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Transcriptome Analysis Reveals Human Cytomegalovirus Reprograms Monocyte Differentiation Towards a M1 Macrophage

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Abstract

Monocytes are primary targets for human cytomegalovirus (HCMV) infection and are proposed to be responsible for haematogenous dissemination of the virus. Monocytes acquire different functional traits during polarization to the classical pro-inflammatory M1 macrophage or the alternative anti-inflammatory M2 macrophage. We hypothesized that HCMV induced a pro-inflammatory M1 macrophage following infection in order to promote viral dissemination because, biologically, a pro-inflammatory state provides the necessary tools to drive infected monocytes from the blood into the tissue. To test this hypothesis of monocyte conversion from a normal quiescent phenotype to an inflammatory phenotype, we utilized Affymetrix Human Microarray to acquire a transcriptional profile of infected monocytes at a time point our data emphasized as being a key temporal regulatory point following infection. We found that HCMV significantly upregulated 583 (5.2%) of the total genes and downregulated 621 (5.5%) of the total genes ≥1.5-fold at 4 hours post infection. Further ontology analysis revealed that genes implicated in classical M1 macrophage activation were stimulated by HCMV infection. Specifically, we found that 65% of genes strictly associated with M1 polarization were upregulated, while only 4% of genes solely associated with M2 polarization were upregulated. Analysis of the monocyte chemokinome at the transcriptional level showed that 44% of M1 and 33% of M2 macrophage chemokines were upregulated, respectively. Proteomic analysis using human chemokine antibody arrays confirmed the secretion of these chemotactic proteins from HCMV-infected monocytes. Overall, the results identify that the HCMV-infected monocyte transcriptome displayed a unique M1/M2 polarization signature that was skewed towards the classical M1 activation phenotype.

Introduction

Infection by human cytomegalovirus (HCMV), a member of the *Herpesviridae* family, leads to morbidity and mortality in immunocompromised individuals including AIDS and organ transplant patients, congenitally infected neonates, and cancer patients undergoing chemotherapy (1-3). HCMV infection causes a wide range of overt organ diseases including retinitis, gastrointestinal disease, hepatitis and interstitial pneumonia due to the broad cellular tropism of the virus *in vivo* (4). Following initial primary infection of host epithelial cells by contact with HCMV-containing bodily fluids, HCMV replicates and spreads to the peripheral blood where viral dissemination to multiple organ systems occurs (4).

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Peripheral blood leukocytes, specifically monocytes, are believed to be responsible for haematogenous spread (4,5). Monocytes are primary *in vivo* targets for HCMV (6), are a site of viral latency and persistence (4,7), are the primary infiltrating cell type found in HCMV-infected organs (8,9) and their aberrant function following HCMV infection is implicated in atherosclerosis, an inflammatory disease whose development and severity is associated with HCMV infection (10,11). Furthermore, animal studies indicate that monocyte-associated viremia is a prerequisite for viral pathogenesis (12,13). In accord with these *in vivo* observations, we previously provided *in vitro* evidence suggesting HCMV utilizes monocytes as a vehicle for spread, infiltration into and persistence in host tissue (14-17). Our studies have demonstrated that primary infection of peripheral blood monocytes by HCMV induces a pro-inflammatory state resulting in increased cell motility, firm adhesion to endothelial cells, and transendothelial migration (14-16). These functional attributes are acquired concomitant with the critical process of monocyte-to-macrophage differentiation, where short-lived monocytes (non-permissive for viral replication) differentiate into long-lived macrophages (permissive for viral replication) (15). Although we have shown increased expression of multiple macrophage markers, including human leukocyte-associated antigen-DR (HLA-DR) and CD68 (15), heterogeneity and plasticity are hallmarks of cells belonging to the mononuclear phagocyte system and overall little is known about the phenotypic characteristics of the HCMV activated monocyte/macrophage.

Monocyte and macrophage plasticity is apparent by the distinct morphological and functional responses to particular tissues and to the immunological microenvironment (18-21). Macrophages can be functionally polarized into classically activated M1 macrophages by treatment with IFN-γ alone or in concert with LPS (22) or alternatively activated M2 macrophages by treatment with IL-4 or IL-10 (18,23). Classically activated macrophages are characterized by an IL-12^{high}, IL-23^{high}, IL-10^{low} phenotype (24) and the production of toxic intermediates (reactive oxygen and nitrogen intermediates) (22) and pro-inflammatory cytokines (IL-1β, and TNF-α) (25). M1 macrophages are potent effector cells efficient at eliminating pathogens and tumour cells. In contrast, alternatively activated macrophages exhibit an IL-12^{low}, IL-23^{low} phenotype (24), produce anti-inflammatory molecules [IL-10 and IL-1 decoy receptor (IL-1Ra)] (21,26) and express high levels of scavenger, mannose and galactose-type receptors (21,23). M2 macrophages sublime the inflammatory response, promote angiogenesis and tissue remodelling (21,26,27). M1 and M2 macrophages, however, are likely representatives of two extremes along a continuum of possible macrophage biological phenotypes.

'Classically' activated monocytes exhibit enhanced antimicrobial activities in a stimulus-dependent [particularly in response to interferon (IFN) γ (28)], but antigen-non-specific manner, through the increased expression of cell surface adhesion receptors and secretion of cytokines and chemokines (29,30). Elevated levels of cell adhesion molecules promote adhesion to the endothelium, while the increased release of cytokines such as TNF-α and IL-1 can activate the endothelium to further promote transendothelial migration (31,32). Similar to other pathogenic agents such as gram negative bacteria (via LPS), we previously showed that HCMV activated monocytes exhibit increased expression of cytokines and chemokines, adhesion to endothelial cells, and transendothelial migration (14,15,33), suggesting a polarization towards a M1 macrophage phenotype. However, unlike other pathogens, HCMV gains a selective replication advantage from a strong host inflammatory response (15,34-37). Moreover, LPS and HCMV activated monocytes are morphologically distinct (15); thus, while it appears that monocytes activated by a different directing stimuli may share some functional traits, HCMV must stimulate a distinct M1/M2 macrophage reprogramming to meet its own specific replicative needs. Indeed, analysis of monocyte/macrophage activation by other stimuli such as CCL5 (38), LPS (38), *E. coli* (39) and *Streptococcus pyogenes* (40) revealed

individually unique expression profiles of macrophage markers characteristic of both the M1 and M2 phenotypes.

To obtain an understanding of the unique changes in monocytes following HCMV infection, particularly in the M1/M2 macrophage reprogramming, we examined the global dysregulation of the infected monocyte transcriptome. Although other studies have examined M1/M2 monocyte/macrophage differentiation induced by various cytokines and bacteria, we for the first time utilized a microarray gene profiling approach to examine the M1/M2 differentiation reprogramming in virally infected monocytes. A cDNA microarray containing 12,626 unique probe sets was used to assess HCMV modulation of genes in peripheral blood monocytes at 4 hours post infection (hpi), which our data has identified as a key temporal point where viral immediate-early proteins are not expressed (15), but a number of critical cellular transcripts associated with early cellular responses are significantly induced (33). HCMV significantly altered the levels of 10.7% (1204 genes) cellular mRNAs in which 5.2% (583 genes) mRNAs were upregulated and 5.5% (621 genes) mRNAs were downregulated. Transcriptional profile comparison analysis revealed a majority of genes strictly associated with the M1 phenotype were induced by HCMV, while a majority of genes associated with the M2 phenotype exhibited no change or a downregulation. The upregulation of monocytic genes implicated in M1 macrophage polarization suggest that HCMV modulates a rapid transition towards a M1 differentiation lineage, supporting our model for haematogenous dissemination of HCMV: HCMV-infection of peripheral blood monocytes forces cells to acquire a M1 pro-inflammatory phenotype to promote infected monocyte infiltration into peripheral tissue, differentiation into long-lived macrophages, and the subsequent establishment of a life-long viral persistence.

Materials and Methods

Virus preparation

HCMV (Towne/E strain; passages 35-45) was cultured as previously described in human embryonic lung (HEL) fibroblasts (33). Virus was purified on a 0.5 M sucrose cushion, resuspended in RPMI 1640 media (Cellgro, Mediatech, Herndon, VA), and used to infect monocytes at a multiplicity of infection (MOI) of 15 for each experiment (14,15,33). Monocytes were diluted in RPMI to prevent homotypic aggregation of monocytes and rocked for 4 hrs at 37°C during infection; thus, a high MOI was utilized to ensure that all monocytes would be infected during this short incubation time. We have shown similar HCMV-induced phenotypic changes in monocytes using MOIs of 0.1 to 20 in previous publications (15).

Human peripheral blood monocyte isolation

Blood was drawn by venipuncture and centrifuged through a Ficoll Histopaque 1077 gradient (Sigma, St. Louis, MO). Mononuclear cells were collected and washed with saline (14,15, 33). Monocytes were then isolated by centrifugation through a Percoll (Pharmacia, Piscataway, NJ) gradient (41). Greater than 95% of isolated peripheral blood mononuclear cells were monocytes as determined by CD14 positive staining (data not shown). Cells were washed and suspended in RPMI 1640 (Cellgro, Mediatech, Herndon, VA) supplemented with 10% human serum (Sigma). University Institutional Review Board and Health Insurance Portability and Accountability Act guidelines were followed for all experimental protocols.

Affymetrix gene array and analysis

Isolated monocytes were HCMV infected or mock infected and incubated nonadherently at 37°C and total RNA harvested 4 hpi with the RNA STAT-60 isolation kit (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. Affymetrix Human Genome U95Av2 arrays (Affymetrix, Santa Clara, CA), which contain 12,626 characterized sequences, were used to examine the cellular changes in primary human monocytes from 6 different human

donors. Total RNA from mock-infected and HCMV-infected monocytes was harvested as described above. A minimum of 5 μg of RNA was used for each array. RNA integrity was assessed by electrophoresis on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA was reverse-transcribed into cDNA and then transcribed *in vitro* to biotin-labelled cRNA. cRNA from each sample was fragmented and hybridized to GeneChip expression arrays following Affymetrix-recommended protocols. After washing and staining, the microarrays were scanned, and the data quantified. Affymetrix Microarray Suite version 5.0 was used to determine changes in gene expression. Data Mining Tool version 3.0 was used to compile data from each of the replicates and one-way analysis of variance (ANOVA) tests performed to calculate p-values. Spotfire DecisionSite 8.1.1 (Somerville, MA) was used to group genes by ontology, generate scatter plots, and calculate correlation coefficients.

RT-PCR analysis

Isolated monocytes were HCMV infected or mock infected and incubated nonadherently at 37°C on an orbital shaker. At 4 hpi, cells were pelleted, and total RNA isolated using the RNA STAT-60 isolation kit (Tel-Test). 0.5 μg of RNA from each sample was reverse-transcribed by mixing RNA with random hexamers (0.1 μg/μl; Invitrogen, Carlsbad, CA), 1 mM deoxynucleotide triphosphates (Amersham Biosciences, Piscataway, NJ), and ddH_20 and incubating at 65°C for 15 minutes. The RNA solution was then chilled for one minute before 1X RT-buffer (Invitrogen), dithiothreitol (DTT; Invitrogen), 80 U of RNasin (Promega, Madison, WI), 400 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen), and ddH_20 were added. After one hour of incubation at 37°C, 2 U of RNaseH (Stratagene, La Jolla, CA) was added, and the samples incubated for an additional 30 minutes. PCR was performed using an iCycler (Bio-Rad Model 1708720, Hercules, CA). 2 μg of template DNA was mixed with 1X iTAQ buffer (Bio-Rad), 15 mM $MgCl_2$ (Invitrogen), 50 μM deoxynucleoside triphosphates (Amersham Biosciences), 1.25 U of TAQ polymerase (Bio-Rad), ddH_20, and 20 μM forward and reverse primers (Integrated DNA Technologies, Coralville, IA). ICAM-1, integrin β8, integrin α1, IL-1β, TNF-α, CCL2 and glyceraldehydephosphate dehydrogenase (GAPDH) samples were incubated for 5 minutes at 95°C and cycled 35 times for one minute at 95°C, 58°C, and 72°C. Samples were then incubated for 7 minutes at 72°C before storage at 4°C. Controls including samples lacking RT in the reverse transcription reactions and template DNA in the PCR reactions were also performed (data not shown). The following forward and reverse primers were designed with the support of Integrated DNA Technologies: ICAM-1, 5′-AAGCCAAGAGGAAGG AGCAAGACT-3′ (forward) and 5′-TGAACCATGATTGCACCACTGCAC-3′ (reverse); integrin β8, 5′-GCTGATTGATGCGCCACAGACTTT-3′ (forward) and 5′-CAGGCAGACAAATGCAGCGGTAAA-3′ (reverse); integrin α1, 5′-ACGCTCAGTGGAGAACAGATTGGT-3′(forward) and 5′-AATTGTGCTGCCGAGATGACCAGC-3′ (reverse); IL-1β, 5′-AACAGGCTGCTCTGGGATTCTCTT-3′ (forward) and 5′-TGAAGGGAAAGAAGGTGCTCAGGT-3′ (reverse); TNF-α, 5′-ACCCTCAACCTCTTCTGGCTCAAA-3′ (forward) and 5′-AGGCCTAAGGTCCACTTGTGTCAA-3′ (reverse); CCL2, 5′-TCGCTCAGCCAGATGCAATCAATG-3′ (forward) and 5′-AGTTTGGGTTTGCTTGTCCAGGTG-3′ (reverse). To confirm equal cDNA loading, GAPDH RNA was amplified with 5′-GAAGGTGAAGGTCGGAGTC-3′ (forward) and 5′-GAAGATGGTGATGG GATTTC-3′ (reversed) primers. DNA bands were resolved on an agarose gel and images captured on a GelDoc System using Quantity One software (Bio-Rad).

RNase protection assays

Monocytes were isolated as described above and HCMV infected or mock infected nonadherently at 37°C. At 4 hpi, cells were centrifuged, and total RNA was isolated with the

RNA STAT-60 isolation kit (Tel-Test). 2 μg of RNA from each sample was hybridized with an [α^{32}P]uridine 5′-triphosphate (UTP)-labelled human cytokine multiprobe (hCK-2b) template set (BD Biosciences, San Diego, CA) for 12 hours. RNase protection assays were performed with the rabiolabelled RNA using the Multi-Probe RNase Protection Assay System (BD Biosciences, 7th edition) according to the manufacturer's protocol. Following hybridization, the samples were digested with RNaseA (Promega) and resolved on a denaturing polyacrylamide gel. The gel was then dried, and the images were captured with a phosphorimager (Bio-Rad).

Human chemokine antibody array analysis

Monocytes were isolated as described above and HCMV infected or mock infected nonadherently for 6 hrs at 37°C on an orbital shaker. Following incubation, cells were removed by centrifugation and the supernatant collected and stored at -80°C until their use in the protein microarray assay. Cell-free culture supernatants were assayed for 37 different chemokines by using a RayBio Human Chemokine Antibody Array (RayBiotech Inc., Norcross, GA) according to the manufacturer's protocol. Data was analyzed by densitometry using Quantity One image analysis software (Bio-Rad).

Phagokinetic track motility assay

Colloidal gold-coated coverslips were prepared as previously described (15). Briefly, glass coverslips were immersed in a 300 Bloom gelatin solution (0.5 g in 300 ml; Sigma), heated at 90°C for 10 min, and dried at 70°C for 45 min. A colloidal gold suspension was prepared by adding 11 ml of tissue culture water (Sigma) and 6 ml Na_2CO_3 (36.5 mM) to 1.8 ml $AuHCl_4$ (14.5 mM; Fisher Scientific), bringing the solution to a boil, and rapidly adding 1.8 ml of 0.1% formaldehyde (Fisher Scientific). While hot, 2 ml of the colloidal gold suspension was added to each coverslip and incubated at 37°C for 1 h. The coverslips were washed and transferred to 24-well plates.

Monocytes were mock infected or HCMV infected and incubated for 45 minutes at 37°C. Cells were then washed extensively with PBS to remove unbound virus and further incubated nonadherently for 6 hours. Supernatants were collected following centrifugation to remove HCMV-infected monocytes. 1 ml of supernatants from mock-infected and HCMV-infected monocytes was added to colloidal gold-covered coverslips in 24 well plates. Next, 500 naïve monocytes from the same donor were added to each well and incubated at 37°C. After 6 hours of incubation, the cells were fixed in 1.5% paraformaldehyde for 15 minutes and mounted onto glass slides with glycerol. Track images of cells were captured using an inverted microscope at 200× original magnification. Average area cleared per cell out of 20 cells per sample was determined by Scion imaging (Scion Corp., Fredrick, MD), and random motility was plotted as mean in arbitrary units (pixels cleared) ± standard error of mean (SEM). The results are representative of 3 independent experiments from different human donors.

Results

Global transcriptome analysis

For compilation of the data from all experiments, the following criteria were used. Genes that had an absent call in more than 2 of the 6 HCMV-infected samples were removed from the pool of genes. The detection algorithm uses probe pair intensities to generate a detection p-value and a predefined Affymetrix threshold was used to assign a present, marginal or absent call. Next, ANOVA tests were performed on the HCMV-infected versus mock-infected samples and p-values were calculated for genes upregulated or downregulated 1.5-fold. A p-value of ≤ 0.05 was used to generate a pool of genes that were statistically significant. Moreover, a fold-change of 1.5 up or down in at least four of six HCMV-infected versus mock-

infected samples were considered to be regulated by infection; thus, genes that may otherwise have been eliminated due to anomalous expression by a single donor were accepted. Analysis of 6 independent donors used minimized the number of false positives, thus allowing for a lower fold-change cutoff threshold than other microarray studies (42,43).

We then tested the validity and reproducibility of hybridization signals from independent preparations of isolated monocyte RNAs from 6 different donors. Scatter plot analysis on the basis of signal was performed on genes from 6 mock-infected and 6 HCMV-infected samples that had a present call in at least 4 of the 6 HCMV-infected samples (Fig. 1). Because of the use of primary cells from different donors, these selection criteria were utilized to account for potential differences in gene expression between donors. Genes from 3 representative pairs of mock-infected vs. mock-infected, HCMV-infected vs. HCMV-infected and mock-infected vs. HCMV-infected sampleswere plotted on the basis of signal and the coefficients of determination ascertained [all possible pairwise analysis were done with similar results obtained (data not shown)]. We found coefficients of determination to be 0.88 or greater between multiple sample comparisons. Conversely, pairwise comparisons of mock-infected and HCMV-infected samples resulted in lower coefficient of determination (~0.74), verifying that the observed changes in mRNA levels by the HCMV-infected monocyte transcriptome were highly reproducible between donors.

At 4 hpi, HCMV significantly altered the levels of 1204 (10.7% of the total genes examined) cellular mRNAs; where 583 (5.2%) genes were upregulated and 621 (5.5%) genes were downregulated ≥1.5-fold in at least 4 of the 6 replicates (see supplemental data S1 for the complete list of altered transcripts). RT-PCR confirmed that HCMV stimulated ICAM-1, integrin β8, integrin α1, IL-1β, TNF-α, and CCL2 mRNA expression by 4 hpi (Fig. 2). Of the 1204 genes modulated in the monocyte transcriptome following infection, 48.4% were upregulated and 51.6% were downregulated (Fig. 3A). To identify the trends in total cellular gene expression, those genes that were upregulated and downregulated ≥1.5-fold following HCMV infection were grouped by their behavioural patterns. Analysis of specific ontology groups in HCMV-infected monocytes showed altered levels of transcripts involved in the anti-viral response (45% of the total anti-viral response genes examined), apoptosis (18%), cell cycle (10%), cellular metabolism (12%), inflammation (31%), signal transduction (11%), and transcription factor activity (10%) (Fig. 3A). Relative to the total number of genes regulated in the monocyte transcriptome during HCMV binding and entry (10.7%), the ontology analysis indicated genes involved in the anti-viral (45%) and inflammatory responses (31%) to be more substantially modulated. Moreover, within these two ontology clusters, genes were disproportionately upregulated (95% and 77% of genes involved in the anti-viral and inflammatory response, respectively). Because we have previously shown that HCMV induces a pro-inflammatory monocyte to promote the required functional changes in the infected cells necessary for haematogenous dissemination into tissue (14-16), we examined in more detail the gene ontologies known to be involved in inflammation (Fig. 3B, Table I). The results presented in Table I are discussed in more detail below, where we specifically focus on genes implicated in monocyte extravasation into peripheral tissue.

HCMV modulates the expression of monocyte transcripts involved in inflammation at 4 hpi

Apoptosis—HCMV encodes a number of immediate-early gene products with anti-apoptotic function (44-46); however, a 3-4 week delay in viral gene expression in HCMV-infected monocytes (15) suggest that HCMV regulates monocyte apoptosis via cellular anti-apoptotic factors. Indeed, detailed examination of transcripts encoding for anti-apoptotic factors revealed 16% were upregulated and only 4% were downregulated at 4 hpi. This data is consistent with previous findings showing that a number of anti-apoptotic related genes such as BCL2A1 (4.9-fold increase) and TNFRSF6 (3.1-fold increase) are specifically upregulated in M1 polarized

macrophages (47). Moreover, NF-κB, a transcription factor responsible for the expression of a number of anti-apoptotic and inflammatory genes (48,49), is upregulated 3.3-fold. These finding suggests that, even in the absence of de novo viral gene expression, HCMV can create a cellular environment skewed towards the inhibition of apoptosis that likely favours monocyte-to-macrophage differentiation and the long term survival of the infected cells.

Cell Adhesion—Adhesion molecules are required for monocyte transendothelial migration during normal and inflammatory processes (50). The tethering, rolling, and firm adhesion of monocytes to the apical surface of vascular endothelial cells is dependent on adhesion molecule expression. Monocytes must first adhere to endothelial cells and then move along the surface of the endothelial cell in search of the endothelial cell junctions (51). Selectins, intercellular adhesion molecules (ICAMs), and integrins are critical mediators of monocyte adhesion to endothelial cells prior to extravasation (52). M1-activated monocytes express significantly higher levels of these receptors; therefore, monocytes with a pro-inflammatory phenotype have a higher propensity to adhere and transmigrate into peripheral tissue (25,29,53). We found ICAM-1, which is necessary for the firm adhesion of monocytes to endothelial cells (54), to be upregulated 4-fold at the transcriptional level following HCMV infection (Table I and supplemental data S1). This observation is in agreement with our previous data showing increased ICAM-1 expression on the surface of monocytes following infection and a number of other studies that have demonstrated strong upregulation of surface ICAM-1 expression on endothelial and epithelial cells following infection (55,56). Additionally, the microarray data indicates that the mRNA of a number of additional cell adhesion molecules such as ninjurin 1, laminin β_3, and integrins α_1, α_6 and β_8 were elevated. Conversely, α_5, α_M, and β_2 integrins were downregulated following infection indicating that HCMV can selectively alter integrin message expression. The specific upregulation and downregulation of integrins by HCMV has been previously observed in HUVECs and fibroblasts; however, it remains unclear of the consequences or reasons for such regulation (55,57,58). Nonetheless, our previous functional studies showing HCMV-infected monocytes exhibited increased firm adhesion to endothelial cells 6 hpi and increased transendothelial migration 24 hpi demonstrate that the net result of the HCMV mediated modulation of cell adhesion molecules is to increase monocyte adhesion to endothelial cells (14,15).

Extracellular Matrix Proteins—An important step in monocyte migration to the tissue, following extravasation, is the degradation of the basal lamina. Matrix metalloproteinases (MMPs) are involved in the breakdown of extracellular matrix components. HCMV upregulated the expression of both MMP1 and MMP10 approximately 12-fold. MMP1 dissolves collagen types I, II, III, (59), and MMP10 breaks down proteoglycans and fibronectin (60). HCMV may upregulate the expression of these proteinases in monocytes to promote migration through the extracellular matrix in route to the tissue.

Cytokine Activity—A classical feature of a M1 pro-inflammatory phenotype is the secretion of a milieu of cytokines and chemokines which promote monocyte activation along with cellular recruitment to sites of inflammation (53). Indeed, we find that HCMV infection significantly alters the levels of 25% of cytokine and chemokine mRNAs in monocytes. Consistent with our previous studies, as well as others, pro-inflammatory cytokines IL-1β, IL-6, IL-12 p40, IL-15, inhibin β_A and TNF-α, which are associated with the classical activation M1 phenotype, were upregulated following infection (14,21,25,33,61). HCMV potently induced IL-6, TNF-α and inhibin β_A mRNA, reaching a 280-, 14.6- and 9.9-fold increase. HCMV infection also led to a downregulation of certain cytokine genes such as vascular endothelial growth factor (VEGF), which can act as a chemoattractant for T-cells, and tumour necrosis factor ligand superfamily member 8 (CD30), which can stimulate T-cell proliferation.

Interestingly, M2-polarization associated anti-inflammatory cytokines, IL-1 receptor antagonist (IL-1Ra) and IL-10 were both upregulated following HCMV infection (21,25).

To confirm this unusual M1/M2 cytokine profile, RNase protection assays examining M1 and M2 associated cytokines were performed on RNA harvested from mock-infected and HCMV-infected monocytes 4 hpi isolated from multiple donors. Consistent with our gene profiling analysis, RNase protection assays show the upregulation of cytokine genes implicated in the M1 phenotype (IL-12B, IL-1β, and IL-6) and the M2 phenotype (IL-10, IL-1Ra and IL-18) (Fig. 4A and B). These data highlight a unique reprogramming induced in monocytes following infection with HCMV. We next examine in depth the polarization gene profile of the HCMV-infected monocyte.

HCMV stimulates monocyte differentiation towards a M1 macrophage phenotype

The rapid production of numerous cytokines is characteristic of a classical M1 monocyte/macrophage activation/differentiation lineage and our transcriptome analysis indicates that HCMV-infected monocytes acquire a pro-inflammatory M1 phenotype. However, the upregulation of transcripts (IL-1Ra and IL-10) implicated in the alternative M2 differentiation lineage suggest a complex and atypical M1/M2 reprogramming of monocytes following infection with HCMV. To further investigate HCMV-induced monocyte-to-macrophage differentiation, we compared the HCMV-infected monocyte transcriptome to the M1/M2 immunophenotypic profile induced by IFN-γ (M1 phenotype) and IL-4 (M2 phenotype) as described by F. Martinez *et al.* (47). Tables II and III lists genes that were found to be strictly associated with M1 or M2 monocyte polarization, respectively. Transcriptional analysis of monocytes following infection with HCMV revealed that 30 (65%) M1 associated genes were upregulated, 14 (30%) genes showed no change and 2 (5%) genes were downregulated by 4 hpi. Moreover, examination of functional categories indicate that the first M1 associated genes to be modulated during HCMV-induced monocyte polarization are membrane receptors (100%), apoptosis-related genes (100%) and cytokines and chemokines (66%). On the other hand, of the genes associated with the anti-inflammatory M2 phenotype only 2 (4%) were upregulated, 37 (84%) displayed no change and 6 (14%) were downregulated. Taken together these data show a clear transcriptional bias towards a M1 macrophage activation phenotype following infection or monocytes with HCMV, although several transcripts implicated in anti-inflammatory responses typical of alternatively activated macrophages (such as IL-1Ra, IL-10, CCL18 and CCL23) were also upregulated.

HCMV induces an atypical M1/M2 macrophage chemokinome

Distinct patterns of chemokines are produced depending on whether a macrophage undergoes a M1 or M2 polarization (47). We found that 16 (44%) chemokine genes were upregulated in monocytes within 4 hpi, while none were downregulated (Table I). Differential analysis showed that 44% and 33% of M1 and M2 associated chemokines to be upregulated, respectively. To confirm that the upregulation of chemokine genes at the level of transcription conferred increased protein production, secretion of chemokines into the supernatant by monocytes 6 hpi was assayed using human chemokine antibody arrays. Examination of 37 chemokines showed that those identified by gene profiling to exhibit elevated gene expression levels also demonstrated the greatest fold increases in protein secretion with the exception of CXCL5 and IL-8, indicating that chemokine protein production strongly correlated with increased gene expression in infected cells (Fig. 5). Contrary, a few chemokines such as CCL19 and CCL9 not identified by the HCMV-infected monocyte transcriptome analysis displayed increased protein secretion, highlighting a complex biological process that likely involves multiple levels of regulation, including transcription, translation and a regulation of the intercellular trafficking. We next examined if the secreted of chemokines from the infected monocyte samples were functional and could induce motility in naïve monocytes. Treatment

of uninfected monocytes with supernatant collected from HCMV-infected monocytes significantly stimulated cell motility (Fig. 6). Overall, although HCMV induces an atypical monocyte chemokine signature that does not strictly adhere to characteristics of the M1 or M2 phenotype, we suggest the overwhelming upregulation of chemoattactants promotes viral spread by aiding infected monocyte migration to peripheral tissue and/or naïve monocyte migration to sites of infection to enhance the infected cell pool.

Discussion

Monocyte extravasation into tissue is a sequential process involving tethering, rolling, adhesion and diapedesis (52). Quiescent monocytes bind to and transmigrate across endothelial cells at very low rates, but the rate of adhesion and transmigration can be significantly increased following classical activation with different pathogenic stimuli including LPS and HCMV (15,62). However, LPS- and HCMV- activated monocytes are morphologically distinct (15); thus, while it appears that monocytes activated with different stimuli display certain similar phenotypical traits, HCMV must stimulate a distinct M1/M2 monocyte/macrophage polarization, which we advocate serves to promote viral survival and persistence. To examine this possibility, we initiated a study to examine the monocyte transcriptome shortly after infection to obtain a global understanding of the rapid M1/M2 reprogramming of HCMV-infected monocytes.

Global DNA microarray analyses demonstrated that HCMV dramatically altered the transcriptional profile of the monocyte transcriptome following infection. The regulation of gene expression was a highly specific process in which 583 mRNAs were significantly upregulated and 621 mRNAs were significantly downregulated. Ontology analysis revealed a majority of the induced genes are associated with classically activated monocytes/macrophages. Generally, inflammation is utilized by the infected host to help clear infection via the release of antiviral regulatory factors such as IL-1β and TNF-α (63,64). However, HCMV targets monocytes *in vivo* (4,5,65) and once infected appear to benefit from the induction of a strong host inflammatory response (15,34-37). Prostaglandin E_2, IL-1β, and TNF-α, key mediators in the inflammatory response, promote viral replication via stimulation of the HCMV IE genes (34,36,37). Moreover, the induction of M1 associated cytokines and chemokines such as IL-1β, IL-6, and TNF-α can also contribute to the shaping of the classically activated or pro-inflammatory macrophage phenotype, which is important for viral replication (15,66,67) and, now as our evidence identifies, for viral dissemination (14,15).

The absence of viral IE gene expression (15) together with the previous observations that UV-inactivated HCMV and the major viral glycoprotein gB exhibited similar pro-inflammatory modulatory effects on monocyte function (33) suggest the involvement of receptor-ligand interactions. Engagement and activation of cellular surface receptors such as toll-like receptor (TLR) 2, epidermal growth factor (EGFR) and integrins by HCMV (68) followed by the subsequent stimulation of cellular NF-κB and PI(3)K activity (14,16,33,69) is likely the major mechanism for the rapid monocyte gene regulation following infection, although we can not preclude the possible involvement of some tegument proteins which exhibit transactivator activity following viral entry (68). Overall, these studies identify that HCMV binding and/or entry is sufficient to induce a pro-inflammatory monocyte state exhibiting increased cytokine/chemokine secretion, motility, endothelial adhesion, transendothelial migration and monocyte-to-macrophage differentiation.

Although transcriptional profile comparisons of genes differentially expressed in the M1 or M2 phenotype reveal a distinct bias towards the classical activation, a number of genes distinctly associated with alternatively activated monocytes were also upregulated. The anti-inflammatory cytokines, IL-1Ra and IL-10 were both upregulated following HCMV infection.

Mice lacking endogenous IL-1Ra were less susceptible to infection by intracellular pathogens supporting the importance of IL-1 in resistance to infection with intracellular organisms (70). HCMV and Epstein Barr virus encode viral homologues to IL-10 (71,72), which can activate signalling pathways that dampen IFN-γ-triggered microbicidal pathways, inhibit antigen processing and presentation by antigen-presenting cells and inhibit T-cell cytokine production and cytotoxic activity (73). Since the HCMV IL-10 homolog is not synthesized in this early time frame in monocytes, we propose that HCMV utilizes host cytokines instead to inhibit T-cell function. Overall, our data hints that HCMV rapidly and selectively modulates M1 and M2 associated factors resulting in an activated monocyte lying between the ends of the M1/M2 polarization spectrum in order to balance viral spread with immune evasion.

In agreement, examination of the HCMV-infected monocyte chemokine signature showed that the upregulation of chemokines implicated both the classically and alternatively activated phenotype, although again a bias towards the M1 phenotype was observed. Thus, our data indicates that HCMV induces multiple chemoattractants regardless of M1 or M2 association, which we speculate promotes viral spread by driving infected monocyte migration into peripheral tissue and/or the recruitment of naïve monocytes to sites of infection to enhance the infected cell pool. Although beneficial to the virus, chronic secretion of these chemokines could have pathological consequences to the host, such as the development of atherosclerosis where HCMV infection is strongly linked to disease development (10,11). For example, chemokine (C-C motif) ligand 2 (4.1-fold increase) is believed to be one of the most powerful inducers of monocyte migration into atherosclerotic lesions (74).

Overall, we show that HCMV infection rapidly induces a pro-inflammatory monocyte. Is this activated phenotype a direct consequence of specific M1/M2 reprogramming by the virus or a general response to viruses? HIV studies including microarray analyses indicate pro-inflammatory gene expression resulting in chronically activated HIV-infected monocytes/macrophages occurs *in vivo* (75-77). Similar to HCMV infected monocytes, release of chemotatic cytokines such as MCP-1 from HIV infected cells enhance dissemination by stimulating recruitment of target CD4 T cells and monocytes/macrophages to sites of infection (78). Furthermore, HIV appears to induce anti-apoptotic and cell cycle associated genes leading to greater cell survival and viral replication, respectively (79-81). Nevertheless, although both HCMV- and HIV-infected monocytes display an activated invasive monocyte phenotype, a comparative analysis of gene expression in monocytes infected with HIV to our results revealed fundamental differences between the two viruses. In HCMV-infected monocytes, expression of inflammatory cytokine genes such as IL-1β, IL-6 and TNF-α were significantly upregulated (presented here) while in HIV-infected monocytes these genes remained unchanged (75). IL-1β and TNF-α have strong anti-viral activity, thus, downregulation by HIV is likely a strategy for immune evasion; however, as mentioned above, HCMV has devised a strategy utilizing the NF-κB activating properties of these cytokines to stimulate its own viral replication (36,37). The chemokine receptor CCR5 is the major entry coreceptor for HIV and is upregulated in HIV (82), but not in HCMV-infected monocytes. These discussed differences are but a few examples to highlight the transcriptional differences that occur following infection of monocytes with HCMV and HIV. Furthermore, HIV does not appear to force monocyte differentiation into macrophages, as we observed for HCMV (15), but rather as monocytes differentiate they become more susceptible to HIV infection (82). Thus, HCMV- and HIV-infected monocytes are not activated identically in the classical sense and lie at different points along the M1/M2 macrophage polarization continuum. By using microarray technology, we can dissect the unique strategies that these different viruses have evolved to promote dissemination and persistence.

The data presented in this global transcriptional profile study is consistent with our previously proposed model of HCMV dissemination, where HCMV infection classically activates

monocytes in order to promote migration into host organ tissue and differentiation into replication permissive macrophages (14-16). Our data also confirms morphological studies suggesting that HCMV infection does not induce all the same characteristics associated with the classically activated M1 phenotype induced by LPS. The HCMV-infected monocyte transcriptome revealed an atypical M1/M2 polarization, with a defined phenotype biased towards the M1 polarization that also incorporated select attributes of the alternatively activated M2 phenotype. Presumably the upregulation of specific genes associated with the anti-inflammatory M2 phenotype, and in particular chemoattractants, would be advantageous for the virus. Overall, our study provides insight into how HCMV reprograms monocytes as a mechanism to promote viral dissemination and demonstrates the complexity of HCMV-induced signaling events, even in the absence of viral gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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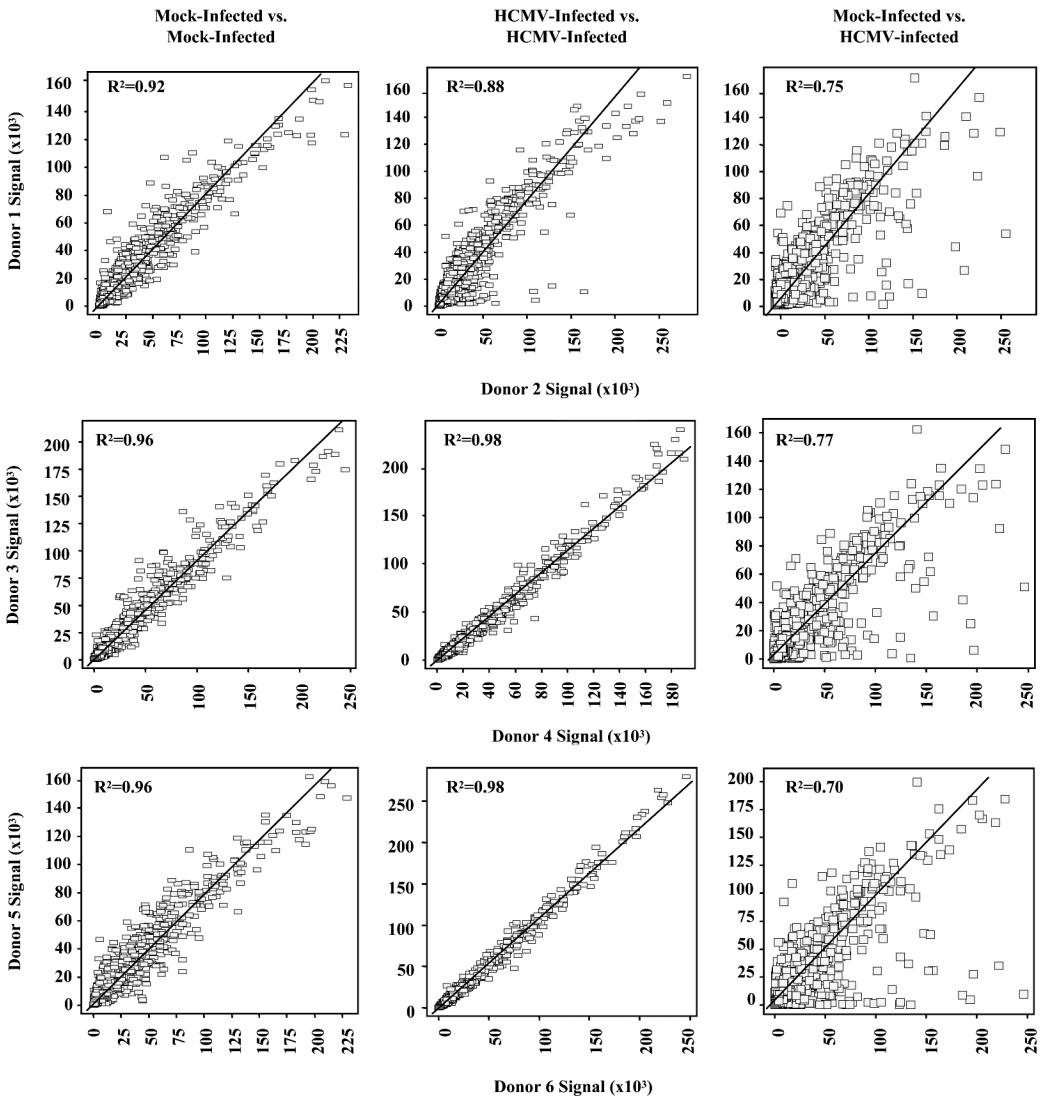

FIGURE 1.

Linear regression analyses of mock-infected and HCMV-infected samples. Monocytes were either mock infected or HCMV infected and incubated nonadherently at 37°C for 4 hours. RNA was harvested and analyzed by Affymetrix gene array. Genes were compiled from six experimental samples and ANOVA tests were performed. P-values were calculated and a p-value cut-off of ≤0.05 was made. To be considered statistically significant, genes had to have a present call in at least 4 of 6 HCMV infected samples and a 1.5-fold or greater change in at least 4 of 6 HCMV samples (see supplemental data S1 for complete list of genes). The significant genes from mock-infected vs. mock-infected, HCMV-infected vs. HCMV-infected and mock-infected vs. HCMV-infected samples were then plotted on the basis of signal. Squares represent individual genes and the “line of best fit” on each graph was used to calculate the coefficients of determination (R^2). These scatter plots were generated with Spotfire DecisionSite software. Three representative pairwise comparisons are illustrated although all pairwise comparisons were performed (data not shown).

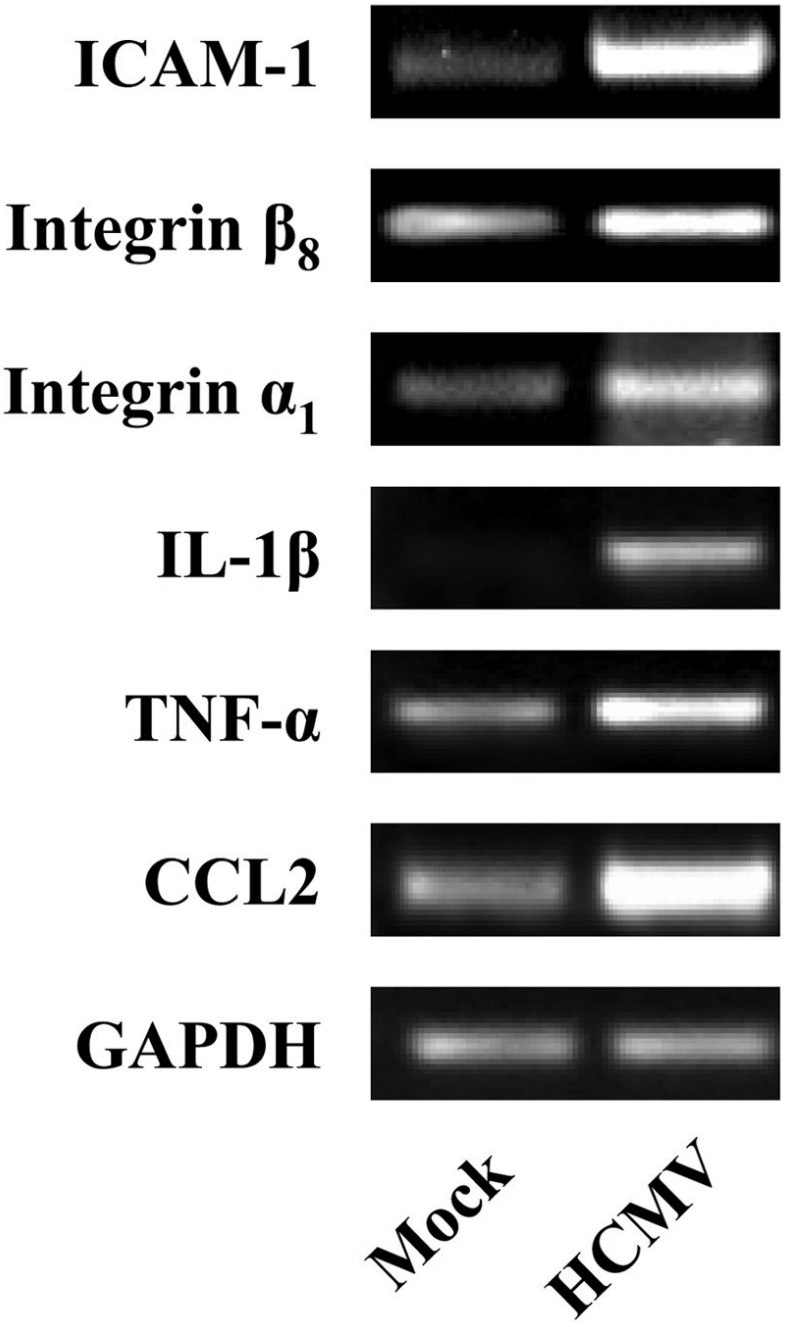

FIGURE 2.

Confirmation of select chemokine and adhesion molecule gene expression by RT-PCR. Monocytes were mock infected or HCMV infected nonadherently for 4 hours at 37°C and RNA was harvested. RT-PCR analysis confirmed that HCMV induced the expression of ICAM-1, integrin β_8, integrin α_1, IL-1β, TNF-α and CCL2. GAPDH expression is shown as a control,

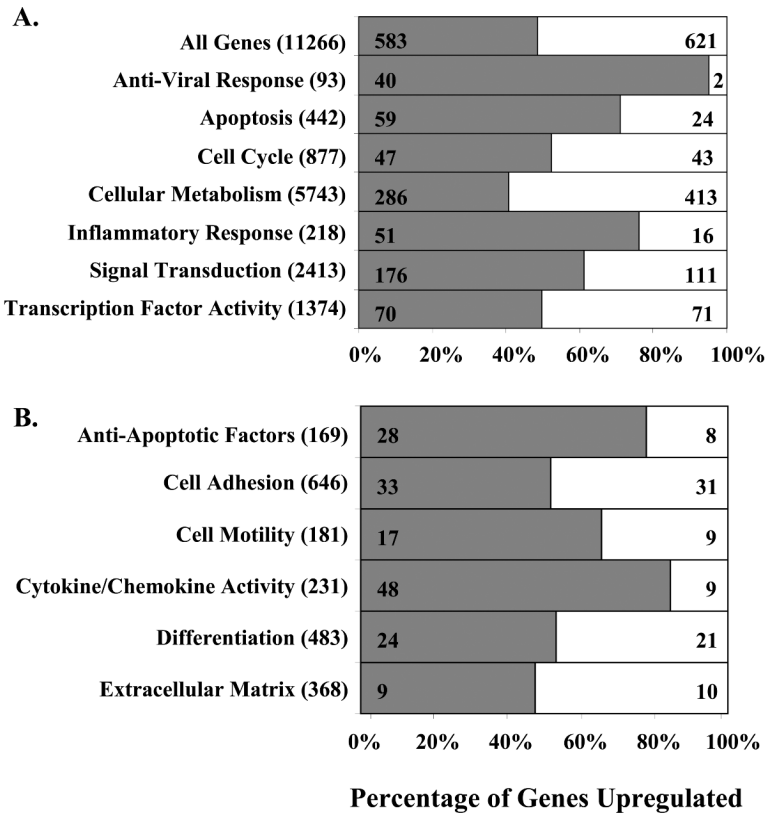

FIGURE 3.

HCMV alters the monocyte transcriptome at 4 hpi. Percentage of genes upregulated (grey bars) and downregulated (white bars) 1.5-fold or more were grouped into (A) functional categories/ontologies or (B) ontologies involved in inflammation using Spotfire DecisionSite software based on the Gene Ontology Consortium data base. Total genes upregulated and downregulated in each grouping are represented within the bar. The total number of genes analyzed in each ontology category is shown within parenthesis. The percentages represent the percentage of genes that were upregulated following infection.

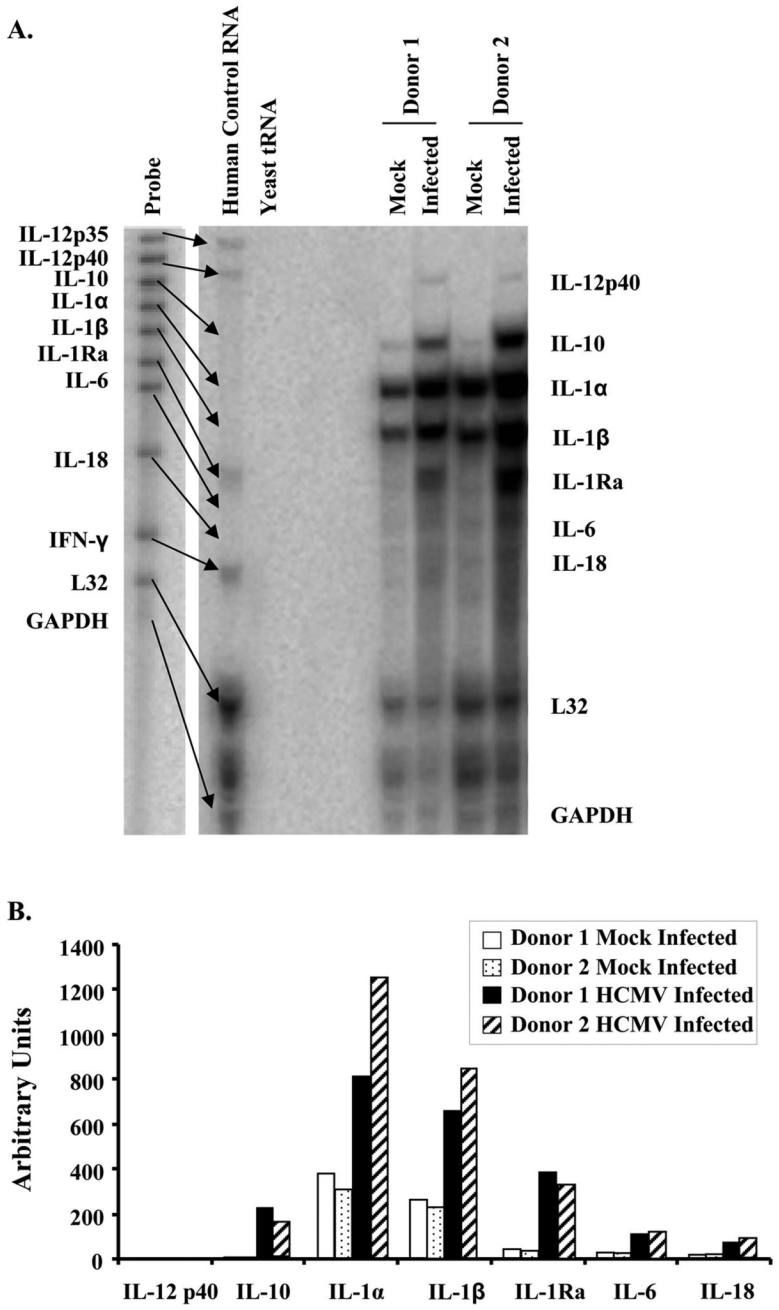

FIGURE 4.

HCMV infection induces cytokine expression. Monocytes were mock infected or HCMV infected nonadherently for 4 hours at 37°C and RNA was harvested. (A) RNA from multiple donors was hybidized with a [α^{32}P]UTP-labelled hCM-8 template set and subjected to RNase protection assay using the Multi-Probe RNase protection assay system according to the manufacturer's protocol. Genes on the left represent the total set of genes probed for with the kit and the genes on the right represent those genes induced following HCMV infection of monocytes. (B) Band intensities from the RNase Protection Assay was determined using by densitometry using Quantity One software (Bio-Rad), normalized to the respective L32 band intensities, and plotted in arbitrary units. Results are representative of 3 independent experiments from 3 donors.

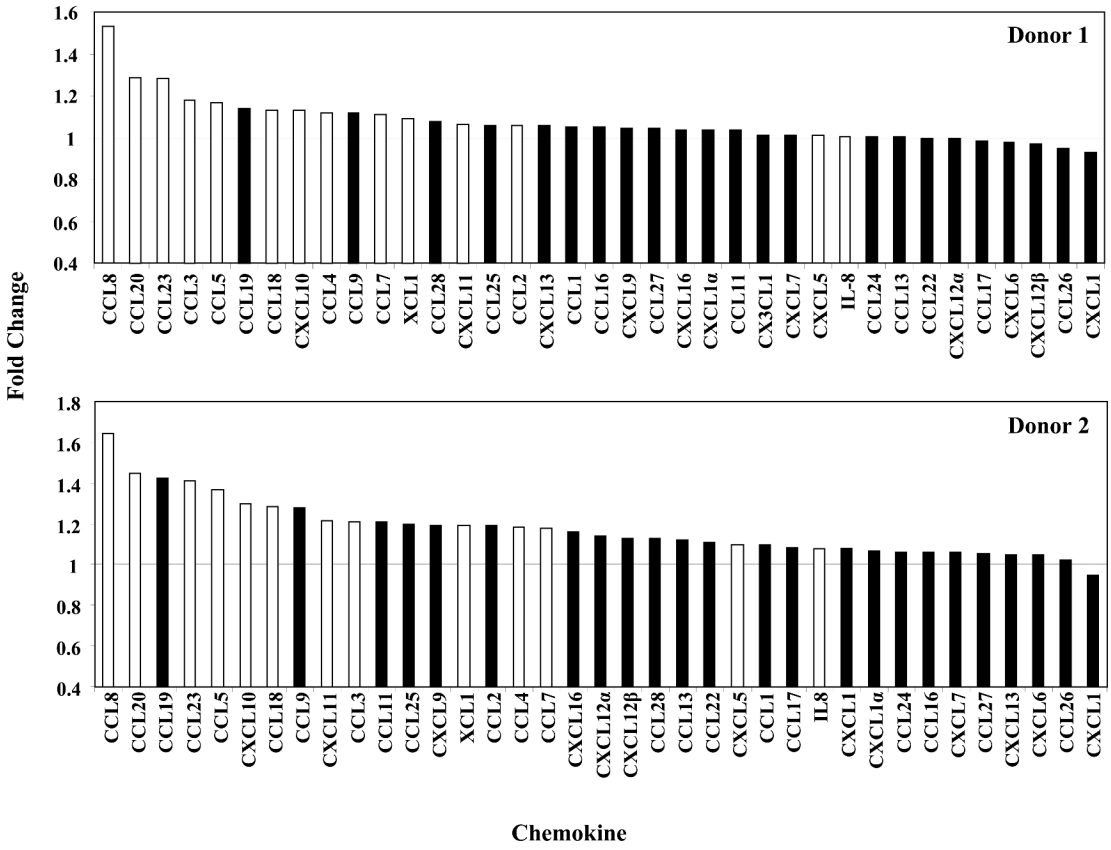

FIGURE 5.

Differential secretion of chemokines by monocytes following infection with HCMV. Monocytes were mock infected or HCMV infected nonadherently for 6 hours at 37°C, and supernatant collected. Cell-free culture supernatants from multiple donors were assayed for 37 different chemokines by using a RayBio Human Chemokine Antibody Array according to the manufacturer's protocol. Densitometry of the Chemokine Antibody array was performed with Quantity One software (Bio-Rad) and fold change plotted. White bars represent chemokines that were identified by transcriptome profiling analysis to be upregulated following infection with HCMV. Black bars represent chemokines not transcriptionally upregulated at 4 hpi. Results are representative of 4 independent experiments from 3 donors (analysis on one donor was repeated).

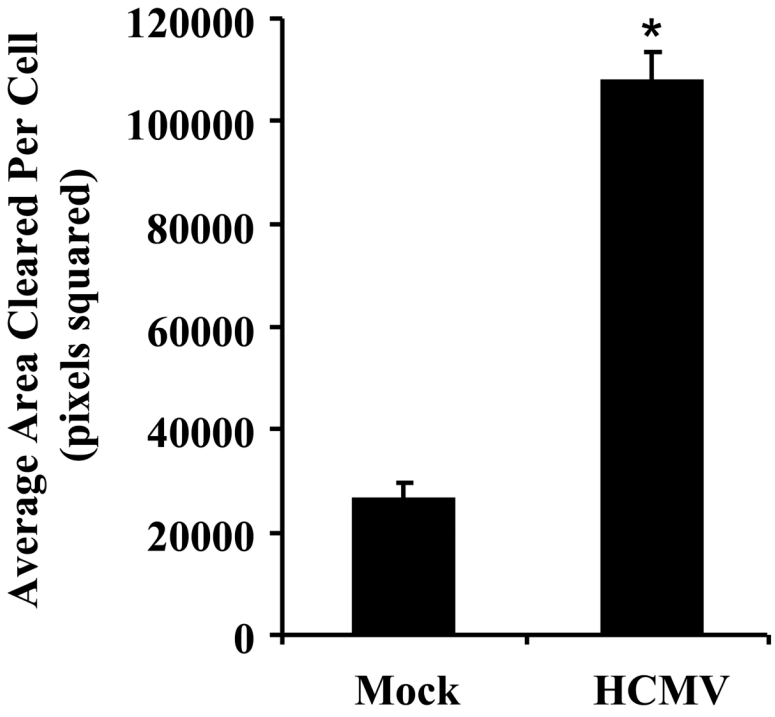

FIGURE 6.

Supernatant harvested from HCMV-infected monocytes stimulate naïve monocyte motility. Monocytes were mock infected or HCMV infected and incubated for 45 minutes. Cells were washed extensively to remove unbound virus and incubated nonadherently for 6 hours. Supernatants were harvested from each of the samples following centrifugation and 1 ml added to colloidal gold-covered coverslips. After 500 naïve monocytes were added to each coverslip and at six hours post-addition of naïve monocytes the cells were fixed with 1.5% paraformaldehyde. The average area (arbitrary units^2) of colloidal gold cleared by 20 monocytes was determined for each experimental arm from the captured images. Random monocyte motility is plotted as a mean ± S.E.M. of 20 cells per experimental arm. The results represent 3 independent experiments with separate human blood donors. Asterisk denotes a significance of $P<0.01$.

Table I

Select statistically significant monocyte mRNAs that increase or decrease ≥1.5-fold 4 hpi with HCMV

Category and Full Gene Name	Gene Title	Probe Set	Fold Change
Anti-Apoptotic Factors			
Tumor necrosis factor (TNF superfamily, member 2)	TNF	1852_at	14.6
Secreted phosphoprotein 1	SPP1	2092_s_at	6.3
Suppressor of cytokine signaling 3	SOCS3	40968_at	5.7
BCL2-related protein A1	BCL2A1	2002_s_at	4.9
Interleukin 10	IL10	1548_s_at	4.6
Chemokine (C-C motif) ligand 2	CCL2	34375_at	4.1
Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	SERPINB2	37185_at	4.0
CASP8 and FADD-like apoptosis regulator	CFLAR	1868_g_at	4.0
Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 9	SERPINB9	34438_at	3.9
Tumor necrosis factor, alpha-induced protein 8	TNFAIP8	33243_at	3.6
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	NFKB1	1378_g_at	3.3
Tumor necrosis factor receptor superfamily, member 6	TNFRSF6	1440_s_at	3.1
Tumor necrosis factor, alpha-induced protein 3	TNFAIP3	595_at	2.9
Heat shock 70kDa protein 1A	HSPA1A	31692_at	2.5
Baculoviral IAP repeat-containing 3	BIRC3	1717_s_at	2.5
Immediate early response 3	IER3	1237_at	2.4
CASP8 and FADD-like apoptosis regulator	CFLAR	32746_at	2.3
Heat shock 70kDa protein 9B (mortalin-2)	HSPA9B	41510_s_at	1.8
Notch homolog 2 (Drosophila)	NOTCH2	38083_at	1.5
V-rel reticuloendotheliosis viral oncogene homolog A	RELA	1271_g_at	1.5
Mucosa associated lymphoid tissue lymphoma translocation gene 1	MALT1	32350_at	-1.6
Transforming growth factor, beta 1 (Camurati-Engelmann disease)	TGFB1	1830_s_at	-1.6
Histone deacetylase 1	HDAC1	38771_at	-1.7
RAS p21 protein activator (GTPase activating protein) 1	RASA1	1675_at	-2.0
Annexin A4	ANXA4	37374_at	-2.3
Dimethylarginine dimethylaminohydrolase 2	DDAH2	38621_at	-3.3
Anti-Viral Response			
Chemokine (C-C motif) ligand 4	CCL4	36674_at	16.2
Tumor necrosis factor (TNF superfamily, member 2)	TNF	1852_at	14.6
Interferon, alpha 10	IFNA10	1075_f_at	10.2
Interferon, alpha 2	IFNA2	1791_s_at	8.0
Chemokine (C-C motif) ligand 8	CCL8	37823_at	7.4
Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	37014_at	5.6
Signal transducer and activator of transcription 1, 91kDa	STAT1	33338_at	5.2
Interferon, alpha 1	IFNA1	1666_at	5.0
Interferon, alpha-inducible protein (clone IFI-15K)	G1P2	1107_s_at	3.9
Interferon-induced protein 35	IFI35	464_s_at	3.0
2′,5′-oligoadenylate synthetase 1, 40/46kDa	OAS1	38388_at	3.0
DnaJ (Hsp40) homolog, subfamily C, member 3	DNAJC3	33208_at	2.8
Tripartite motif-containing 22	TRIM22	36825_at	2.5
Interferon stimulated gene 20kDa	ISG20	33304_at	2.4
Myxovirus (influenza virus) resistance 2 (mouse)	MX2	879_at	2.3
Interferon, gamma-inducible protein 16	IFI16	1456_s_at	2.2
Interferon regulatory factor 7	IRF7	36412_s_at	2.1
Signal transducer and activator of transcription 2, 113kDa	STAT2	36770_at	2.0
Chemokine (C-C motif) ligand 5	CCL5	1404_r_at	1.8
Interferon (alpha, beta and omega) receptor 2	IFNAR2	1589_s_at	1.8
Interferon-stimulated transcription factor 3, gamma 48kDa	ISGF3G	38517_at	1.6
Protein kinase, interferon-inducible double stranded RNA dependent	PRKR	1008_f_at	1.7
Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	APOBEC3G	34947_at	1.7
V-rel reticuloendotheliosis viral oncogene homolog A	RELA	1271_g_at	1.5
Protein tyrosine phosphatase, receptor type, C	PTPRC	40520_g_at	-1.6
Interferon gamma receptor 1	IFNGR1	1038_s_at	-1.6
Cell Adhesion			
Chemokine (C-C motif) ligand 4	CCL4	36674_at	16.2
Tumor necrosis factor, alpha-induced protein 6	TNFAIP6	1372_at	14.7
Tumor necrosis factor (TNF superfamily, member 2)	TNF	1852_at	14.6
Integrin, beta 8	ITGB8	889_at	9.3
Fasciculation and elongation protein zeta 1 (zygin I)	FEZ1	37743_at	9.1
Secreted phosphoprotein 1	SPP1	2092_s_at	6.3
ADAM-like, decysin 1	ADAMD1	34974_at	6.2
Pleckstrin homology domain containing, family C (with FERM domain) member 1	PLEKHC1	36577_at	5.2
Chemokine (C-C motif) ligand 2	CCL2	34375_at	4.1
Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	ICAM1	32640_at	4.0
Ninjurin 1	NINJ1	41475_at	3.7
CD44 antigen (homing function and indian blood group system)	CD44	1125_s_at	3.0

Category and Full Gene Name	Gene Title	Probe Set	Fold Change
Laminin, beta 3	LAMB3	36929_at	2.9
Scavenger receptor class F, member 1	SCARF1	40034_r_at	2.7
Integrin, alpha 1	ITGA1	120_at	2.5
Integrin, alpha 6	ITGA6	41266_at	2.4
Interleukin 8	IL8	35372_r_at	2.4
Interleukin 18 (interferon-gamma-inducing factor)	IL18	1165_at	2.0
CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	CD47	37890_at	1.9
Thrombospondin 1	THBS1	867_s_at	1.9
Heat shock 60kDa protein 1 (chaperonin)	HSPD1	37720_at	1.7
Chemokine (C-C motif) ligand 5	CCL5	1405_i_at	1.7
Lectin, galactoside-binding, soluble, 1 (galectin 1)	LGALS1	33412_at	-1.5
Colony stimulating factor 3 receptor (granulocyte)	CSF3R	596_s_at	-1.6
Integrin-linked kinase	ILK	35365_at	-1.7
Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	ITGA5	39753_at	-1.7
Rho GDP dissociation inhibitor (GDI) beta	ARHGDIB	1984_s_at	-1.7
Chemokine (C-C motif) receptor 1	CCR1	1128_s_at	-1.7
Angiogenic factor VG5Q	VG5Q	35067_at	-1.7
PTK2B protein tyrosine kinase 2 beta	PTK2B	2009_at	-1.8
Myosin X	MYO10	35362_at	-1.8
Integrin, beta 2 (antigen CD18 (p95)	ITGB2	37918_at	-1.8
Calcium and integrin binding 1 (calmyrin)	CIB1	1020_s_at	-1.9
RAS p21 protein activator (GTPase activating protein) 1	RASA1	1675_at	-2.0
Calsyntenin 1	CLSTN1	41498_at	-2.1
HSPCO34 protein	LOC51668	36032_at	-2.1
Collagen, type VII, alpha 1 (epidermolysis bullosa, dystrophic, dominant and recessive)	COL7A1	32123_at	-2.1
PTK2B protein tyrosine kinase 2 beta	PTK2B	33804_at	-2.3
Signal-induced proliferation-associated gene 1	SIPA1	36843_at	-2.3
Pleckstrin homology, Sec7 and coiled-coil domains, binding protein	PSCDBP	39604_at	-2.4
Zyxin	ZYX	36958_at	-2.6
CD9 antigen (p24)	CD9	39389_at	-2.7
Catenin (cadherin-associated protein), delta 1	CTNND1	40444_s_at	-2.9
Stabilin 1	STAB1	38487_at	-2.9
Endoglin (Osler-Rendu-Weber syndrome 1)	ENG	32562_at	-3.2
Integrin, alpha M	ITGAM	38533_s_at	-3.4
Transforming growth factor, beta-induced, 68kDa	TGFBI	1385_at	-10.1
Chondroitin sulfate proteoglycan 2 (versican)	CSPG2	31682_s_at	-14.4
Platelet/endothelial cell adhesion molecule (CD31 antigen)	PECAM1	37398_at	-14.7
Cell Motility			
Chemokine (C-X-C motif) ligand 10	CXCL10	431_at	69.3
Chemokine (C-C motif) ligand 4	CCL4	36674_at	16.2
Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2	1069_at	15.5
Chemokine (C-C motif) ligand 3	CCL3	36103_at	10.4
Thioredoxin	TXN	36992_at	3.7
Myristoylated alanine-rich protein kinase C substrate	MARCKS	32434_at	2.8
Plasminogen activator, urokinase receptor	PLAUR	189_s_at	2.4
Interleukin 8	IL8	35372_r_at	2.4
Serine (or cysteine) proteinase inhibitor, clade E, member 2	SERPINE2	41246_at	2.0
Vanin 2	VNN2	34498_at	2.0
Jagged 1 (Alagille syndrome)	JAG1	35414_s_at	1.9
Thrombospondin 1	THBS1	867_s_at	1.9
Chemokine (C-C motif) ligand 5	CCL5	1405_i_at	1.7
Chloride intracellular channel 4	CLIC4	33891_at	1.5
Actinin, alpha 4	ACTN4	41753_at	-1.6
Actin related protein 2/3 complex, subunit 1B, 41kDa	ARPC1B	39043_at	-1.6
Rho GDP dissociation inhibitor (GDI) beta	ARHGDIB	1984_s_at	-1.7
Mitogen-activated protein kinase 14	MAPK14	37733_at	-2.0
Lymphocyte-specific protein 1	LSP1	36493_at	-2.5
CD9 antigen (p24)	CD9	39389_at	-2.7
Coronin, actin binding protein, 1A	CORO1A	38976_at	-3.0
Formyl peptide receptor-like 2	FPRL2	33092_at	-7.8
Platelet/endothelial cell adhesion molecule (CD31 antigen)	PECAM1	37398_at	-14.7
Cytokine/Chemokine			
Interleukin 6 (interferon, beta 2)	IL6	38299_at	280.8
Chemokine (C-X-C motif) ligand 10	CXCL10	431_at	69.3
Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	CCL18	32128_at	39.4
Interleukin 12B	IL12B	563_at	33.7
Colony stimulating factor 3	CSF3	1334_s_at	27.4
Chemokine (C-C motif) ligand 20	CCL20	40385_at	16.5

Category and Full Gene Name	Gene Title	Probe Set	Fold Change
Chemokine (C-C motif) ligand 4	CCL4	36674_at	16.2
Chemokine (C-X-C motif) ligand 11	CXCL11	35061_at	15.2
Tumor necrosis factor (TNF superfamily, member 2)	TNF	1852_at	14.6
Chemokine (C-C motif) ligand 3	CCL3	36103_at	10.4
Colony stimulating factor 2 (granulocyte-macrophage)	CSF2	1400_at	10.3
Interferon, alpha 10	IFNA10	1075_f_at	10.2
Inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	40357_at	9.9
Chemokine (C motif) ligand 1	XCL1	31496_g_at	8.7
Interferon, alpha 2	IFNA2	1791_s_at	8.0
Chemokine (C-C motif) ligand 8	CCL8	37823_at	7.4
Colony stimulating factor 2 (granulocyte-macrophage)	CSF2	1401_g_at	6.8
Chemokine (C-X-C motif) ligand 2	CXCL2	408_at	6.3
Secreted phosphoprotein 1	SPP1	2092_s_at	6.3
Chemokine (C-X-C motif) ligand 3	CXCL3	34022_at	6.0
Interleukin 1 receptor antagonist	IL1RN	37603_at	5.7
Tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	1715_at	5.8
Interleukin 15	IL15	38488_s_at	5.3
Interferon, alpha 1	IFNA1	1666_at	5.0
Interleukin 10	IL10	1548_s_at	4.6
Chemokine (C-C motif) ligand 2	CCL2	34375_at	4.1
Chemokine (C-C motif) ligand 23	CCL23	36444_s_at	3.5
Chemokine (C-X-C motif) ligand 5	CXCL5	35025_at	3.2
Interleukin 1, beta	IL1B	39402_at	3.2
Tumor necrosis factor (ligand) superfamily, member 14	TNFSF14	31742_at	3.1
Chemokine (C-C motif) ligand 7	CCL7	39802_at	2.5
Interleukin 8	IL8	35372_r_at	2.4
Interleukin 7	IL7	33966_at	2.0
Interleukin 18 (interferon-gamma-inducing factor)	IL18	1165_at	2.0
Oncostatin M	OSM	1579_at	2.0
Pre-B-cell colony enhancing factor 1	PBEF1	33849_at	1.9
Lymphotoxin beta (TNF superfamily, member 3)	LTB	40729_s_at	1.8
Chemokine (C-C motif) ligand 5	CCL5	1405_i_at	1.7
CD27-binding (Siva) protein	SIVA	39020_at	-1.5
IK cytokine, down-regulator of HLA II	IK	218_at	-1.6
Endothelial cell growth factor 1 (platelet-derived)	ECGF1	36879_at	-1.8
Granulin	GRN	41198_at	-1.9
Tumor necrosis factor (ligand) superfamily, member 8	TNFSF8	33012_at	-2.5
Vascular endothelial growth factor	VEGF	36101_s_at	-2.5
Differentiation			
Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)	IL12B	563_at	33.7
Colony stimulating factor 3 (granulocyte)	CSF3	1334_s_at	27.4
Aryl-hydrocarbon receptor nuclear translocator 2	ARNT2	35352_at	14.0
Fms-related tyrosine kinase 1	FLT1	1545_g_at	13.8
Histone deacetylase 9	HDAC9	37483_at	10.4
Inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	40357_at	9.9
Fasciculation and elongation protein zeta 1 (zygin I)	FEZ1	37743_at	9.1
Secreted phosphoprotein 1	SPP1	2092_s_at	6.3
Fms-related tyrosine kinase 1	FLT1	1963_at	5.0
CD80 antigen (CD28 antigen ligand 1, B7-1 antigen)	CD80	35015_at	4.7
Interleukin 10	IL10	1548_s_at	4.6
Tumor necrosis factor, alpha-induced protein 2	TNFAIP2	38631_at	3.5
Growth arrest and DNA-damage-inducible, beta	GADD45B	39822_s_at	3.3
Epiregulin	EREG	34476_r_at	2.7
Interferon, gamma-inducible protein 16	IFI16	1456_s_at	2.2
Interleukin 7	IL7	33966_at	2.0
Serine (or cysteine) proteinase inhibitor, clade E, member 2	SERPINE2	41246_at	2.0
Agrin	AGRN	33454_at	1.8
Metallothionein 3 (growth inhibitory factor (neurotrophic))	MT3	870_f_at	1.6
Notch homolog 2 (Drosophila)	NOTCH2	38083_at	1.6
Chloride intracellular channel 4	CLIC4	33891_at	1.5
Purine-rich element binding protein A	PURA	35221_at	-1.5
Protein tyrosine phosphatase, receptor type, C	PTPRC	40520_g_at	-1.6
Leukocyte specific transcript 1	LST1	37967_at	-1.6
Endothelial cell growth factor 1 (platelet-derived)	ECGF1	36879_at	-1.8
Angiogenic factor VG5Q	VG5Q	35067_at	-1.8
CCAAT/enhancer binding protein (C/EBP), gamma	CEBPG	39219_at	-1.8
RAS p21 protein activator (GTPase activating protein) 1	RASA1	1675_at	-2.0
PDZ and LIM domain 7 (enigma)	PDLIM7	39530_at	-2.1

Category and Full Gene Name	Gene Title	Probe Set	Fold Change
V-ski sarcoma viral oncogene homolog (avian)	SKI	41499_at	-2.2
Alanyl (membrane) aminopeptidase	ANPEP	39385_at	-2.2
Filamin B, beta (actin binding protein 278)	FLNB	38078_at	-2.3
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	YWHAH	1424_s_at	-2.5
Vascular endothelial growth factor	VEGF	36101_s_at	-2.5
Bridging integrator 1	BIN1	459_s_at	-2.6
Ubiquitin-conjugating enzyme E2 variant 1	UBE2V1	36959_at	-2.6
CD9 antigen (p24)	CD9	39389_at	-2.7
CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)	CD86	36270_at	-3.4
Extracellular Matrix			
Matrix metalloproteinase 10 (stromelysin 2)	MMP10	1006_at	12.1
Matrix metalloproteinase 1 (interstitial collagenase)	MMP1	38428_at	9.2
Secreted phosphoprotein 1	SPP1	2092_s_at	6.3
Matrix metalloproteinase 14 (membrane-inserted)	MMP14	34747_at	5.9
Laminin, beta 3	LAMB3	36929_at	2.9
Protease inhibitor 3, skin-derived (SKALP)	PI3	41469_at	2.7
Agrin	AGRN	33454_at	1.8
Collagen, type VII, alpha 1 (epidermolysis bullosa, dystrophic, dominant and recessive)	COL7A1	32123_at	-2.1
Tissue inhibitor of metalloproteinase 2	TIMP2	1375_s_at	-2.1
Vascular endothelial growth factor	VEGF	36101_s_at	-2.5
Transforming growth factor, beta-induced, 68kDa	TGFBI	1385_at	-10.0
Chondroitin sulfate proteoglycan 2 (versican)	CSPG2	31682_s_at	-14.4

Table II

Comparison of M1 genes expressed in HCMV-infected monocytes/macrophages[a]

Category and Full Gene Name	Gene Title	Probe Set	Fold Change
Membrane Receptors			
Interleukin 15 receptor, alpha	IL15RA	41677_at	15.3
Chemokine (C-C motif) receptor 7	CCR7	1097_s_at	3.0
interleukin 2 receptor, gamma (severe combined immunodeficiency)	IL2RG	1506_at	1.9
Interleukin 7 receptor	IL7R	1370_at	1.7
Cytokine and Chemokines			
Interleukin 6 (interferon, beta 2)	IL6	38299_at	280.8
Chemokine (C-X-C motif) ligand 10	CXCL10	431_at	69.3
Interleukin 12B	IL12B	563_at	33.7
Chemokine (C-C motif) ligand 20	CCL20	40385_at	16.5
Chemokine (C-X-C motif) ligand 11	CXCL11	35061_at	15.2
Tumor necrosis factor (TNF superfamily, member 2)	TNF	1852_at	14.6
Tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	1715_at	5.8
Interleukin 15	IL15	1036_at	4.0
Pre-B-cell colony enhancing factor 1	PBEF1	33849_at	1.9
Chemokine (C-C motif) ligand 5	CCL5	1403_s_at	1.6
Endothelial cell growth factor 1 (platelet-derived)	ECGF1	36879_at	-1.8
Chemokine (C-C motif) ligand 19	CCL19	36067_at	nc
Chemokine (C-X-C motif) ligand 9	CXCL9	37219_at	nc
Chemokine (C-C motif) ligand 15	CCL15	33789_at	nc
Apoptosis-related Genes			
BCL2-related protein A1	BCL2A1	2002_s_at	4.9
XIAP associated factor-1	HSXIAPAF1	35583_at	3.4
Tumor necrosis factor receptor superfamily, member 6	TNFRSF6	1440_s_at	3.1
Baculoviral IAP repeat-containing 3	BIRC3	1717_s_at	2.5
Growth arrest and DNA-damage-inducible, alpha	GADD45A	1911_s_at	2.1
Solute Carriers			
Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	SLC7A5	32186_at	1.6
Solute carrier family 31 (copper transporters), member 2	SLC31A2	34749_at	nc
Enzymes			
Adenylate kinase 3	AK3	32331_at	9.3
Indoleamine-pyrrole 2,3 dioxygenase	INDO	36804_at	4.1
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	PFKFB3	37111_g_at	3.6
2′-5′-oligoadenylate synthetase 2, 69/71kDa	OAS2	39263_at	3.4
Proteasome (prosome, macropain) activator subunit 2 (PA28 beta)	PSME2	1184_at	1.8
Proteasome (prosome, macropain) subunit, beta type, 9	PSMB9	38287_at	1.7
2′-5′-oligoadenylate synthetase-like	OASL	269_at	nc
Chitinase 3-like 1 (cartilage glycoprotein-39)	CHI3L1	36197_at	nc
Hydroxysteroid (11-beta) dehydrogenase 1	HSD11B1	35702_at	nc
Phosphofructokinase, platelet	PFKP	39175_at	nc
Proteasome (prosome, macropain) subunit, alpha type, 2	PSMA2	41240_at	nc
Extracellular Matrix			
Pentaxin-related gene, rapidly induced by IL-1 beta	PTX3	1491_at	13.5
Inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	40357_at	9.9
Chondroitin sulfate proteoglycan 2 (versican)	CSPG2	31682_s_at	-14.4
Insulin-like growth factor binding protein 4	IGFBP4	1737_s_at	nc
Apolipoprotein L, 1	APOL1	35099_at	nc
Platelet-derived growth factor alpha polypeptide	PDGFA	1109_s_at	nc
DNA-binding Factors			
Interferon regulatory factor 1	IRF1	669_s_at	3.3
Interferon regulatory factor 7	IRF7	36412_s_at	2.1
Homeo box (expressed in ES cells) 1	HESX1	35463_at	nc
Activating transcription factor 3	ATF3	287_at	nc

[a]Examined M1 genes are based on the transcriptional profiling work of Martinez *et al*. (47).

Table III

Comparison of M2 genes expressed in HCMV-infected monocytes/macrophages[a]

Category and Full Gene Name	Gene Title	Probe Set	Fold Change
Membrane Receptors			
Chemokine (C-X-C motif) receptor 4	CXCR4	649_s_at	-2.5
Transforming growth factor, beta receptor II (70/80kDa)	TGFBR2	1814_at	-2.7
Histamine receptor H1	HRH1	35384_at	nc
Toll-like receptor 5	TLR5	34473_at	nc
Type I transmembrane C-type lectin receptor DCL-1	DCL-1	34760_at	nc
Macrophage scavenger receptor 1	MSR1	39981_at	nc
G protein-coupled receptor 105	GPR105	33462_at	nc
CD209 antigen-like	CD209L	39270_at	nc
CD36 antigen (collagen type I receptor, thrombospondin receptor)	CD36	36656_at	nc
Mannose receptor, C type 1	MRC1	36908_at	nc
Cytokine and Chemokines			
Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	CCL18	32128_at	39.4
Chemokine (C-C motif) ligand 23	CCL23	36444_s_at	3.5
Insulin-like growth factor 1 (somatomedin C)	IGF1	1501_at	nc
Chemokine (C-C motif) ligand 13	CCL13	37454_at	nc
Solute Carriers			
Solute carrier family 4, sodium bicarbonate cotransporter, member 7	SLC4A7	34936_at	-1.5
Solute carrier family 38, member 6	SLC38A6	36758_at	nc
Enzymes			
Leukotriene A4 hydrolase	LTA4H	38081_at	-5.5
Cathepsin C	CTSC	133_at	nc
Hexosaminidase B (beta polypeptide)	HEXB	34888_at	nc
Lipase A, lysosomal acid, cholesterol esterase (Wolman disease)	LIPA	38745_at	nc
Adenosine kinase	ADK	168_at	nc
Histamine N-methyltransferase	HNMT	37604_at	nc
Tyrosylprotein sulfotransferase 2	TPST2	35172_at	nc
Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	HS3ST1	41555_at	nc
Carbonic anhydrase II	CA2	40095_at	nc
Arachidonate 15-lipoxygenase	ALOX15	34636_at	nc
Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	HS3ST1	41555_at	nc
Extracellular Matrix			
Transforming growth factor, beta 1 (Camurati-Engelmann disease)	TGFB1	1830_s_at	-1.6
Fibrinogen-like 2	FGL2	39593_at	-6.7
Selenoprotein P, plasma, 1	SEPP1	34363_at	nc
Chimerin (chimaerin) 2	CHN2	33244_at	nc
Fibronectin 1	FN1	31719_at	nc
DNA-binding Factors			
Growth arrest-specific 7	GAS7	33387_at	nc
Early growth response 2 (Krox-20 homolog, Drosophila)	EGR2	37863_at	nc
V-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	41504_s_at	nc

[a] Examined M2 genes are based on the transcriptional profiling work of Martinez *et al*. (47).