

# Transcriptome Analysis Reveals Human Cytomegalovirus Reprograms Monocyte Differentiation toward an M1 Macrophage

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Monocytes are primary targets for human CMV (HCMV) infection and are proposed to be responsible for hematogenous dissemination of the virus. Monocytes acquire different functional traits during polarization to the classical proinflammatory M1 macrophage or the alternative antiinflammatory M2 macrophage. We hypothesized that HCMV induced a proinflammatory M1 macrophage following infection to promote viral dissemination because, biologically, a proinflammatory state provides the 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 used Affymetrix Microarray to acquire a transcriptional profile of infected monocytes at a time point our data emphasized is a key temporal regulatory point following infection. We found that HCMV significantly up-regulated 583 (5.2%) of the total genes and down-regulated 621 (5.5%) of the total genes  $\geq 1.5$ -fold at 4 h postinfection. Further ontology analysis revealed that genes implicated in classical M1 macrophage activation were stimulated by HCMV infection. We found that 65% of genes strictly associated with M1 polarization were up-regulated, while only 4% of genes solely associated with M2 polarization were up-regulated. Analysis of the monocyte chemokine at the transcriptional level showed that 44% of M1 and 33% of M2 macrophage chemokines were up-regulated. Proteomic analysis using chemokine Ab 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 toward the classical M1 activation phenotype. *The Journal of Immunology*, 2008, 181: 698–711.

Infection by human CMV (HCMV),<sup>3</sup> 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).

Peripheral blood leukocytes, specifically monocytes, are thought to be responsible for hematogenous spread of HCMV (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 uses monocytes as a vehicle for spreading, infiltrating into, and persisting in host tissue (14–17). Our studies have demonstrated that primary infection of peripheral blood monocytes by HCMV induces a proinflammatory 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 (nonpermissive for viral replication) differentiate into long-lived macrophages (permissive for viral replication) (15). Although we have shown increased expression of multiple macrophage markers, including 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- $\gamma$  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<sup>high</sup>, IL-23<sup>high</sup>, IL-10<sup>low</sup> phenotype (24) and the production of toxic intermediates (reactive oxygen and nitrogen

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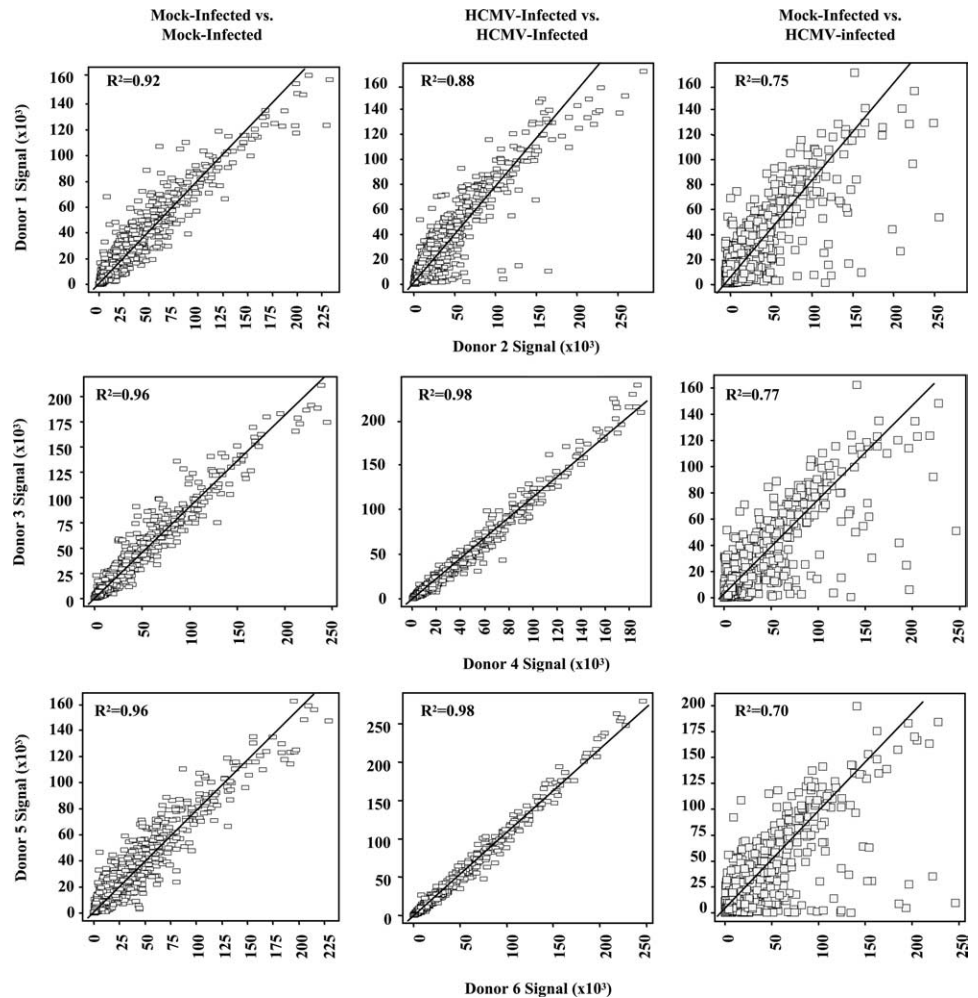
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<sup>3</sup> Abbreviations used in this paper: HCMV, human CMV; hpi, hours postinfection; IL-1Ra, IL-1 receptor antagonist; MMP, matrix metalloproteinase; MOI, multiplicity of infection.

**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 h. RNA was harvested and analyzed by Affymetrix gene array. Genes were compiled from six experimental samples and ANOVA tests were performed. Values of  $p$  were calculated and a  $p$  value cut-off of  $\leq 0.05$  was made. To be considered statistically significant, genes had to have a present call in at least four of six HCMV-infected samples and a 1.5-fold or greater change in at least four of six HCMV-infected 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).



intermediates) (22) and proinflammatory cytokines (IL-1 $\beta$  and TNF- $\alpha$ ) (25). M1 macrophages are potent effector cells efficient at eliminating pathogens and tumor cells. In contrast, alternatively activated macrophages exhibit an IL-12<sup>low</sup>, IL-23<sup>low</sup> phenotype (24), produce antiinflammatory molecules (IL-10 and IL-1 receptor antagonist (IL-1Ra)) (21, 26), and express high levels of scavenger, mannose, and galactose-type receptors (21, 23). M2 macrophages counteract inflammatory response and promote angiogenesis and tissue remodeling (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 IFN- $\gamma$ ) (28) but Ag-nonspecific 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- $\alpha$  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 toward an 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), *Escherichia 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 used 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 h postinfection (hpi), which our data has identified as a key temporal point when viral immediate-early proteins are not expressed (15) but when 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 up-regulated and 5.5% (621 genes) mRNAs were down-regulated. Transcriptional profile comparison analysis revealed a majority of genes strictly associated with the M1 phenotype were induced by HCMV, while most genes associated with the M2 phenotype exhibited no change or a

down-regulation. The up-regulation of monocytic genes implicated in M1 macrophage polarization suggest that HCMV modulates a rapid transition toward an M1 differentiation lineage, supporting our model for hematogenous dissemination of HCMV; that is, HCMV infection of peripheral blood monocytes forces cells to acquire an M1 proinflammatory 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 fibroblasts (33). Virus was purified on a 0.5 M sucrose cushion, resuspended in RPMI 1640 medium (Cellgro/Mediatech), and used to infect monocytes at a multiplicity of infection (MOI) of 15 for each experiment (14, 15, 33). Monocytes were diluted in RPMI 1640 medium to prevent homotypic aggregation of monocytes and rocked for 4 h at 37°C during infection; thus, a high MOI was used 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 0.1–20 in previous publications (15).

### Human peripheral blood monocyte isolation

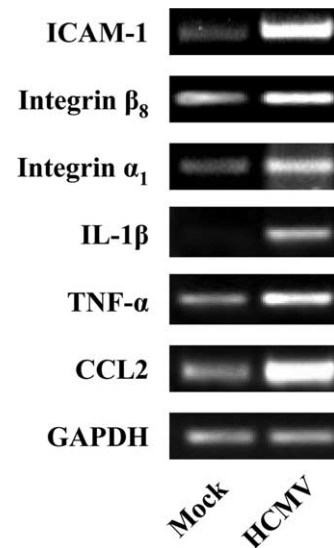
Blood was drawn by venipuncture and centrifuged through a Ficoll-Histopaque 1077 gradient (Sigma-Aldrich). Mononuclear cells were collected and washed with saline (14, 15, 33). Monocytes were then isolated by centrifugation through a Percoll (Pharmacia) gradient (41). More than 95% of isolated PBMC were monocytes as determined by CD14-positive staining (data not shown). Cells were washed and suspended in RPMI 1640 medium (Cellgro/Mediatech) supplemented with 10% human serum (Sigma-Aldrich). 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 was harvested 4 hpi with the RNA STAT-60 isolation kit (Tel-Test) according to the manufacturer's protocol. Affymetrix human genome U95Av2 arrays, which contain 12,626 characterized sequences, were used to examine the cellular changes in primary human monocytes from six 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). RNA was reverse-transcribed into cDNA and then transcribed in vitro to biotin-labeled 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 ANOVA tests were performed to calculate *p* values. Spotfire DecisionSite 8.1.1 was used to group genes by ontology, generate scatter plots, and calculate correlation coefficients. The GEO accession number for these data is GSE11408.

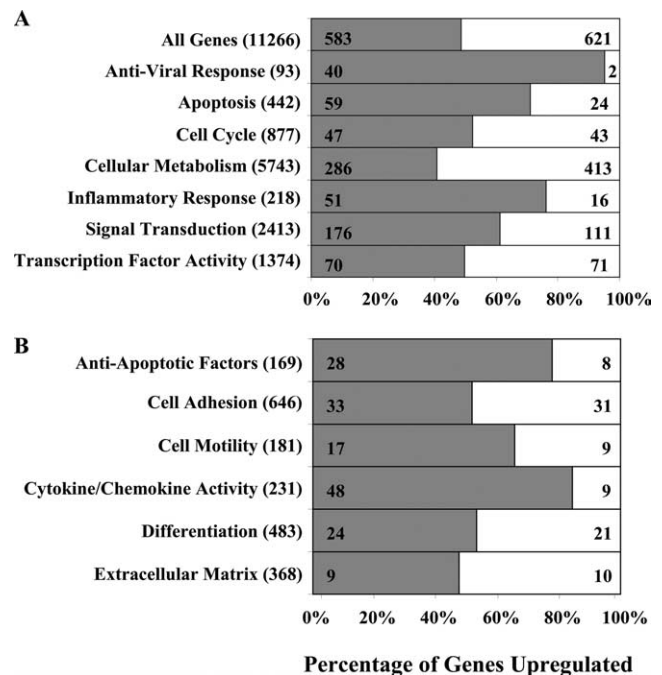
### RT-PCR analysis

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 was isolated using the RNA STAT-60 isolation kit. RNA (0.5 µg) from each sample was reverse-transcribed by mixing RNA with random hexamers (0.1 µg/µl; Invitrogen), 1 mM deoxynucleotide triphosphates (Amersham Biosciences), and double-distilled H<sub>2</sub>O and incubating at 65°C for 15 min. The RNA solution was then chilled for 1 min before 1 × RT-buffer (Invitrogen), DTT (Invitrogen), 80 U of RNasin (Promega), 400 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen), and double-distilled H<sub>2</sub>O were added. After 1 h of incubation at 37°C, 2 U of RNaseH (Stratagene) was added and the samples were incubated for an additional 30 min. PCR was performed using an iCycler (Bio-Rad model 1708720). Template DNA (2 µg) was mixed with 1 × iTAQ buffer (Bio-Rad), 15 mM MgCl<sub>2</sub> (Invitrogen), 50 µM deoxynucleoside triphosphates (Amersham Biosciences), 1.25 U of TAQ polymerase (Bio-Rad), double-distilled H<sub>2</sub>O, and 20 µM forward and reverse primers (Integrated DNA Technologies). ICAM-1, integrin β<sub>8</sub>, integrin α<sub>1</sub>, IL-1β, TNF-α,



**FIGURE 2.** Confirmation of selected chemokine and adhesion molecule gene expression by RT-PCR. Monocytes were mock infected or HCMV infected nonadherently for 4 h at 37°C and RNA was harvested. RT-PCR analysis confirmed that HCMV induced the expression of ICAM-1, integrin β<sub>8</sub>, integrin α<sub>1</sub>, IL-1β, TNF-α, and CCL2. GAPDH expression is shown as a control.

CCL2, and GAPDH samples were incubated for 5 min at 95°C and cycled 35 times for 1 min at 95°C, 58°C, and 72°C. Samples were then incubated for 7 min at 72°C before storage at 4°C. Controls including samples lacking reverse transcriptase in the reverse transcription reactions and template DNA in the PCR reactions were also performed (data not shown). The



**FIGURE 3.** HCMV alters the monocyte transcriptome at 4 hpi. Percentage of genes up-regulated (gray bars) and down-regulated (open bars)  $\geq 1.5$ -fold were grouped into (A) functional categories/ontologies or (B) ontologies involved in inflammation using Spotfire DecisionSite software based on the Gene Ontology Consortium database. Total genes up-regulated and down-regulated in each grouping are represented within the bar. The total numbers of genes analyzed in each ontology category are shown within parentheses. Percentages represent genes that were up-regulated following infection.

Table I. Selected statistically significant monocyte mRNAs that increase or decrease  $\geq 1.5$ -fold 4 hpi with HCMV

Category and Full Gene Name	Gene Title	Probe Set	Fold Change
<b>Antiapoptotic factors</b>			
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 70 kDa 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 70 kDa protein 9B (mortalin-2)	HSPA9B	41510_s_at	1.8
Notch homolog 2 ( <i>Drosophila</i> )	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
<b>Antiviral response</b>			
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, 91 kDa	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, 40/46 kDa	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, 20 kDa	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, 113 kDa	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, 48 kDa	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
<b>Cell adhesion</b>			
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
Laminin, beta 3	LAMB3	36929_at	2.9
Scavenger receptor class F, member 1	SCARF1	40034_r_at	2.7

(Table continues)

Table I. (Continued)

Category and Full Gene Name	Gene Title	Probe Set	Fold Change
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 60 kDa 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, 68 kDa	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, 41 kDa	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
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

(Table continues)

Table I. (Continued)

Category and Full Gene Name	Gene Title	Probe Set	Fold Change
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 ( <i>Drosophila</i> )	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
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

(Table continues)

Table I. (Continued)

Category and Full Gene Name	Gene Title	Probe Set	Fold Change
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
Agtrin	AGRIN	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, 68 kDa	TGFBI	1385_at	-10.0
Chondroitin sulfate proteoglycan 2 (versican)	CSPG2	31682_s_at	-14.4

following forward and reverse primers were designed with the support of Integrated DNA Technologies: ICAM-1, 5'-AAGCCAAGGAAGGAGCAAGACT-3' (forward) and 5'-TGAACCATGATGCACCACTGCAC-3' (reverse); integrin  $\beta_8$ , 5'-GCTGATTGATGCCACAGACTTT-3' (forward) and 5'-CAGGCAGACAAATGCAGCGTAAA-3' (reverse); integrin  $\alpha_1$ , 5'-ACGCTCAGTGGAGAACAGATTGGT-3' (forward) and 5'-AATTGTGCTGCCAGATGACCAGC-3' (reverse); IL-1 $\beta$ , 5'-AACAGGCTGCTGGGATTCCTT-3' (forward) and 5'-TGAAGGAAAG AAGGTGCTCAGGT-3' (reverse); TNF- $\alpha$ , 5'-ACCCTCAACCTCTCTG GCTCAA-3' (forward) and 5'-AGGCCTAAGGTCCACTTGTGCAA-3' (reverse); CCL2, 5'-TCGCTCAGCCAGATGCAATCAATG-3' (forward) and 5'-AGTTTGGGTTTGGCTTGCCAGGTG-3' (reverse). To confirm equal cDNA loading, GAPDH RNA was amplified with 5'-GAAGGTGAAGGTCCGAGTC-3' (forward) and 5'-GAAGATGGTGA TGGGATTC-3' (reverse) 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. RNA (2  $\mu$ g) from each sample was hybridized with a [ $\alpha^{32}$ P]UTP-labeled human cytokine multiprobe (hCK-2b) template set (BD Biosciences) for 12 h. RNase protection assays were performed with the radiolabeled RNA using the Multiprobe 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 Ab array analysis

Monocytes were isolated as described above and HCMV infected or mock infected nonadherently for 6 h at 37°C on an orbital shaker. Following incubation, cells were removed by centrifugation and the supernatant was collected and stored at -80°C until use in the protein microarray assay. Cell-free culture supernatants were assayed for 37 different chemokines by using a RayBio human chemokine Ab array (RayBiotech) according to the manufacturer's protocol. Data were 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-Aldrich), 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-Aldrich) and 6 ml Na<sub>2</sub>CO<sub>3</sub> (36.5 mM) to 1.8 ml AuHCl<sub>4</sub> (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 min at 37°C. Cells were then washed extensively with PBS to remove unbound virus and further incubated nonadherently for 6 h. Supernatants were collected following centrifugation to remove HCMV-infected monocytes. One milliliter of supernatants from mock-infected and HCMV-infected monocytes was added to colloidal gold-covered coverslips in 24-well plates. Next, 500 naive monocytes from the same donor were added to each well and incubated at 37°C. After 6 h of incubation, the cells were fixed in 1.5% paraformaldehyde for 15 min and mounted onto glass slides with glycerol. Track images of cells were captured using an inverted microscope at  $\times 200$  original magnification. Average area cleared per cell out of 20 cells per sample was determined by Scion imaging, and random motility was plotted as mean in arbitrary units (pixels cleared)  $\pm$  SEM. The results are representative of three 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 two of the six 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 vs mock-infected samples and *p* values were calculated for genes up-regulated or down-regulated 1.5-fold. A *p* value of  $\leq 0.05$  was used to generate a pool of genes that was statistically significant. Moreover, a fold change of 1.5 up or down in at least four of six HCMV-infected vs mock-infected samples was 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 six independent donors minimized the number of false-positives, thus allowing for a lower fold change cutoff threshold than for other microarray studies (42, 43).

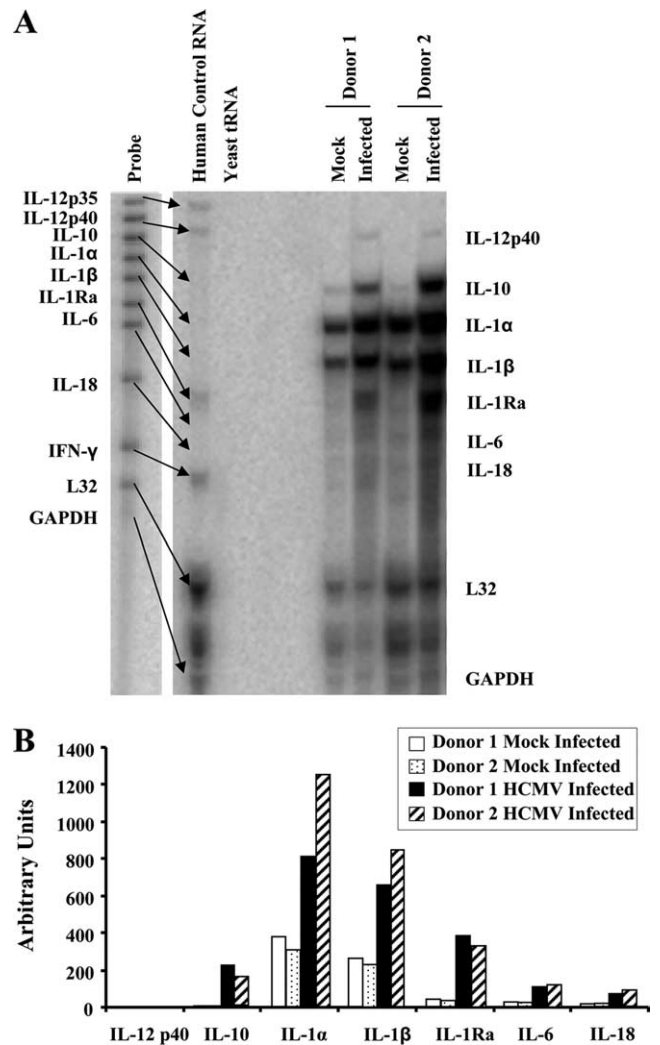
We then tested the validity and reproducibility of hybridization signals from independent preparations of isolated monocyte RNAs from six different donors. Scatter plot analysis on the basis of signal was performed on genes from six mock-infected and six HCMV-infected samples that had a present call in at least four of the six HCMV-infected samples (Fig. 1). Because of the use of primary cells from different donors, these selection criteria were

used to account for potential differences in gene expression between donors. Genes from three representative pairs of mock-infected vs mock-infected, HCMV-infected vs HCMV-infected, and mock-infected vs HCMV-infected samples were plotted on the basis of signal and the coefficients of determination ascertained (all possible pairwise analyses were done with similar results obtained (data not shown)). We found coefficients of determination to be  $\geq 0.88$  between multiple sample comparisons. Conversely, pairwise comparisons of mock-infected and HCMV-infected samples resulted in a lower coefficient of determination ( $\sim 0.74$ ), 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 up-regulated and 621 (5.5%) genes were down-regulated  $\geq 1.5$ -fold in at least four of the six replicates (see supplemental data S1 for the complete list of altered transcripts).<sup>4</sup> RT-PCR confirmed that HCMV stimulated ICAM-1, integrin  $\beta_8$ , integrin  $\alpha_1$ , IL-1 $\beta$ , TNF- $\alpha$ , and CCL2 mRNA expression by 4 hpi (Fig. 2). Of the 1204 genes modulated in the monocyte transcriptome following infection, 48.4% were up-regulated and 51.6% were down-regulated (Fig. 3A). To identify the trends in total cellular gene expression, those genes that were up-regulated and down-regulated  $\geq 1.5$ -fold following HCMV infection were grouped by their behavioral patterns. Analysis of specific ontology groups in HCMV-infected monocytes showed altered levels of transcripts involved in the antiviral response (45% of the total antiviral 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 antiviral (45%) and inflammatory responses (31%) to be more substantially modulated. Moreover, within these two ontology clusters, genes were disproportionately up-regulated (95% and 77% of genes involved in the antiviral and inflammatory responses, respectively). Because we have previously shown that HCMV induces a proinflammatory monocyte to promote the required functional changes in the infected cells necessary for hematogenous 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 antiapoptotic function (44–46); however, a 3–4-wk delay in viral gene expression in HCMV-infected monocytes (15) suggests that HCMV regulates monocyte apoptosis via cellular antiapoptotic factors. Indeed, detailed examination of transcripts encoding for antiapoptotic factors revealed that 16% were up-regulated and only 4% were down-regulated at 4 hpi. These data are consistent with previous findings showing that a number of antiapoptotic-related genes such as BCL2A1 (4.9-fold increase) and TNFRSF6 (3.1-fold increase) are specifically up-regulated in M1-polarized macrophages (47). Moreover, NF- $\kappa$ B, a transcription



**FIGURE 4.** HCMV infection induces cytokine expression. Monocytes were mock infected or HCMV infected nonadherently for 4 h at 37°C and RNA was harvested. *A*, RNA from multiple donors was hybridized with a [ $\alpha^{32}$ P]UTP-labeled hCM-8 template set and subjected to RNase protection assay using the Multiprobe 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 were determined by densitometry using Quantity One software, normalized to the respective L32 band intensities, and plotted in arbitrary units. Results are representative of three independent experiments from three donors.

factor responsible for the expression of a number of antiapoptotic and inflammatory genes (48, 49), is up-regulated 3.3-fold. These findings suggest that, even in the absence of de novo viral gene expression, HCMV can create a cellular environment skewed toward the inhibition of apoptosis that likely favors 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 are 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,

<sup>4</sup> The online version of this article contains supplemental material.



Table II. Comparison of M1 genes expressed in HCMV-infected monocytes/macrophages<sup>a</sup>

Category and Full Gene Name	Gene Title	Probe Set	Fold Change
<b>Membrane receptors</b>			
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
<b>Cytokine and chemokines</b>			
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
<b>Apoptosis-related genes</b>			
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
<b>Solute carriers</b>			
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
<b>Enzymes</b>			
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, 69/71 kDa	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
<b>Extracellular matrix</b>			
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
<b>DNA-binding factors</b>			
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

<sup>a</sup> Examined M1 genes are based on the transcriptional profiling work of Martinez et al. (47). nc, No change.

ICAMs, and integrins are critical mediators of monocyte adhesion to endothelial cells before extravasation (52). M1-activated monocytes express significantly higher levels of these receptors; therefore, monocytes with a proinflammatory 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 up-regulated 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, as well as with a number of other studies that have demonstrated strong up-regulation of surface ICAM-1 expression on endothelial and epithelial

cells following infection (55, 56). Additionally, the microarray data indicate that the mRNA of a number of additional cell adhesion molecules such as ninjurin 1, laminin  $\beta_3$ , and integrins  $\alpha_1$ ,  $\alpha_6$ , and  $\beta_8$  were elevated. Conversely,  $\alpha_5$ ,  $\alpha_M$ , and  $\beta_2$  integrins were down-regulated following infection, indicating that HCMV can selectively alter integrin message expression. The specific up-regulation and down-regulation of integrins by HCMV have 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 that 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

Table III. Comparison of M2 genes expressed in HCMV-infected monocytes/macrophages<sup>a</sup>

Category and Full Gene Name	Gene Title	Probe Set	Fold Change
<b>Membrane receptors</b>			
Chemokine (C-X-C motif) receptor 4	CXCR4	649_s_at	-2.5
Transforming growth factor, beta receptor II (70/80 kDa)	TGFB2	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
<b>Cytokine and chemokines</b>			
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
<b>Solute carriers</b>			
Solute carrier family 4, sodium bicarbonate cotransporter, member 7	SLC4A7	34936_at	-1.5
Solute carrier family 38, member 6	SLC38A6	36758_at	nc
<b>Enzymes</b>			
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
<b>Extracellular matrix</b>			
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
<b>DNA-binding factors</b>			
Growth arrest-specific 7	GAS7	33387_at	nc
Early growth response 2 (Krox-20 homolog, <i>Drosophila</i> )	EGR2	37863_at	nc
V-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	MAF	41504_s_at	nc

<sup>a</sup> Examined M2 genes are based on the transcriptional profiling work of Martinez et al. (47). nc, No change.

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 up-regulated the expression of both MMP1 and MMP10 ~12-fold. MMP1 dissolves collagen types I, II, and III, (59), and MMP10 breaks down proteoglycans and fibronectin (60). HCMV may up-regulate the expression of these proteinases in monocytes to promote migration through the extracellular matrix en route to the tissue.

**Cytokine activity.** A classical feature of an M1 proinflammatory phenotype is the secretion of a milieu of cytokines and chemokines that 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 with others, proinflammatory cytokines IL-1 $\beta$ , IL-6, IL-12p40, IL-15, inhibin  $\beta_A$ , and TNF- $\alpha$ , which are associated with the classical activation M1 phenotype, were up-regulated following infection (14, 21, 25, 33, 61). HCMV potently induced IL-6, TNF- $\alpha$ , and inhibin  $\beta_A$  mRNA, reaching a 280-, 14.6- and 9.9-fold increase. HCMV infection

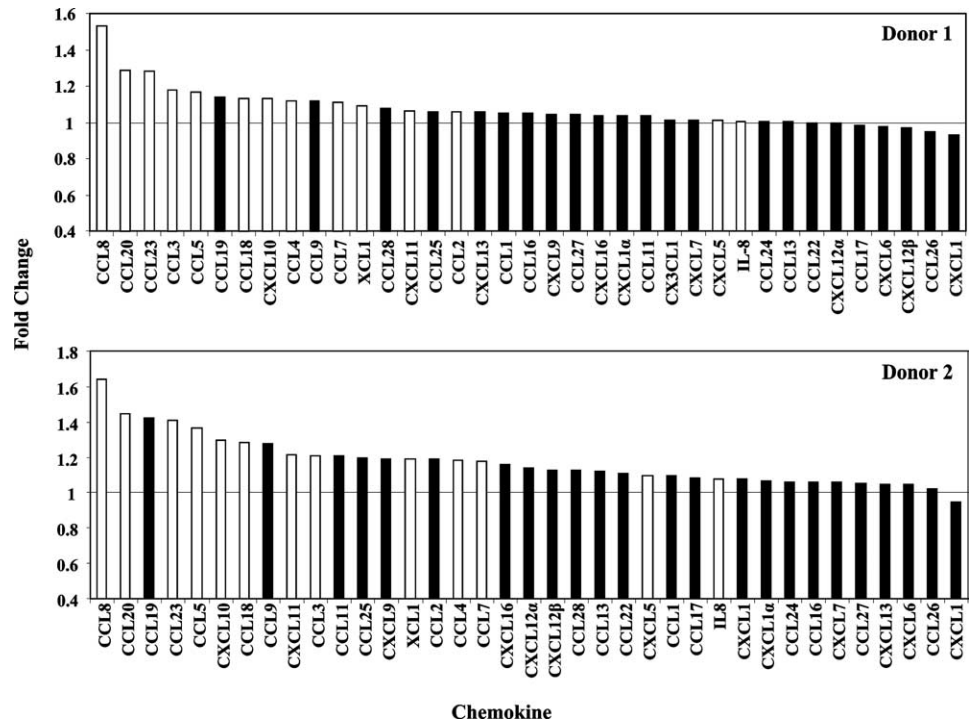
also led to a down-regulation of certain cytokine genes such as vascular endothelial growth factor, which can act as a chemoattractant for T cells, and TNF ligand superfamily member 8 (CD30), which can stimulate T cell proliferation. Interestingly, M2 polarization-associated anti-inflammatory cytokines IL-1Ra and IL-10 were both up-regulated 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 up-regulation of cytokine genes implicated in the M1 phenotype (IL-12B, IL-1 $\beta$ , and IL-6) and the M2 phenotype (IL-10, IL-1Ra, and IL-18) (Fig. 4). 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 toward an M1 macrophage phenotype*

The rapid production of numerous cytokines is characteristic of a classical M1 monocyte/macrophage activation/differentiation

**FIGURE 5.** Differential secretion of chemokines by monocytes following infection with HCMV. Monocytes were mock infected or HCMV infected nonadherently for 6 h at 37°C and supernatant was collected. Cell-free culture supernatants from multiple donors were assayed for 37 different chemokines by using a Ray-Bio human chemokine Ab array according to the manufacturer's protocol. Densitometry of the chemokine Ab array was performed with Quantity One software and fold change plotted. Open bars represent chemokines that were identified by transcriptome profiling analysis to be up-regulated following infection with HCMV. Filled bars represent chemokines not transcriptionally up-regulated at 4 hpi. Results are representative of four independent experiments from three donors (analysis on one donor was repeated).

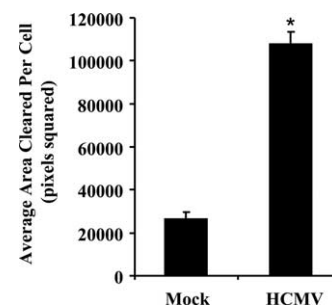


lineage, and our transcriptome analysis indicates that HCMV-infected monocytes acquire a proinflammatory M1 phenotype. However, the up-regulation of transcripts (IL-1Ra and IL-10) implicated in the alternative M2 differentiation lineage suggests a complex and atypical M1/M2 reprogramming of monocytes 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- $\gamma$  (M1 phenotype) and IL-4 (M2 phenotype) as described by Martinez et al. (47). Tables II and III list 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 up-regulated, 14 (30%) genes showed no change, and 2 (5%) genes were down-regulated 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%). In contrast, of the genes associated with the antiinflammatory M2 phenotype, only 2 (4%) were up-regulated, 37 (84%) displayed no change, and 6 (14%) were down-regulated. Taken together, these data show a clear transcriptional bias toward an M1 macrophage activation phenotype following infection of monocytes with HCMV, although several transcripts implicated in antiinflammatory responses typical of alternatively activated macrophages (such as IL-1Ra, IL-10, CCL18, and CCL23) were also up-regulated.

#### *HCMV induces an atypical M1/M2 macrophage chemokine*

Distinct patterns of chemokines are produced depending on whether a macrophage undergoes an M1 or M2 polarization (47). We found that 16 (44%) chemokine genes were up-regulated in monocytes within 4 hpi, while none was down-regulated (Table I). Differential analysis showed that 44% and 33% of M1- and M2-associated chemokines to be up-regulated, respectively. To confirm that the up-regulation of chemokine genes at the level of tran-

scription conferred increased protein production, secretion of chemokines into the supernatant by monocytes 6 hpi was assayed using human chemokine Ab 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). Conversely, 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



**FIGURE 6.** Supernatant harvested from HCMV-infected monocytes stimulate naive monocyte motility. Monocytes were mock infected or HCMV infected and incubated for 45 min. Cells were washed extensively to remove unbound virus and incubated nonadherently for 6 h. Supernatants were harvested from each of the samples following centrifugation and 1 ml was added to colloidal gold-covered coverslips. After 500 naive monocytes were added to each coverslip and at 6 h postaddition of naive monocytes, the cells were fixed with 1.5% paraformaldehyde. The average area (arbitrary units<sup>2</sup>) 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  $\pm$  SEM of 20 cells per experimental arm. The results represent three independent experiments with separate human blood donors. \*,  $p < 0.01$ .

trafficking. We next examined whether the secreted chemokines from the infected monocyte samples were functional and could induce motility in naive 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 up-regulation of chemoattractants promotes viral spread by aiding infected monocyte migration to peripheral tissue and/or naive 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 up-regulated and 621 mRNAs were significantly down-regulated. Ontology analysis revealed that most of the induced genes are associated with classically activated monocytes/macrophages. Generally, inflammation is used by the infected host to help clear infection via the release of antiviral regulatory factors such as IL-1 $\beta$  and TNF- $\alpha$  (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<sub>2</sub>, IL-1 $\beta$ , and TNF- $\alpha$ , 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-1 $\beta$ , IL-6, and TNF- $\alpha$  can also contribute to the shaping of the classically activated or proinflammatory macrophage phenotype, which is important for viral replication (15, 66, 67) and, as our present 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 proinflammatory modulatory effects on monocyte function (33) suggest the involvement of receptor-ligand interactions. Engagement and activation of cellular surface receptors such as TLR2, epidermal growth factor, and integrins by HCMV (68) followed by the subsequent stimulation of cellular NF- $\kappa$ B and PI3K activity (14, 16, 33, 69) are likely the major mechanisms for the rapid monocyte gene regulation following infection, although we cannot 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 proinflammatory 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 toward the classical activation, a number of genes distinctly associated with alternatively activated monocytes were also up-regulated. The antiinflammatory cytokines IL-1Ra and IL-10 were both up-regulated 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 EBV encode viral homologs to IL-10 (71, 72), which can activate signaling pathways that dampen IFN- $\gamma$ -triggered microbicidal pathways, inhibit Ag processing and presentation by APCs, and inhibit T cell cytokine production and cytotoxic activity (73). Because the HCMV IL-10 homolog is not synthesized in this early timeframe in monocytes, we propose that HCMV uses host cytokines instead to inhibit T cell function. Overall, our data hint 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 to balance viral spread with immune evasion.

In agreement with this idea, examination of the HCMV-infected monocyte chemokine signature showed that the up-regulation of chemokines implicated both the classically and alternatively activated phenotype, although again a bias toward the M1 phenotype was observed. Thus, our data indicate 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 naive 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 thought to be one of the most powerful inducers of monocyte migration into atherosclerotic lesions (74).

Overall, we show that HCMV infection rapidly induces a proinflammatory 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 that proinflammatory gene expression resulting in chronically activated HIV-infected monocytes/macrophages occurs *in vivo* (75–77). Similar to HCMV-infected monocytes, release of chemotactic cytokines such as MCP-1 from HIV-infected cells enhances dissemination by stimulating recruitment of target CD4 T cells and monocytes/macrophages to sites of infection (78). Furthermore, HIV appears to induce antiapoptotic 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-1 $\beta$ , IL-6, and TNF- $\alpha$  were significantly up-regulated (presented herein), whereas in HIV-infected monocytes these genes remained unchanged (75). IL-1 $\beta$  and TNF- $\alpha$  have strong antiviral activity, and thus down-regulation by HIV is likely a strategy for immune evasion; however, as mentioned above, HCMV has devised a strategy using the NF- $\kappa$ B-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 up-regulated in HIV (82) but not in HCMV-infected monocytes. These differences are but a few examples used 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 are consistent with our previously proposed model of HCMV dissemination, where HCMV infection classically activates monocytes to promote migration into host organ tissue and differentiation into replication permissive macrophages (14–16). Our data also confirm morphological studies suggesting that HCMV infection does not induce all of 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 toward the M1 polarization that also incorporated selected attributes of the alternatively activated M2 phenotype. Presumably the up-regulation of specific genes associated with the antiinflammatory 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.

## Disclosures

The authors have no financial conflicts of interest.

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