Signal Transduction to Potentiate Inducible Inflammatory Responses

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Abstract

Circulating monocytes carrying human cytomegalovirus (HCMV) migrate into tissues, where they differentiate into HCMV-infected resident macrophages that upon interaction with bacterial products may potentiate tissue inflammation. Here we investigated the mechanism by which HCMV promotes macrophage-orchestrated inflammation using a clinical isolate of HCMV (TR) and macrophages derived from primary human monocytes. HCMV infection of the macrophages, which was associated with viral DNA replication, significantly enhanced TNF-α, IL-6 and IL-8 gene expression and protein production in response to TLR4 ligand (lipopolysaccharide, LPS) stimulation compared with mock-infected LPS-stimulated macrophages during a 6-day *in vitro* infection. HCMV infection also potentiated TLR5 ligand-stimulated cytokine production. To elucidate the mechanism by which HCMV infection potentiated inducible macrophage responses, we show that infection by HCMV promoted the maintenance of surface CD14 and TLR4 and 5, which declined over time in mock-infected macrophages, and enhanced both the intracellular expression of adaptor protein MyD88 and the inducible phosphorylation of IκBα and NF-κB. These findings provide additional information toward elucidating the mechanism by which HCMV potentiates bacteria-induced NF-κB-mediated macrophage inflammatory responses, thereby enhancing organ inflammation in HCMV-infected tissues.

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Introduction

Human cytomegalovirus (HCMV) is an important cause of disease in immunocompromised hosts and the most commonly acquired intrauterine infection in humans (1–3). The clinical syndromes associated with these disease manifestations can be correlated with the level of virus replication and respond to treatment with antiviral agents (3). In contrast, some chronic diseases associated with HCMV infection appear not to have high levels of virus replication as a correlate of disease (1, 3). Perhaps the most studied associations with chronic disease are the accelerated rate of coronary artery and carotid artery disease in patients with serologic evidence of HCMV infection (4–10). Similarly, the association between HCMV infection and exacerbations of inflammatory bowel disease is well-described (11–17). The mechanism(s) responsible for the chronic inflammation and exacerbation of ongoing inflammatory disease in subjects with HCMV infection are not well described but have not been associated with the loss of immune control of virus replication. Thus, understanding the role of HCMV persistent infection and inflammation could provide insight into mechanisms of disease in chronic conditions such as inflammatory bowel disease.

HCMV infects mononuclear phagocytes at the hematopoietic stem cell stage of differentiation (18), permitting the release of HCMV-infected monocytes into the circulation (19, 20). In myeloid progenitor cells and during the early stages of differentiation, the viral genome can persist without replication, allowing circulating $CD14^+$ monocytes to serve as a reservoir for latent HCMV (21). Circulating HCMV-infected monocytes migrate into organ tissues, where they differentiate into resident macrophages and dendritic cells (DCs) (22, 23). Infection with HCMV enhances monocyte trans-endothelial migration and motility (24, 25), promoting the dissemination of infected monocytes into the tissues. Non-productive infection is maintained by repression of the immediate early (IE) genes that drive lytic transcription (26, 27). Repression of these genes in undifferentiated myeloid cells appears to be achieved through histone suppression of the major IE promotor, but, when the cells differentiate, changes in the histone-modified chromatin structure associated with the IE genes initiate gene expression and the lytic transcription program, resulting in release of viral progeny. In compromised immunological or physiological conditions that impair immune surveillance, the differentiation of newly recruited HCMV-infected monocytes into tissue macrophages can activate viral gene expression, leading to the local production and release of viral progeny (21, 28–30). Thus, HCMV promotes monocyte dissemination into the tissues, where differentiation-dependent activation of macrophages leads to virus expression and the release of HCMV throughout the body.

HCMV has been identified in all major leukocyte populations in peripheral blood (31), but the infection of blood monocytes is particularly relevant to organ inflammatory disease, since blood monocytes are the source of macrophages in many tissues (32, 33). After recruited monocytes take up residence in tissues such as the intestinal mucosa and differentiate into macrophages (34), they are positioned to participate in or orchestrate HCMV-associated organ inflammation, especially in response to tissue-invading bacteria or bacterial products. In this connection, we (35–38) and others (39, 40) have reported that HCMV infection promotes pro-inflammatory cytokine and chemokine production by monocytes and macrophages. Further elucidation of the immunobiology of HCMV-induced

inflammatory responses in macrophages could provide new insight into the mechanism of HCMV-associated organ inflammatory disease, a clinical problem of increasing frequency due to the expanding use of immunosuppressive therapies (41–46).

Here we show that HCMV infection of monocytes during their differentiation into macrophages significantly enhanced Toll-like receptor (TLR)-induced macrophage inflammatory responses. HCMV infection was associated with the maintenance of surface CD14 and TLR4 and 5, which declined over time in mock-infected macrophages. HCMV also enhanced macrophage expression of MyD88 and the inducible phosphorylation of both IκBα and NF-κB, enhancing inducible inflammatory cytokine production. These results offer a mechanism, at least in part, by which HCMV infection potentiates macrophage responses to bacterial components.

Materials and Methods

Virus

HCMV strain TR (a gift of J. Nelson, Oregon Health and Sciences University, to WJ Britt) was originally isolated from the eye of an AIDS patient with HCMV retinitis (47). The virus was propagated (<3 passages) in human foreskin fibroblasts, harvested at 100% cytopathic effect, and isolated by centrifugation at 16,000x*g* for 2 h at 4°C (48). Viral pellets were resuspended in RPMI plus antibiotics and 10% human AB serum and stored at −80°C in single use aliquots. Only virus passaged <6 times was used in the experiments described here. Virus titers were determined using our previously described assay based on the detection of HCMV immediate early antigen 1 (IE1) (49). Control HCMV included (a) UV-inactivated HCMV and (b) HCMV-free fibroblast culture supernatant. For inactivated HCMV, virus was exposed to ultraviolet (UV) radiation at 150 mJ in a cross-linking chamber (Bio-Rad, Hercules CA), as described (50). For HCMV-free fibroblast culture supernatant, culture supernatant from HCMV-infected fibroblasts was ultracentrifuged (UC) at 30,000x*g* for 1 h, and the absence of infectious virus in the culture supernatant was confirmed by incubating the supernatant on foreskin fibroblasts. UV-inactivated HCMV and HCMV-free UC fibroblast culture supernatants were stored at −80°C.

Monocytes and HCMV infection of monocyte-derived macrophages

Mononuclear cells were isolated from blood donated by healthy HCMV-seronegative donors by Ficoll Hypaque sedimentation, enumerated by automated cell counter (Beckman Coulter, Fullerton, CA) and plated in serum-free RPMI in 24-well plates or for immunofluorescence analysis onto glass converslips inserted into 24-well plate wells at a concentration of 2×10^6 monocytes/well (see Detection of HCMV below) (51). After 1 h incubation, the non-adherent lymphocytes were removed by washing, and the media was replaced with RPMI containing 10% human AB serum, 1% penicillin-streptomycin, and 50 μg/mL gentamicin (complete media) plus HCMV at an MOI of either 0.5 or 1.0, depending on the experiment. The adherent cells displayed morphological features of macrophages (large size, eccentric and concave nuclei, phagocytic vacuoles, and cord-like pseudopods extending from the surface), expressed mRNA transcript and protein for the monocyte/macrophage markers CD13, HLA-DR and CD14, and did not differentiate into T cells, B cells, NK cells or DCs

(Supplemental Fig. 1). After 2 h at 37°C, the media was removed and replaced with HCMV-free complete media. Parallel cultures of macrophages were mock-infected with either a) complete media, b) control fibroblast culture supernatant in which the HCMV had been removed by ultracentrifugation, or c) control UV-inactivated HCMV (MOI=1). The cells then were washed and cultured for up to 6 days in complete media in 24-well plates. At 2, 4 or 6 days post-infection, cells were harvested by scraping, enumerated by automated cell counter and replated in 96-well plates (0.2 mL at 1×10^6 cells/well) in the presence or absence of smooth LPS 1 μg/mL (*Salmonella abortus equi*; Alexis, Amgen, Thousand Oaks, CA) and harvested after either 4 h for cytokine mRNA analysis or 24 h for cytokine protein determination (see below).

Detection of HCMV infection

Parallel cultures of monocytes (2×10^6) bound to coverslips in 24-well plates were exposed to HCMV or media, as described above. On day 4, HCMV- and mock-infected macrophages were fixed in 4% paraformaldehyde and permeabilized using 0.1% Triton X-100. HCMV-infected macrophages were detected by immunofluorescence using the antibody p63-27, specific for IE1 antigen (UL123), the major immediate early gene product, and a FITC-conjugated goat anti-mouse IgG antibody, as we have previously described (50). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and cells were enumerated by fluorescence microscopy.

HCMV replication also was evaluated using quantitative DNA PCR. Monocytes and primary human foreskin fibroblasts were plated at 2×10^5 cells/well in 96-well plates, inoculated with HCMV (MOI 1.0) and washed, after which the cells and supernatants were harvested on days 2, 4 and 6. Total DNA was isolated from each sample using the Qiagen QiaAMP DNA Kit according to the manufacturer's protocol. Quantitative DNA PCR was performed by amplification of a fragment of the HCMV UL55 open reading frame (ORF) and quantified by comparison to a standard curve generated by amplification of a plasmid encoding a fragment of the HCMV UL55 ORF in serial dilution, as we have described (50). Samples were run in triplicate using the described two-step amplification protocol on the ABI StepOnePlus Real-Time PCR cycler. Copy numbers were calculated per cells/well.

Flow cytometric analysis

Monocyte-derived macrophages (2×10^6) were stained with allophycocyanin-, phycoerythrin- or FITC-conjugated antibodies to CD14 (BD BioSciences, San Jose, CA), TLR2, 4 and 5 (eBiosciences, San Diego, CA) and pNF-κB p65 (BD-Phosflow, BD BioSciences), respectively, or irrelevant antibodies of the same isotype and fluorochrome, and analyzed by flow cytometry, as previously described (52). Data were evaluated with Cellquest.

Real-time PCR

HCMV- and mock-infected monocyte-derived macrophages (1×10^6 cells/mL) incubated for 4 h with LPS or media were harvested, RNA isolated (QIA RNeasy kit; Qiagen, Valencia, CA) and cDNA generated from total RNA (Transcriptor First Strand cDNA Synthesis Kit; Roche, Indianapolis, IN). Genes were amplified in 25 μL mixtures containing TaqMan

Universal PCR Master Mix and FAM/MGB-labeled primer-probe sets for TNF-α, IL-6, MyD88 and control gene GAPDH (Life Technologies, Carlsbad, CA), as previously described (52). Real-time PCR was run for 40 cycles (15 sec 95°C, 60 sec 60°C) on a Chromo4 PCR system (Biorad, Hercules, CA) and analyzed with Opticon Monitor™ software, version 3.1. All PCR reactions were performed twice, once with each reference gene, and data are presented as the geometric mean of both reactions. Relative expression rates of target genes in stimulated versus unstimulated cells were calculated using the method of Pfaffl (53) and presented as relative RNA expression.

Cytokine protein and gene expression analysis

HCMV- and mock-infected macrophages (1×10^6/mL) were cultured for 24 h in the absence or presence of smooth LPS (1 μg/mL) or TLR1-9 ligands (InvivoGen, San Diego, CA). TLR ligands included TLR1: Pam3CSK4 (1 μg/mL); TLR2: Heat-killed *Listeria monocytogenes* (HKLM; 10^8/mL); TLR3: polyinosinic:polycytidylic acid (Poly(1:C); 10 μg/mL); TLR4: LPS (1 μg/mL); TLR5: *S*. Typhimurium flagellin (*S*. Typhimurium flag; 1 μg/mL); TLR6: Pam2CaDPKH PKSF (FLS1; 1 μg/mL); TLR7: Imiquimod (1 μg/mL); TLR8: ssRNA40 (1 μg/mL); TLR9: ODN2006 (5 μM). Culture supernatants were analyzed for TNF-α and IL-6 protein by immunoassay (R&D Systems, Minneapolis, MN).

Total cellular RNA was extracted (RNeasy kit; QIAGEN) from blood monocytes prior to HCMV infection, synthesized (Superscript Choice system; Gibco Life Technologies) into cDNA utilizing an oligo(dT_{24}) primer, from which biotinylated cRNA was generated using a BioArray HighYield RNA transcription labeling kit (ENZO Diagnostics) and purified through RNeasy nucleic acid columns, using our previously described protocol (52). After scanning, fluorescence data were processed by the GeneChip operating system (version 1.1; Affymetrix). Background correction, normalization, generation of expression values, and analysis of differential gene expression were performed using dChip analysis software (DNA-Chip analyzer [dChip], version 1.3; Harvard University) in compliance with Minimal Information About a Microarray Experiment (MIAME) guidelines (http://www.ncbi.nlm.nih.gov/geo/info/MIAME.html) and data was presented as fluorescence intensity.

NF-κB p65 and IκBα detection

Whole cell extracts were prepared from 10×10^6 mock-infected or HCMV-infected macrophages cultured in the presence or absence of LPS (1 μg/mL) for 15 min at 37°C using the RIPA Lysis Buffer Kit (Santa Cruz Biotechnology, Santa Cruz, CA). Phosphorylated NF-κB p65 and IκBα were analyzed using the InstantOne ELISA (eBioscience), which detects total and phosphorylated NF-κB p65 and IκBα attached to consensus binding sites in a 96-well plate using the TMB colorimetric substrate and optical density at 450 nm (EL 800 ELISA reader, Biotek Instruments, Inc., Winooski, VT). Data is presented as phosphorylated NF-κB p65 and IκBα at OD 450 nm/10 μg protein.

Electron microscopy

The starting population of monocyte-derived macrophages (prior to HCMV infection) was prepared and examined using a Zeiss EM 10A electron microscope, as previously described (54).

Western blot

The expression of MyD88 protein in HCMV-infected macrophages (10×10^6/mL) isolated on day 4 of the infection cycle was determined by immunoblotting using antibodies to MyD88 and actin (Santa Cruz Biotechnology, Santa Cruz, CA), as previously described in detail (36).

Statistical Analysis

Data were analyzed by t-test/Mann-Whitney U test, where appropriate. Data are expressed as mean +SEM.

Results

HCMV infects monocytes as they differentiate into macrophages

The inflammatory lesion in HCMV-infected tissues such as the intestinal mucosa is characterized by the local accumulation of HCMV-infected macrophages (22, 35, 55). To explore the mechanism by which HCMV infection enhances macrophage inflammatory responses, we first established a reproducible *in vitro* system to infect monocyte-derived macrophages with HCMV. Adherent blood monocytes, which displayed the features of macrophages (Supplemental Fig. 1), from HCMV-seronegative donors were incubated with a clinical isolate of HCMV (TR strain; <6 passages) at a pre-determined optimal MOI of 0.5 or 1.0 and allowed to differentiate into macrophages. HCMV was detected in the cells by immunofluorescence analysis for IE1, and HCMV DNA was quantified by quantitative PCR. As shown in Fig. 1A, HCMV IE1 gene product was detected in macrophage nuclei as non-diffuse staining, consistent that reported by Soderberg-Naucler et al. (29), on day 4 post-exposure to HCMV but not in mock-infected cells. Based on the presence of HCMV IE1 in the macropahges, we routinely achieved an infection rate of >50% (n=9) by day 4 of infection (Fig. 1B). Progressive and substantial increase in the number of copies of HCMV DNA in both the macrophages and culture supernatant during the 6-day infection cycle (Fig. 1C) confirmed the replication of viral DNA and its release by infected cells. The cells retained a macrophage phenotype with negligible CD3, CD19, CD69 and CD83 expression in the absence or presence of HCMV and/or M-CSF (Supplemental Fig. 1D), and the number and viability (>85%) of HCMV-infected macrophages were maintained during infection (Supplemental Fig. 1B).

HCMV infection of macrophages promotes inducible inflammatory responses

We next analyzed the effect of HCMV infection on macrophage inflammatory cytokine gene expression and protein production. On day 4 of infection, macrophages infected with HCMV expressed 3-fold and 100-fold more TNF-α and IL-6 mRNA, respectively, than mock-infected macrophages (Fig. 2A). Predictably, LPS stimulated cytokine-specific

mRNA expression by mock-infected macrophages, but when the macrophages were pre-infected with HCMV, LPS stimulation induced several hundred- to several thousand-fold more mRNA for both inflammatory cytokines compared with mock-infected, LPS-stimulated macrophages (Fig. 2A). Consistent with HCMV-enhanced inducible gene expression, HCMV infection significantly increased LPS-stimulated TNF-α protein production on days 2, 4 and 6 ($P<0.01$ to $P<0.001$) and IL-6 and IL-8 on days 4 and 6 ($P<0.01$ to $P<0.001$) of the infection cycle. HCMV infection alone up-regulated TNF-α and IL-6 gene transcription (Fig. 2A), but TNF-α and IL-6 protein production by HCMV-infected macrophages did not occur unless the cells were subsequently exposed to LPS (Fig. 2B), suggesting that HCMV-induced cytokine gene transcription required a second signal for translation. The ability of HCMV infection to potentiate inducible cytokine production by macrophages was due to the infection itself, since culture supernatant from HCMV-infected macrophages did not enhance cytokine production by non-infected bystander cells (Supplemental Fig. 2). Inducible cytokine production also was not significantly affected when the macrophages were generated in the presence of M-CSF (Supplemental Fig. 3). Macrophages exposed to UC HCMV and UV HCMV, similar to mock-infected macrophages, did not express cytokine-specific mRNA or protein (data not shown). These findings indicate that HCMV infection primes macrophages for enhanced LPS-induced production of inflammatory cytokines and that this response is regulated at the level of gene transcription and translation.

HCMV infection promotes maintenance of macrophage CD14 and TLR4 expression

To explore the mechanism by which HCMV infection enhances macrophage responsiveness to LPS, we first assessed the effect of HCMV infection on macrophage expression of CD14 and TLR4, the two major components of the LPS receptor complex. As shown in the representative experiment in Fig. 3A, on day 0, 81.2% of mock-infected monocytes expressed surface CD14 and 54.9% expressed TLR4. During the subsequent 6-day culture period, mock-infected macrophages showed progressive declines in CD14 and TLR4 expression, consistent with previous reports (56, 57). However, HCMV-infected macrophages derived from the same donor continued to express high levels of both CD14 and TLR4 at each time point. By day 6, 6.6% of mock-infected cells expressed CD14 and 10.8% expressed TLR4, whereas 45.1% of HCMV-infected cells expressed CD14 and 53.9% expressed TLR4. The ability of HCMV-infected macrophages to maintain expression of these components of the LPS receptor was not donor-specific, as significantly higher proportions of HCMV-infected monocytes from four separate donors continued to express both CD14 and TLR4, especially on days 4 and 6 of the infection cycle (Fig. 3B).

HCMV infection promotes TLR ligand-induced cytokine production

We next investigated whether the effect of HCMV on macrophage TLR4 gene expression and ligand-induced cytokine production extended to other TLRs. HCMV infection of monocyte-derived macrophages was associated with higher levels of TLR3, 5, 7 and 9 compared with mock-infected macrophages, as shown for cells on day 4 of an infection cycle (Fig. 4A). In addition, infection with HCMV enhanced TLR2 ligand (heat-killed *Listeia* monocytogenes, HKLM)- and TLR5 ligand (*Salmonella* Typhimurium flagellin)-stimulated production of TNF-α and IL-6 compared with mock-infected, TLR2- and TLR5-

stimulated macrophages (Fig. 4B). Thus, HCMV infection potentiated the production of key macrophage pro-inflammatory cytokines in response to stimulation by bacterial components. The absence of detectable TLR7 and 9 ligand-specific responses by mock- and HCMV-infected monocyte-derived macrophages (Fig. 4B) is consistent with the absent to near-absent TLR7-9-stimulated responses by monocytes and intestinal macrophages that we previously reported (52).

HCMV infection promotes macrophage TLR2 and 5 expression

To begin to elucidate the mechanism by which HCMV infection enhances TLR-mediated inflammatory responses, we evaluated HCMV-infected macrophages for the expression of TLR2 and TLR5. Similar to CD14 and TLR4 expression (Fig. 3), mock infection of macrophages was associated with a progressive decline in TLR2 and 5 during a 6-day infection (Fig. 5). However, HCMV infection promoted the up-regulation of TLR5, but not TLR2, especially on days 4 and 6 (Fig. 5).

HCMV enhances LPS-stimulated NF-κB signal transduction and nuclear translocation

The inability of HCMV to enhance monocyte-derived macrophage TLR2 expression (Fig. 4A) despite enhancing TLR2-stimulated cytokine production (Fig. 4B) suggested that HCMV potentiated TLR responses through downstream signaling. In the canonical LPS-induced signal cascade, the binding of LPS to its receptor activates the recruitment of adaptor proteins, including MyD88, the master adaptor molecule in the NF-κB signal cascade that initiates all TLR, except TLR3, signaling (52, 58–63). In this connection, HCMV infection did not enhance TLR3-mediated responses (Fig. 4B **inset**). MyD88 binds to the cytoplasmic Toll-IL-1 (TIR) domain, which triggers the phosphorylation of IL-1 receptor-associated kinase 4 (IRAK4) with subsequent recruitment and phosphorylation of IRAK1, causing the release of tumor necrosis factor receptor-associated factor 6 (TRAF-6) and propagation of the NF-κB signaling cascade (58–63). Therefore, we examined the effect of HCMV infection on the expression of MyD88 in monocyte-derived macrophages. HCMV infection induced substantial increases in MyD88 mRNA (Fig. 6A,B) and protein (Fig. 6C) in the macrophages. In addition, infection caused a slight increase in the phosphorylation of IκBα compared with mock-infected macrophages, but infected macrophages stimulated with LPS displayed a larger increase in IκBα phosphorylation compared with mock-infected LPS-stimulated cells on day 4 (Fig. 6D, **left panel**) and day 6 (not shown) in a representative infection. Similarly, HCMV infection alone did not induce significant NF-κB phosphorylation, reflected in nearly the same levels of pNF-κB p65 in HCMV- and mock-infected cells during the infection cycle shown in Fig. 6D, **right panel**, consistent with the inability of HCMV infection alone to induce inflammatory cytokine release. However, when HCMV-infected macrophages were subsequently stimulated with LPS, the level of pNF-κB p65 increased substantially, especially on days 4 and 6, compared with mock-infected LPS-stimulated macrophages ($P<0.001$ and $P<0.03$, respectively) (Fig. 6D, **right panel**). HCMV infection plus LPS stimulation also enhanced the proportion of macrophages that contained pNF-κB p65 compared with mock-infected LPS-stimulated cells, as detected by flow cytometry (Fig. 6E). Thus, HCMV infection of macrophages induced MyD88 expression and enhanced inducible phosphorylation of IκBα and NF-κB.

Discussion

Monocytes are an important reservoir for latent HCMV infection and, after their differentiation into macrophages in the tissues, may contribute to HCMV-associated inflammatory disease (64, 65). Using a clinical isolate of HCMV to investigate the mechanism of HCMV-induced macrophage-mediated inflammation, we showed that macrophages infected with HCMV continued to express CD14 and TLR4 and 5 during a 6-day infection cycle, whereas mock-infected cells displayed a progressive decline in CD14 and TLR expression. HCMV infection promoted ligand-inducible pro-inflammatory cytokine mRNA expression and TNF-α, IL-6 and IL-8 protein production. Coincident with the enhanced pro-inflammatory response, HCMV infection promoted expression of the adapter protein MyD88 and potentiated inducible phosphorylation of both IκBα and NF-κB, indicating a mechanism for the increased inducible pro-inflammatory cytokine production.

The ability of HCMV to promote the continued expression of surface CD14 and TLR4 and TLR5 on monocytes as they differentiate into macrophages is relevant to mucosal macrophages in particular. Circulating monocytes, the exclusive source of human intestinal macrophages (32), lose CD14 as they differentiate into mucosal macrophages (32, 54). Intestinal macrophages also do not activate NF-κB, leading to inflammation anergy (34, 52, 54, 66). Thus, our finding that HCMV infection causes monocytes to maintain CD14 and TLR4 expression and up-regulate inducible IκBα and NF-κB phosphorylation, thereby potentiating inducible pro-inflammatory responses by macrophages, could contribute to the pathogenesis of HCMV-associated mucosal inflammation and is the subject of ongoing investigation.

The enhanced pro-inflammatory response of stimulated macrophages may benefit both host and virus. In a novel mouse model, Barton et al. (67) showed that both latent infection with Epstein-Barr virus and murine CMV conferred enhanced resistance to the bacterial pathogens *L. monocytogenes* and *Yersinia pestis*, suggesting that latent herpesvirus infection provides symbiotic protection against at least some bacterial infections. Consistent with murine CMV induction of cross-protection against bacteria in mice (67), the ability of HCMV to enhance the inflammatory capability of human macrophages suggests that HCMV-enhanced inflammation may promote containment of bacterial, and possibly fungal, pathogens and, through the induction of inflammatory chemokines [26], recruit HCMV-susceptible target cells to the inflammatory lesion, thereby amplifying host protection against certain pathogens while perpetuating HCMV infection.

The binding of envelope glycoproteins gB (UL55) and gH (UL75) from high-passaged Towne strain HCMV to embryonic lung fibroblasts *in vitro* has been shown to activate NF-κB gene transcription within 30–60 min of binding (68, 69). The binding of HCMV (Towne strain) gB and gH to human monocytes also has been reported to up-regulate NF-κB, IκBα and IL-1 gene transcription after 4 h (40). Others have shown that the Towne and AD169 strains inhibit NF-κB signaling in human foreskin fibroblasts (70). In contrast to these studies, we show here that HCMV infection of human macrophages potentiated inducible TLR ligand-induced phosphorylation of both IκBα and the transcription factor NF-κB, leading to enhanced gene and protein expression for pro-inflammatory cytokines. However,

the binding of gB or gH alone was not responsible for the phenotype observed in our studies, as exposure of macrophages to UV-inactivated virions did not induce the changes caused by incubation with infectious virions. Our finding that HCMV infection enhanced inducible inflammatory responses is important because the enhanced responses were potentiated by a low-passaged clinical isolate of the virus, persisted throughout the infection cycle and pertained to primary macrophages, cells that play a fundamental role in mediating tissue inflammation during HCMV infection (23, 35, 55).

The ability of HCMV to potentiate inducible macrophage release of IL-6 is noteworthy for two reasons. First, in the presence of TGF-β, IL-6 induces the differentiation of Th17 cells (71), which mediate tissue inflammation through the induction of IL-17. In mice, for example, IL-17 plays a key role in promoting murine CMV-induced interstitial pneumonia (72). Thus, HCMV-induced IL-6, as reported here, and the HCMV-induced TGF-β production that we (37) and others (73, 74) have reported, could together promote IL-17-mediated tissue inflammation such as that characteristic of Crohn's disease (75). Second, IL-6 can reactivate HCMV latently infected cells (21), resulting in viral replication and the release of infectious progeny from otherwise quiescent macrophages; notably, the virions released in response to IL-6-mediated reactivation may be more infectious. Thus, HCMV-induced IL-6 may potently influence both inflammatory and virological responses. Further elucidation of the immunobiology of HCMV infection of human macrophages should provide new insights into HCMV pathogenesis and help identify novel therapeutic strategies for HCMV-associated inflammatory disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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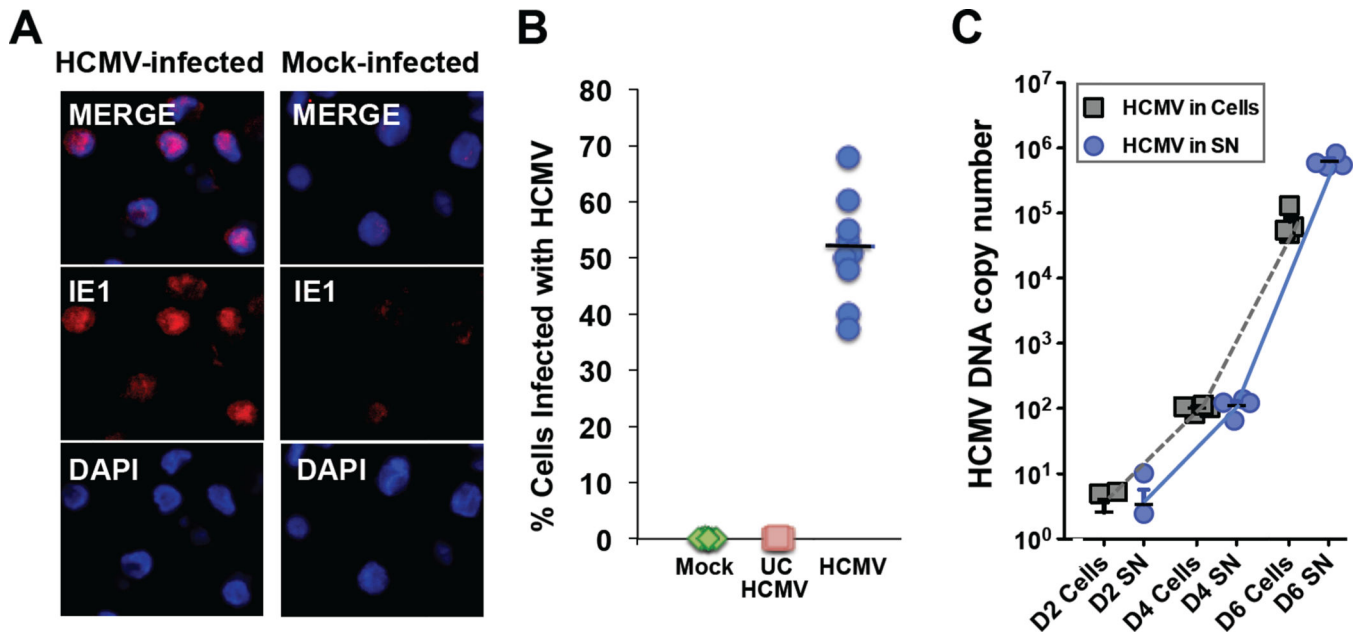

Fig. 1. HCMV infection of monocyte-derived macrophages

Freshly adherent blood monocytes were exposed to media (Mock HCMV), ultracentrifuged culture supernatant from HCMV-infected human foreskin fibroblasts (UC HCMV, MOI 0.5), or HCMV also propagated in foreskin fibroblasts (MOI 0.5), cultured and analyzed for HCMV infection and DNA replication. **(A)** Immunofluorescence analysis of mock-infected and HCMV-infected macrophages shows nuclear localization of IE1 in a representative experiment on day 4 (n=3; red=IE1; blue=DAPI-stained nuclei) (40x). **(B)** Percent macrophages that contained IE1 on day 4 (n=9). **(C)** Quantitative PCR analysis for HCMV DNA in macrophages (Cells) and culture supernatant (SN) on days (D) 2, 4 and 6 of infection (n=4).

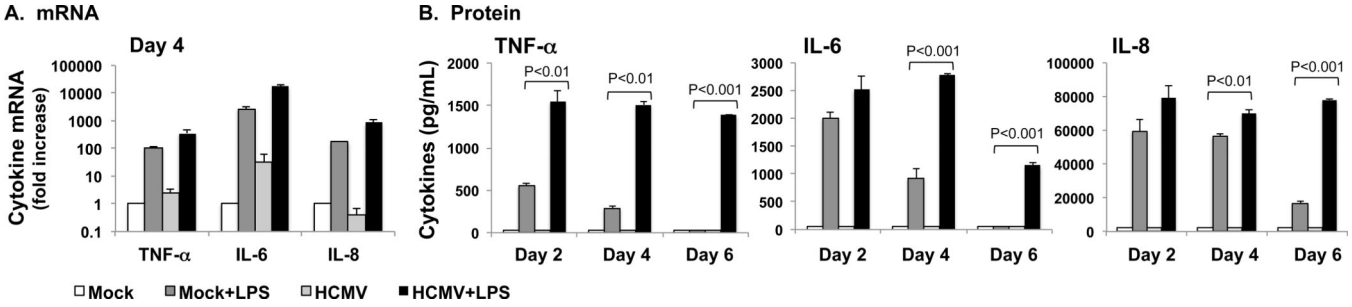

Fig. 2. HCMV infection of macrophages enhances LPS-induced cytokine-specific mRNA expression and protein production

(A) Macrophages were mock- or HCMV-infected, cultured for 4 days, treated with or without LPS (1 μg/mL) for 4 h, harvested and analyzed for cytokine-specific mRNA by real-time PCR and expressed as fold-increase. Data are the mean ± SEM from two separate cell preparations. **(B)** Macrophages were mock- or HCMV-infected, cultured for 2, 4 or 6 days, treated with LPS (1 μg/mL) for 24 h on the indicated day, and the culture supernatants harvested and analyzed for the indicated cytokine. Data represent the mean ± SEM from 3 independent experiments.

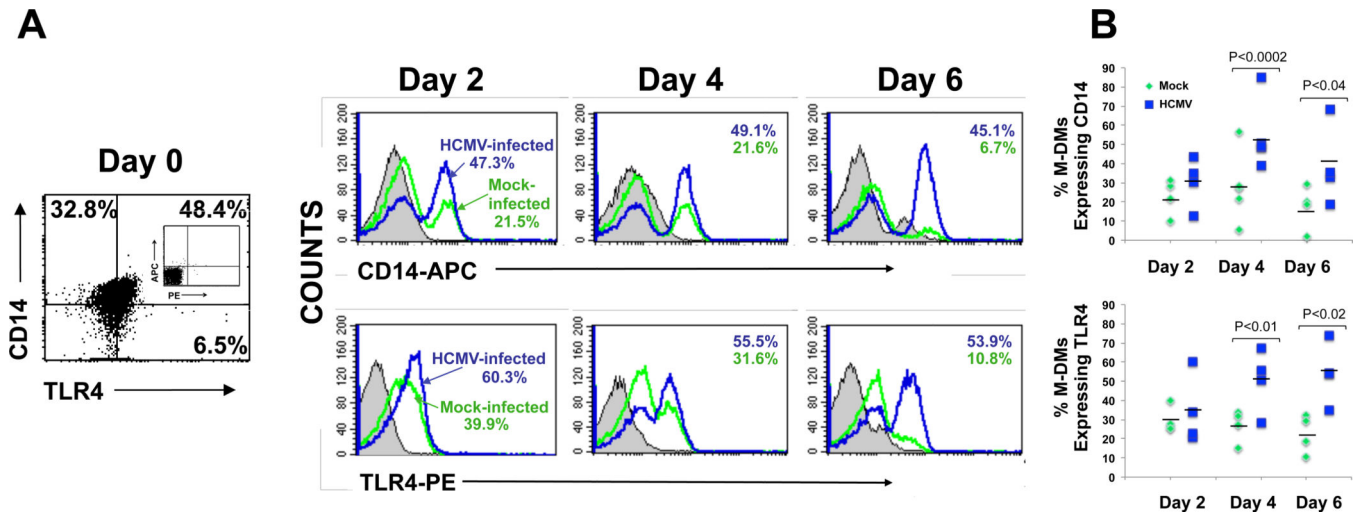

Fig. 3. HCMV infection induces macrophage CD14 and TLR4 expression

Monocyte-derived macrophages were mock- or HCMV-infected, harvested on days 2, 4 and 6 of the infection cycle and then analyzed by flow cytometry for surface CD14 and TLR4 by gating on the $CD13^+$ monocyte-derived macrophage population. (**A**) Macrophages from a representative donor were examined before infection on day 0 and on days 2, 4 and 6 post-infection for CD14 and TLR4 by flow cytometry. (**B**) Mock- and HCMV-infected macrophages from four additional donors were analyzed for CD14 and TLR4. Insert in (**A**) and gray histograms in (**B**) correspond to isotype controls. Horizontal bars in **B** = mean values.

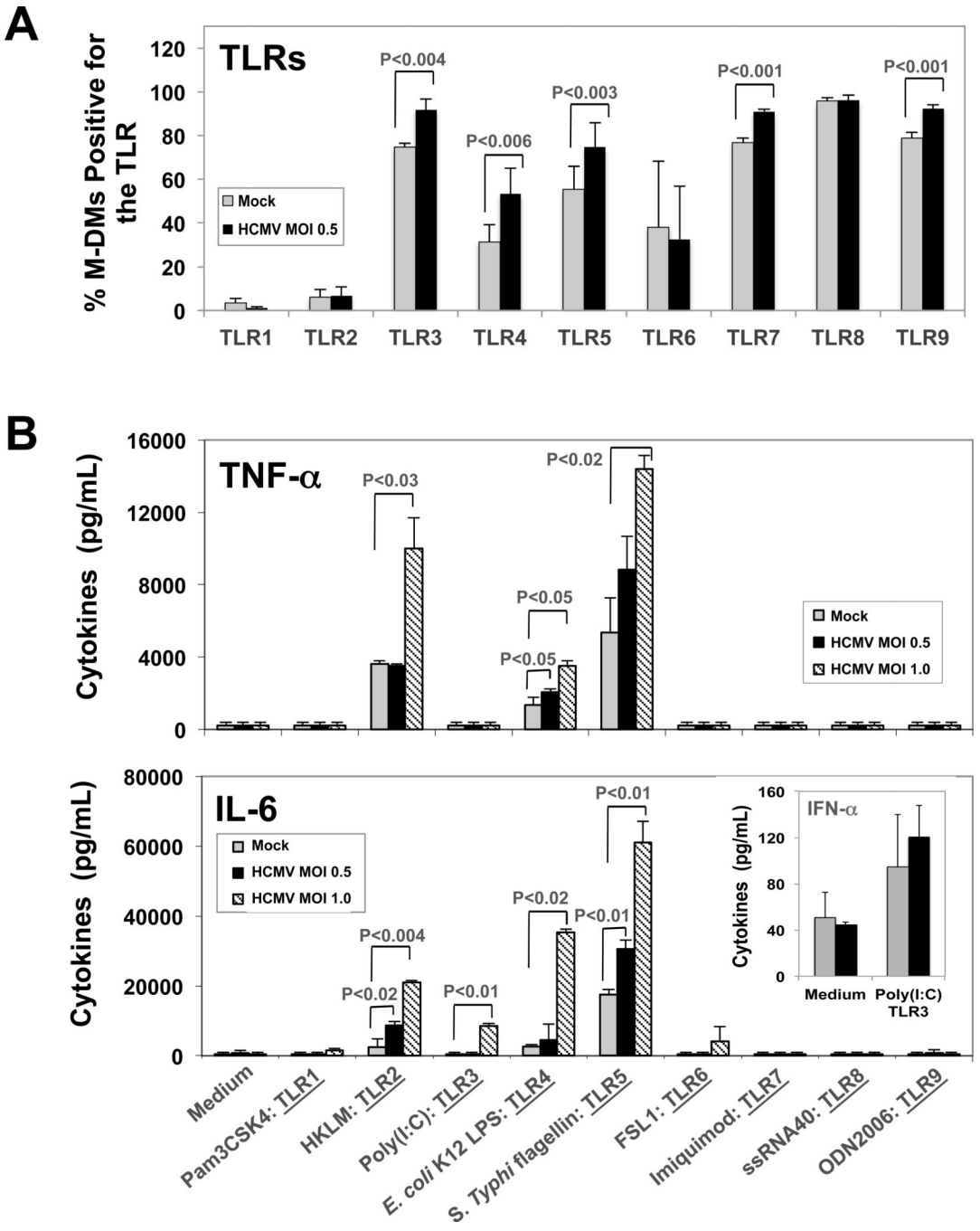

Fig. 4. HCMV infection potentiates macrophage TLR expression and TLR ligand-induced cytokine production

(A) Mock- and HCMV-infected (MOI 0.5) monocyte-derived macrophages from three separate donors were cultured for 4 days and analyzed for the indicated TLR. Data are the mean % macrophages that expressed the indicated TLR. **(B)** Monocyte-derived macrophages from three separate donors were treated with optimal concentrations of the indicated TLR ligands on day 4, and 24 h later culture supernatants were harvested and analyzed for TNF-α and IL-6. Cytokine levels are the mean ±SEM pg/mL for the three

donors. Lower panel inset shows the level of IFN-α produced by mock- and HCMV-infected (MOI 0.5) macrophages after 24 h stimulation with poly(I:C).

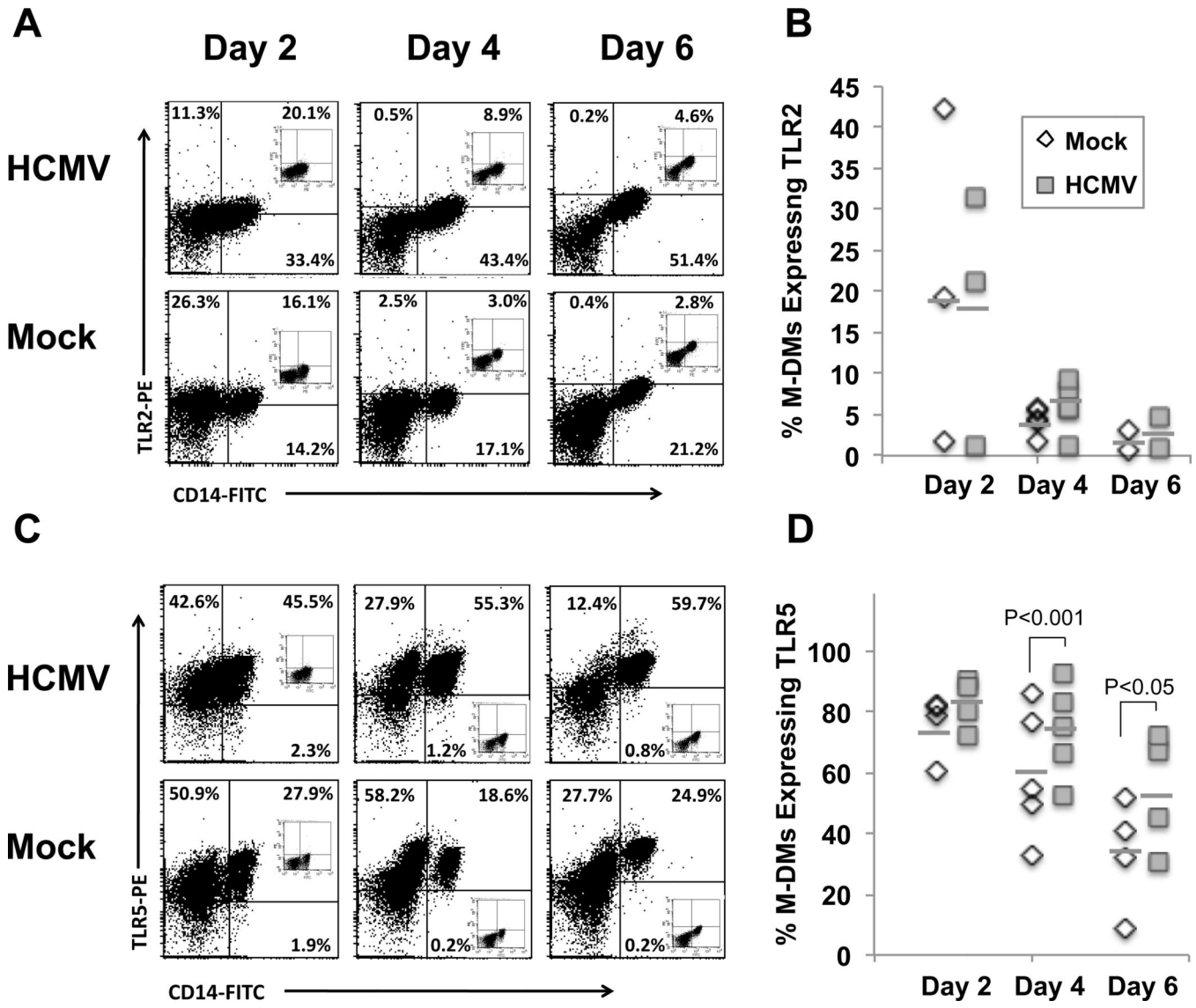

Fig. 5. Effect of HCMV infection on macrophage TLR2 and 5 expression

HCMV-infected (MOI 0.5) and mock-infected monocyte-derived macrophages from **(A)** a representative donor were cultured for 4 days and from **(B)** four additional donors were cultured for 2, 4 and 6 days and analyzed for CD14, TLR2 and TLR5 by flow cytometry. Insets (panels A and C) show isotype control staining.

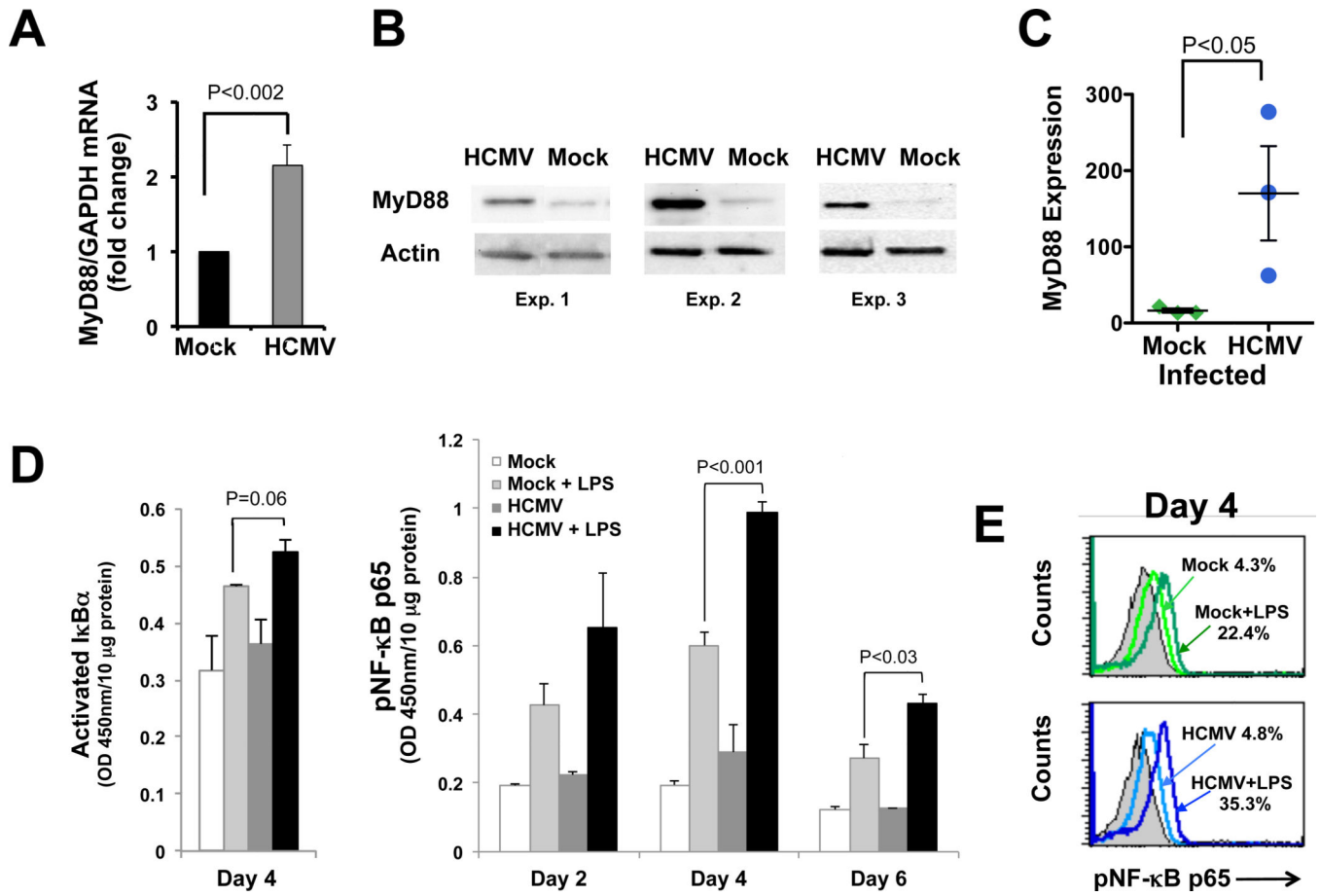

Fig. 6. HCMV infection enhances LPS-stimulated NF-κB signal protein expression and NF-κB nuclear translocation in macrophages

(A) Mock- and HCMV-infected (MOI 0.5) monocyte-derived macrophages were cultured for 4 days and analyzed for MyD88 mRNA by real-time PCR using GAPDH as a control and expressed as fold change ± SEM (n=3). MyD88 protein in HCMV- and mock-infected monocyte-derived macrophages from three separate donors were harvested on day 4 of an infection cycle and analyzed by **(B)** Western blot and **(C)** densitometric comparison of the bands. Mock- and HCMV-infected (MOI 0.5) macrophages were treated on the indicated day with media or LPS (1 μg/mL, 15 min), harvested, and cell extracts analyzed by ELISA for pIκBα **(D, left panel)** and pNF-κBp65 **(D, right panel)**. Data are the mean ±SEM values for triplicate cultures from 3 experiments. **(E)** Mock- and HCMV-infected (MOI 0.5) macrophages (day 4) were analyzed for pNF-κB p65 by flow cytometry.