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Human Cytomegalovirus Binding to Human Monocytes Induces Immunoregulatory Gene Expression

Andrew D. Yurochko* and Eng-Shang Huang*†‡¶

To continue our investigation of the cellular events that occur following human CMV (HCMV) infection, we focused on the regulation of cellular activation following viral binding to human monocytes. First, we showed that viral binding induced a number of immunoregulatory genes (IL-1β, A20, NF-κB-p105/p50, and IκBα) in unactivated monocytes and that neutralizing Abs to the major HCMV glycoproteins, gB (UL55) and gH (UL75), inhibited the induction of these genes. Next, we demonstrated that these viral ligands directly up-regulated monocyte gene expression upon their binding to their appropriate cellular receptors. We then investigated if HCMV binding also resulted in the translation and secretion of cytokines. Our results showed that HCMV binding to monocytes resulted in the production and release of IL-1β protein. Because these induced gene products have NF-κB sites in their promoter regions, we next examined whether there was an up-regulation of nuclear NF-κB levels. These experiments showed that, in fact, NF-κB was translocated to the nucleus following viral binding or purified viral ligand binding. Changes in IκBα levels correlated with the changes in NF-κB translocation. Lastly, we demonstrated that p38 kinase activity played a central role in IL-1β production and that it was rapidly up-regulated following infection. These results support our hypothesis that HCMV initiates a signal transduction pathway that leads to monocyte activation and pinpoints a potential mechanism whereby HCMV infection of monocytes can result in profound pathogenesis, especially in chronic inflammatory-type conditions.


Monocytes are vital effector cells responsible for protecting the body from infections and tumors through their release of soluble factors or cytokines, direct action toward invaders or aberrant cells, and interaction with T lymphocytes (1). However, in many disease states these cells are dysregulated or are the target of invading pathogens, resulting in an aberrant immune response (2–5). Of particular interest to our laboratory is the common theme that viruses often target these cells during viral infection. HIV and human CMV (HCMV) are two examples of viruses that target these cells. During HCMV infection, for instance, monocytes are one of the primary targets in vivo (4, 6–11). Understanding the results of this infection is important because 1) monocytes appear to be a site of latency or persistent infection for HCMV (7, 8, 11–13); 2) they are responsible for the dissemination of the virus throughout the body (4, 7, 10, 12); and 3) their infection has been hypothesized to be important in the manifestation of many HCMV-associated diseases (2, 4, 6, 14–16). Previously, it has been shown that HCMV infection of adherent monocytes caused a sustained expression of IL-1β message and intracellular protein levels (6). In addition, it has been shown using monocytes or monocytic cell lines that HCMV infection or its immediate-early (IE) gene products can stimulate the expression of various monocyte-mediator gene products and transactivate the promoters of these genes (6, 16–22). These results taken together suggest that HCMV infection of monocytes could be associated with the immune dysregulation seen in HCMV-infected individuals, whether it be immunosuppression (4, 23–25) or hyperactivation of the immune system (2, 4, 6, 16, 19, 24).

Previously, we hypothesized that the hallmark of HCMV infection—the induction and dysregulation of the tightly regulated cellular transcription factor system (4, 26–32)—was responsible for the wide range of clinical symptoms and viral pathogenesis observed in HCMV-infected individuals (4, 14, 32). We and others have demonstrated that infection results in the rapid mobilization of NF-κB to the nucleus and increases the expression of the two NF-κB subunits, p105/p50 and p65, in human fibroblasts (27, 29, 32, 33) and that these changes were mediated initially by the binding of the major HCMV envelope glycoproteins, gB (UL55) and gH (UL75), to their cognate cellular receptors (32). This initial cellular activation occurred in a protein synthesis-independent manner (27, 29, 32). In addition, we showed that beginning at early times after infection the promoters for p105/p50 and p65 were transactivated in a protein synthesis-dependent manner and that the major viral IE gene products along with the induced cellular factors (such as Sp1) played a role in this transactivation (26, 27).

The importance of the induction of these host cell factors for the virus stems from their critical role during infection in the regulation of the major IE promoter (MIEP), the regulation of the IE genes, as well as the regulation of viral cellular genes, and, consequently, the entire gene cascade (26, 27, 30, 34, 35). Specifically, NF-κB binds to and is essential for the transactivation of the MIEP (30, 35). The essential role for NF-κB in the viral life cycle is
further underscored by the fact that, to date, no other documented stimulus induces the normally constitutively expressed p65 gene product (27, 33).

In addition to the up-regulation of the viral life cycle, these documented viral-mediated changes in cellular factors would have profound effects on the infected host, because the induced transcription factors would up-regulate a wide array of important cellular genes (not only the ones needed by the virus). This might be particularly important during infection of nonpermissive cells, such as monocytes where all cells can be infected (9), but where viral gene expression is usually limited to only the IE genes (10, 12). Thus, the importance of understanding the up-regulation of these transcription factors and their ultimate downstream targets not only would reveal the overall regulation of the viral life cycle, but also their potential role in viral pathogenicity. As mentioned, we hypothesized that the induction of host cell factors may be central to disease progression in HCMV-infected individuals, and that this would be especially true of immune cells. Therefore, in our next line of study we wanted to determine the effect of viral infection on monocytes because of the physiological role infection of these cells plays in HCMV-associated diseases (4, 6, 14). In particular, we wanted to address the regulation of monocyte activation following viral binding. Thus, we chose to examine the possibility that viral binding to these cells through the major HCMV glycoproteins, gB and gH, could up-regulate the activity of these cells, a scenario that could be envisioned to occur in vivo during cases of active HCMV viremia or perhaps in a localized environment in a persistently infected individual. This possibility is supported by the studies that show that the IE events that take place following HCMV infection including Ca2+ flux, phospholipid turnover, induction of second messengers, up-regulation of transcription factors, and induction of the protooncogenes are similar to the events that occur during known receptor/ligand interactions (27–29, 32, 36–38).

Here we show that HCMV binding up-regulates the steady-state message levels of a number of important monocyte mediator genes (IL-1β, A20, NF-xB-p105/p50, and IκBo). In addition, we show that viral binding activates the monocyte because neutralizing Abs block viral-mediated message induction, and, more importantly, purified viral glycoprotein ligands (gB and gH) also increase monocyte mediator mRNA. We also show that the increase in cytokine message expression correlates with an increase in cytokine secretion. We went on to investigate the signaling cascade and demonstrated that this transcriptional up-regulation is probably due to an increase in cellular transcription factors as viral binding also induces the nuclear mobilization of NF-xB, which has been reported to up-regulate many of these induced monocyte-mediator genes (39). Lastly, we showed that p38, a mitogen-activated protein kinase (MAPK) family member (40), is activated by viral genes (39) and is reported to up-regulate many of these induced monocyte-mediator genes (27). Moreover, double density gradient centrifugation as described previously (27). Monocytes were further purified from the contaminating lymphocytes by centrifugation through a Percoll (Pharmacia, Uppsala, Sweden) gradient. The cells were then counted and treated as described in the experimental results section.

HCMV infection

Methods for the culturing of our HCMV Towne strain (passage 43–44 in human fibroblasts have been described (27)). Monocytes were only gradient-purified virus at a multiplicity of infection (M.O.I.) of 2–3 and incubated at 37°C in 1% human serum in endotoxin-free RPMI 1640 in 6% CO2 incubators for the lengths of time described. HCMV was gradient purified and used to infect cells as previously described (32). UV-inactivated virus was prepared as previously described (31, 32) and was used in the same manner as “live” virus. The UV-inactivated virus did not replicate or produce any detectable levels of IE gene products (data not shown). For the studies in which inhibitors of the various kinases were used, monocytes were pretreated for 1 h before the addition of virus. The inhibitors were used SB203580 (Calbiochem, San Diego, CA), which has been previously shown to inhibit p38 activation (40) and to block HCMV (41) and HIV (43) replication, and PD98059 (Calbiochem), which has been previously shown to inhibit MAP kinase kinase (MEK) activity (44).

Abs and cellular activation

We used the same protocol for the use of the various Abs or purified ligands in this manuscript as we previously described (32). For the various experiments involving neutralizing Abs, virus (gradient-purified “live” or UV-irradiated virus) was preincubated with a protein G affinity-purified murine monoclonal anti-gB (4 μg of 15D8 (45) of the IgG2a isotype) and anti-gH Ab (4 μg of IgG4 (46) of the IgG1 isotype) or a protein G affinity-purified murine monoclonal control Ab (4 μg of an anti-human prostate-specific Ag (PSA) Ab (anti-PSA or αPSA of the IgG2a isotype) for 1 h at 4°C. The 15D8 and 1G6 Abs were a generous gift from Dr. L. Rasmussen (Division of Infectious Diseases, Stanford School of Medicine, Stanford, CA). For neutralization with the 1G6 monoclonal Ab, 2% guinea pig complement (Accurate Scientific, Westbury, NY) was also added. Complement alone had no effect on monocytes (data not shown). The neutralizing Abs used in the experiments prevented viral-mediated cytopathic effects and viral gene expression in fibroblasts as confirmed by side-by-side experiments in which these variables were assessed (data not shown). In addition, purified viral gB (1, 20, and 100 ng/ml) or purified viral gH block viral infection with Abs (4 μg of 15D8 or the control Ab) were used in some studies to stimulate cells. In other studies, 15 μg of the 4-3-5 affinity-purified murine anti-idiotypic (αδδ) IgM Ab (47, 48), which mimics the HCMV viral gH glycoprotein and is thus specific for the 92.5-kDa cellular gH receptor (49), or 15 μg of an IgM control Ab (affinity-purified mouse IgM (Southern Biotechnology Associates, Birmingham, AL)) plus 3 μg of an appropriate cross-linking Ab (goat anti-mouse IgM Ab (Southern Biotechnology Associates)) was used to stimulate cells as previously described (32, 38, 50). The 4-3-5 Ab and the appropriate control anti-serum were a generous gift from Dr. S. Key (Research Services, The Department of Veteran Affairs Medical Center, University of Maryland School of Medicine, Baltimore, MD). In all cases of cellular stimulation (purified virus, purified gB, αδδ Ab, or the various control products), the cells were treated the same, washed similarly, incubated in an equal volume of product and media, incubated for the same length of time, and harvested in the same manner) to allow for comparisons between groups. In all cases, samples labeled “Mock” were treated in a similar manner to the test samples (except no virus, purified viral ligands, etc., were added) and they were incubated for the length of the stated experiment. In addition, in some experiments monocytes were treated with 10 ng/ml of PMA or 1 μg/ml LPS as a positive control (42, 51). All experiments were repeated.

RNA isolation and Northern blot analysis

Total cellular RNA was collected in guanidinium isothiocyanate and isolated by cesium chloride centrifugation (42). Then, 5 μg of total cellular RNA was electrophoresed on a 1% denaturing formaldehyde agarose gel and transferred overnight to nitrocellulose (Immobiion-NC; Millipore, Bedford, MA). Expression of specific messages were detected by Northern blot hybridization. RNA levels were equivalent based on 18S and 28S rRNA levels. All data presented in this manuscript are from multiple probes of the same blot; however, all experiments were repeated two to five times.
times. Nitrocellulose blots were probed with nick-translated (Boehringer Mannheim, Indianapolis, IN) 32P-labeled cDNA sequences specific for p105/p50 (52), p65 (53), IxBα (54), IL-1β (42), and A20 (42). The blots were hybridized overnight in a 50% formamide solution at 42°C. The blots were then washed to a stringency of 0.2× SSC at 56°C and developed for 1–6 days with intensifier screens at ~70°C.

ELISA

Monocyte supernatant was harvested following the treatment of cells as stated in the results section. The supernatant was centrifuged three times to remove any cellular debris and then frozen at ~70°C until needed. The ELISA was then performed using the manufacturers protocol (Endogen, Cambridge, MA). The samples were run in duplicate along with the appropriate IL-1β standards. Experiments were repeated and the data shown is from representative experiments.

Cytoplasmic and nuclear extract isolation

Isolation of nuclear extracts have been described elsewhere (27, 33). Briefly, collected cell pellets were incubated for 4 min on ice with a cytoplasmic isolation buffer (10 mM Hepes, pH 7.6, 60 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT, 1 mM PMSF (Sigma), 2 mM phenanthroline (Sigma), 250 μM dHCO (Sigma), 100 μM E-64 (Sigma), and 10 μM pepstatin A (Sigma)). After collection by centrifugation, the nuclear pellets (the cytoplasmic extracts were clarified by centrifugation and stored at ~70°C) were washed in a cytoplasmic isolation buffer without Nonidet P-40, spun, and then incubated for 10 min on ice with a nuclear isolation buffer (20 mM Tris-HCl, pH 8.0, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM PMSF (Sigma), 25% glycerol, 2 mM Phenanthroline (Sigma), 250 μM dHCO (Sigma), 100 μM E-64 (Sigma), and 10 μM pepstatin A (Sigma)). Supernatants containing the nuclear extracts were collected and stored at ~70°C.

Electrophoretic mobility shift assay (EMSA)

Collected nuclear extracts were incubated for 15 min in an EMSA buffer containing: a binding buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, and 1 mM DTT); 7.5 mM MgCl2; 0.1 μM polydeoxyinosinic-deoxycytidylic acid (dIdC); and a 32P-labeled double-stranded oligonucleotide probe containing a wild-type MHC class I κ binding site (5'-CTTTTTTTTTTGAGATTCCCCA-3') or a wild-type activation transcription factor (ATF)/cAMP-response-element-binding protein (CREB) binding site (5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3') for experiments examining NF-κB activity and ATF/CREB activity, respectively. The annealed double stranded oligo probes with "T" overhangs and "C" ends were labeled by filling in the recessed 3' ends of the oligo with [α-32P]dATP (ICN, Irvine, California) using Klenow (Boehringer Mannheim, followed by a chase with cold dATP and dGTP, and then finally G-25 Sephadex (Boehringer Mannheim) column purified. The samples were electrophoresed on a 5% polyacrylamide gel, dried, and dehydrated with intensifier screens at ~70°C. Abs were used to supershift the specific complexes of interest by pretreating the extracts for 15 min to 1 h at 4°C with 1 μg of Ab before their addition to the binding buffer, MgCl2, dIdC, and labeled probes. Specific Ab to p50 and p65, as well as preimmune serum, and their appropriate blocking peptides (a generous gift from Dr. A. S. Baldwin, Jr., Lineberger Cancer Center, University of North Carolina, Chapel Hill, NC) were used in the supershift experiments. Preimmune serum and blocking peptides were used to show the specificity of the super shifts (data not shown).

Western blot analysis

For the IxBα and β Western blot analyses, cytoplasmic proteins were used and were harvested as stated above. For the p38 Western blot analyses, whole cell extract was used. The various harvested proteins were collected and added to an SDS-PAGE sample buffer, boiled, and then stored at ~70°C. Equal protein amounts were added to each lane. The samples were run on a 12% SDS-PAGE gel and transferred to nitrocellulose (Immobilon-P, Millipore) overnight. The blots were blocked for 1 h in a 5% skim milk, 0.1% Tween 20, PBS solution for 1 h, and then incubated with the primary Ab (1:2000 dilution of the anti-IxBα and anti-1xBβ Abs; Santa Cruz Biotechnology, Santa Cruz, CA; or 1:1000 dilution of the phospho-specific anti-p38 MAPK Ab; New England Biolabs, Beverly, MA) for 1 h in the same blocking solution. The blots were washed, incubated for 1 h with a 1:2500 dilution of goat anti-rabbit-horseradish peroxidase-conjugated secondary Ab in the same blocking solution, washed again, and finally incubated with the developing agents and developed as per the enhanced chemiluminescence protocol (Amersham Life Sciences, Arlington Heights, IL or New England Biolabs).

FIGURE 1. HCMV infection results in the induction of monocyte mediator genes. A Northern blot analysis was performed on total RNA harvested from monocytes treated in a variety of ways. The experiment was repeated and a representative experiment is shown. All the blots shown are from multiple proberings of the same blot. Monocytes were treated for 4 h in the following manner before harvesting: 1) NAD monocytes were untreated or mock infected; 2) and 3) NAD monocytes were treated with 10 ng/ml of PMA or 1 μg/ml of LPS; 4) NAD monocytes were incubated with gradient-purified HCMV (M.O.I. = 2–3); 5) monocytes were adhered to tissue culture plastic (“ADH”) in the absence of virus or other stimuli; or 6) ADH monocytes were incubated with gradient-purified HCMV (M.O.I. = 2–3). The membranes were probed with 32P-labeled cDNA probes specific for IL-1β, A20, p105/p50, p65, and IxBα. 28S ribosome levels are shown as a control.

Results

HCMV infection and virus binding up-regulate monocyte gene expression

To examine the role of HCMV binding and infection in the activation of monocytes, we initially determined whether there was an up-regulation of monocyte mediators following infection of monocytes with HCMV. As seen in Fig. 1, isolated nonadherent (NAD) monocytes showed a dramatic increase in the steady-state mRNA expression of various monocyte effectors (IL-1β, A20, p105/p50, and IxBα) following a 4-h viral infection (with gradient-purified virus) or incubation with known activating agents (10 ng/ml of PMA or 1 μg/ml of LPS) as compared with the unstimulated control (“NAD”). Expression of p65, an NF-κB subunit, appeared to change only slightly during the treatments shown in Fig. 1. This was the only gene product tested that showed donor variability; some donors showed no effect and some donors showed a small change in expression. This in itself is not surprising, as p65 message expression is not induced by any known stimulus except HCMV infection and usually only occurs at later times postinfection (27). In addition, we also examined if monocyte adhesion (a known activating stimuli (37, 51), a process that occurs during extravasation of monocytes from the blood to an inflammatory site (37, 51, 55, 56), and a process that has hypothesized to be affected...
by HCMV infection (57–59) was affected by HCMV infection. As seen in Fig. 1, monocyte adhesion (“ADH”) caused an increase in message expression and HCMV infection further enhanced the signal (“ADH+HCMV”). Furthermore, it was shown that viral infection can up-regulate at least IL-1β expression as early as 30 min to 1 h postinfection (data not shown). Interestingly, this time frame is similar to the induction of IL-1β and other monocyte regulatory products following adhesion to extracellular matrix components or treatment of monocytes with Abs to various adhesion receptors (42, 60, 61).

**Neutralizing Abs to the major HCMV viral glycoproteins prevent monocyte gene induction**

Next, we wanted to narrow down the nature of the viral-mediated induction of monocyte gene products. Previously, we showed that a receptor/ligand-mediated process was responsible for transcription factor induction in human fibroblasts following viral binding (32). Therefore, we decided to test if this type of process—a receptor/ligand-mediated event—was responsible for the activation of monocytes as measured by an increase in mRNA expression. To demonstrate that viral binding mediated cellular activation, we examined the role of the two major HCMV glycoproteins, gB and gH, by preincubating the gradient-purified virus with neutralizing monoclonal Abs (4 μg of anti-gB [15D8 (45)] and anti-gH [1G6 (46)], labeled “v-gBgH”) or an appropriate control Ab (4 μg of anti-PSA, labeled “r-PSA”, isotype and species matched (32)). The effect of UV-irradiated HCMV is also shown (“UV-HCMV”). The lane labeled only “NAD” is the untreated or mock-infected control. A representative experiment is shown and the experiment was repeated. All the blots shown are from multiple probings of the same blot. The membranes were probed with 32P-labeled cDNA probes specific for IL-1β, A20, p105/p50, p65, and IκBα. 28S ribosome levels are shown as a control.

![FIGURE 2. Neutralizing Abs to gB and gH block the induction of monocyte message expression. A Northern blot analysis of total RNA harvested from NAD monocytes infected for 4 h with gradient-purified HCMV (M.O.I. = 2–3) or HCMV pretreated for 1 h with neutralizing Ab (4 μg of anti-gB [15D8 (45)] and -gH [1G6 (46)], labeled “v-gBgH”) or an appropriate control Ab (4 μg of anti-PSA, labeled “r-PSA”, isotype and species matched (32)). The effect of UV-irradiated HCMV is also shown (“UV-HCMV”). The lane labeled only “NAD” is the untreated or mock-infected control. A representative experiment is shown and the experiment was repeated. All the blots shown are from multiple probings of the same blot. The membranes were probed with 32P-labeled cDNA probes specific for IL-1β, A20, p105/p50, p65, and IκBα. 28S ribosome levels are shown as a control.](http://www.jimmunol.org/)

As shown in Fig. 2, an increase in message expression following the 4-h incubation of cells with virus was observed (compare the untreated lanes “NAD” to the infected lanes “NAD+HCMV”). In contrast, when the virus was preincubated with neutralizing Abs, the increase in IL-1β, A20, p105/p50, p65, and IκBα expression was blocked (the “NAD+HCMV/v-gBgH” lane), while the control Ab had no effect on signaling (the “NAD+HCMV/r-PSA” lane). In comparison experiments, the neutralizing function of these Abs was shown to prevent cytopathic effects and viral gene expression in human fibroblasts (results not shown). It is important to note that neutralizing Abs to gB or gH alone can block viral-mediated signaling in human fibroblasts, while nonneutralizing Abs have no effect (32). We also wanted to show that UV-irradiated virus, which can bind to cells but cannot replicate, could activate monocytes. As shown in Fig. 2, irradiated virus also increased the expression of the examined gene products (the “NAD+UV-HCMV” lane). This signaling was also blocked by the anti-gB and -gH neutralizing Abs in the same manner as was seen with “live” virus (data not shown). Thus, these results support the hypothesis that viral binding itself is sufficient to activate monocyte gene expression.

**Purified HCMV gB up-regulated message expression**

The results from Figs. 1 and 2, as well as our previous results (32), present the possibility that the initial HCMV-mediated cellular activation involves a viral-ligand/receptor interaction. Therefore, we next addressed whether our purified HCMV gB (32) also up-regulated the expression of the different monocyte gene products. Purified HCMV gB was incubated under similar conditions to that
used for viral infections. A 4-h incubation of various concentrations of purified HCMV gB (1, 20, and 100 ng/ml) with the isolated monocytes (lanes marked “NAD+gB”) induced IL-1β, A20, p105/p50, and IkBa expression activity above that seen in the control untreated “NAD” monocytes (Fig. 3). The 20-ng/ml concentration of purified gB showed the largest increase in message expression. However, this amount of gB did not show the largest increase in IL-1β protein secretion (see Fig. 5B). As the use of all human donors, there is variability, and we attribute these sorts of subtle differences to donor variability. In addition, we used a control lysate (labeled as “Control”), which was isolated from extracts from the parental 293T cells (no transfected gB) by the same procedure used to purify the gB. This control lysate (100 ng/ml) showed no ability to activate cellular transcription factors, thus demonstrating that the protocol used to purify gB did not in itself have a mitogenic effect. Furthermore, the effects mediated by the purified gB (100 ng/ml) could be blocked by pretreatment with anti-GB Ab (15D8; compare lanes treated with Ab, “αgB”, to those without), but not by pretreatment with an isotype-matched control Ab (αPSA). The activation of monocytes following incubation with purified gB and its inhibition by an anti-gB Ab demonstrates that the specific gB/receptor interaction directly initiates the signaling process.

Incubation of monocytes with an αdAb, which mimics the HCMV gH glycoprotein activated monocytes

To show that the viral gH product also played a role in monocyte activation, we used a well-characterized affinity-purified monoclonal αdAb (38, 47–50), which mimics the HCMV gH glycoprotein and binds to the same 92.5-kDa cell membrane protein as affinity-purified gH (49), to stimulate our purified monocytes. We incubated the cells under the same conditions as with purified gB or “live” or UV-irradiated virus. The αdAb (4-3-5) or the appropriate control (a nonspecific IgM) were preincubated with 4 μg of an anti-gB Ab or its control. C, NAD monocytes were left untreated or were treated with an αdAb (“αd+X-L”) or its control Ab (“αdM+X-L”), D, ADH monocytes were incubated with or without gradient-purified virus for various times (4, 24, and 48 h). Time zero (TO) in these experiments represents the supernatant collected from the incubated cells at the start of the incubation time.
IL-1β protein secretion is induced by the binding of the virus or of the viral glycoproteins to their appropriate cell surface receptors

Next, we wanted to examine if the increase in steady-state mRNA of the monocyte mediators (specifically IL-1β) correlated with an increase in the production and secretion of proteins. To accomplish this goal, supernatants were harvested from monocytes following their treatment for 4 h with the various stimulatory products ("live" or UV-inactivated virus or the purified viral ligands) and then examined by ELISA for IL-1β levels. As shown in Fig. 5A, HCMV infection as well as treatment with UV-irradiated virus significantly increased IL-1β protein levels. In addition, neutralizing Abs blocked this increase ("gBgH"), while the isotype- and species-matched control Ab ("opSA") had no effect. Treatment of monocytes with our purified gB (32) increased IL-1β levels in a dose-dependent fashion (1, 20, and 100 ng/ml; Fig. 5B). In contrast, treatment of cells with the control lysate (100 ng/ml) or purified gB (100 ng/ml) pretreated with an anti-gB Ab did not cause an increase in secreted IL-1β levels. The control Ab ("opSA") had no effect. Treatment of monocytes with an old Ab ("old+X-L"), which mimics gH, also could increase the secretion of IL-1β in treated cells (Fig. 5C), while the control Ab ("oldG+X-L") had no effect. Viral infection of adherent monocytes also resulted in an increase in IL-1β levels (Fig. 5D). Monocytes were infected at the time of adherence to tissue culture plates. It can be seen that there is a large increase in IL-1β levels in 24-h and 48-h infected cells, with no activity seen in 4-h infected cells. It should be noted that in at least one donor there was an increase in IL-1β protein secreted at 4 h after virus addition (data not shown). This difference in the regulation of protein secretion in adherent vs nonadherent cells is not surprising as it has been previously documented to be differences in the signaling in these cells under these different conditions (42, 51).

HCMV binding up-regulates NF-κB activity in monocytes

Because many of the gene products (IL-1β, A20, p105/p50, and IκBα) that we examined contain NF-κB binding sites in their promoter regions and we are very interested in viral-mediated signal transduction, we next addressed if NF-κB activity was also up-regulated in monocytes following viral binding. We examined by EMSA the induction of monocyte NF-κB activity following HCMV infection (gradient-purified "live" or UV-inactivated virus) or incubation with purified viral ligands for 1 h (Fig. 6A). The results of this study showed that there was an increase in NF-κB DNA binding activity following HCMV infection (compare lanes 1 and 2) and following the incubation of monocytes with our purified gB (compare lanes 1 and 4). In contrast, the control lysate had no effect on NF-κB activity (lane 5). UV-inactivated virus...
peptides and preimmune serum to show the specificity of the 

bands marked with an asterisk). We also used appropriate blocking 

(p50/p65) heterodimers and p50/p50 homodimers (supershifted 

1

HCMV

mediated induction of NF-

B could be blocked by neutralizing 

Abs (anti-gB and 

gH ("+HCMV+gBgH") or a control Ab ("+HCMV+αPSA"); 10) and 11) 
cells treated with 15 μg of an αdAb plus 3 μg of the cross-linking Ab 

("αdM+X-L") or 15 μg of a control Ab plus 3 μg of its appropriate 
cross-linking Ab ("+αd+X-L"). After incubation, monocytes were 

harvested and the extract was run on an SDS-PAGE gel and analyzed 

by Western blot analysis using Santa Cruz anti-IκBα and IκBβ anti-sera. A, Western blot of IκBα levels. B, Densitometry analysis of the blot shown in A, C, Western blot of IκBβ levels. D, Densitometry analysis of the blot shown in C. A representative experiment is shown.

IkBα, but not IkBβ, levels decrease during NF-κB mobilization to the nucleus following HCMV-mediated activation of monocytes. Western blot analyses of cytoplasmic extracts from monocytes following activation. Monocytes were treated by incubating the cells for 1 h nonadherently under different conditions: 1) mock infected or untreated cells ("NAD"); 2) cells incubated with gradient-purified HCMV (M.O.I. = 2–3; "+HCMV"); 3) cells incubated with UV-inactivated virus ("+UV-

HCMV"); 4) cells treated with 100 ng/ml of purified gB ("+gB"); 5) cells treated with 100 ng/ml of the control lysate (+Control); or in the other experiment 6) untreated cells (NAD); 7) cells incubated with gradient-purified HCMV ("+HCMV"); 8) and 9) cells incubated with gradient-purified HCMV preincubated with 4 μg of neutralizing Abs (anti-gB and 
gH ("+HCMV+gBgH") or a control Ab ("+HCMV+αPSA"); 10) and 11) 
cells treated with 15 μg of an αdAb plus 3 μg of the cross-linking Ab 

("αdM+X-L") or 15 μg of a control Ab plus 3 μg of its appropriate 
cross-linking Ab ("+αd+X-L"). After incubation, monocytes were 

harvested and the extract was run on an SDS-PAGE gel and analyzed by Western blot analysis using Santa Cruz anti-IκBα and IκBβ anti-sera. A, Western blot of IκBα levels. B, Densitometry analysis of the blot shown in A, C, Western blot of IκBβ levels. D, Densitometry analysis of the blot shown in C. A representative experiment is shown.

IkB regulation correlates with NF-κB induction in activated monocytes

To further correlate the increase in NF-κB that was seen above, we also examined the cytosolic lysates for IkBα and IkBβ expression following various treatments of monocytes (Fig. 7). As seen in Fig. 7A showing a Western blot analysis of IkBα protein levels, there was a significant decrease in IkBα levels following cellular activation (compare lanes 2, 3, 4, 7, 9, and 10), while there was no change in IkBβ levels in the control-treated cells (lanes 1, 5, 6, 8, and 11). Fig. 7B shows a graph of the levels of IkBα as measured by densitometry. We also examined changes in IkBβ levels under the various activating conditions. As seen in Figs. 7, C and D (Western blot and densitometry analyses), there was no detectable change in IkBβ levels. These results are thus consistent with the virus acting as an acute activating signal because previously described agents that act as acute activating signals have been shown to only down-regulate IkBα, not IkBβ (39, 63, 64).

Blocking p38 function prevents viral-mediated induction of IL-1β message expression

To further characterize the viral-mediated signal transduction cascade in monocytes, we next wanted to focus on the role of the MAPK family member p38 (40). To initially examine the role of

FIGURE 8. p38 kinase activity plays a role in the viral-mediated induction of IL-1β message expression. A Northern blot analysis was performed on total RNA harvested from NAD monocytes pretreated with the kinase inhibitors, SB203580 and PD98059. A representative experiment is shown. Monocytes were pretreated for 1 h with 20 μM SB203580 ("+SB203580"), 20 μM PD98059 ("+PD98059"), or 0.1% DMSO as a control. NAD monocytes ("NAD") were then incubated with gradient-purified HCMV ("+HCMV", M.O.I. = 2–3) for 1 h and then harvested. The membrane was probed with a 32P-labeled cDNA probe specific for IL-1β. 28S ribosome levels are shown as a control.

FIGURE 9. p38 is rapidly up-regulated following viral binding. A Western blot analysis was performed on whole cell lysate from harvested NAD monocytes treated with gradient-purified virus (M.O.I. = 2–3). Monocytes were either harvested immediately after purifying (T0), 15 min postinfection with the corresponding mock-treated control, or 1 h postinfection with the corresponding mock-treated control. Harvested monocyte extracts were run on an SDS-PAGE gel and analyzed by Western blot analysis using New England Biolabs anti-phospho-specific p38 anti-sera. A representative experiment is shown.

(level 3) also up-regulated NF-κB activity. In addition, the viral-mediated induction of NF-κB could be blocked by neutralizing Abs to gB and gH (the control Ab had no effect (compare lanes 6, 8, and 9)). Lastly, we showed that an αd Ab (which mimics gH) also stimulated NF-κB activity in monocytes (compare lanes 6, 10, and 11). These results support our work using fibroblasts in which we reported an increase in nuclear NF-κB activity following the engagement of the HCMV glycoproteins gB and gH with their cellular receptors (32). Furthermore, we also examined in the same nuclear extracts the induction of ATF/CREB DNA binding activity, which only was reported to be increased at late times of infection (62), to demonstrate the specificity of the transcription factor induction (Fig. 6B). The results of these experiments showed that ATF/CREB binding activity was not induced during IE times of infection in monocytes. As shown in Fig. 6C, supershifting Abs showed that the shifted bands from Fig. 6A were "classic" NF-κB (p50/p65) heterodimers and p50/p50 homodimers (supershifted bands marked with an asterisk). We also used appropriate blocking peptides and preimmune serum to show the specificity of the supershifting Abs (data not shown). This rapid induction of NF-κB by HCMV fits our model of the viral-ligand/cellular-receptor-mediated process being responsible for an early step of cellular acti-
p38, we used an inhibitor of p38 activity and then looked at mono
cyte IL-1β message expression. Monocytes were pretreated for 1 h 
with the inhibitors (20 μM SB203580, a p38 inhibitor (a dose we 
have shown inhibits HCMV replication (41) and Shapiro et al. 
showed blocks HIV replication (43)), and 20 μM PD98059, a 
MEK inhibitor (44)) before their 1-h incubation with gradient-
purified virus. Because the inhibitors were dissolved in DMSO and 
were used such that the final concentration of DMSO was 0.1%, an 
equivalent amount of DMSO was added to the control groups. In 
addition, in none of the samples did DMSO or the various inhib-
itors affect cell viability (data not shown). Following incubation 
with the inhibitors and the virus, monocytes were harvested and 
Northern blots performed (Fig. 8). As shown, only the blocking of 
p38 function had a major effect on IL-1β expression (>95% in-
hibition). The MEK inhibitor only slightly blocked the IL-1β sig-
nal (5–15% decrease). We also performed titrations and showed 
that higher doses had no additional effects and that the drugs effects 
were titratable (data not shown). In addition, we also examined 
these drugs in fibroblasts at a number of different concentrations 
for toxicity and none of the doses used in our experiments were 
toxic to these cells in long-term studies. Our finding on the role of 
p38 in viral-mediated signaling in monocytes is apparently 
uniquely monocytic in nature as parallel experiments in fibroblasts 
showed no effect (our unpublished observations).

Phosphorylated p38 is rapidly induced following viral binding

Lastly, because the above mentioned data suggested a role for p38 
in viral-mediated signaling, we next wanted to examine if HCMV 
infection up-regulated p38 activity. Because it is well documented 
that phosphorylated p38 correlates with kinase activity (65), we 
performed Western blot analyses of whole cell extracts using a 
phospho-specific Ab (recognizes phosphorylated Thr180 and 
Tyr182). The results as shown in Fig. 9 demonstrate that p38 is 
phosphorylated very rapidly (within 15 min). Following induction, 
phosphorylated p38 levels begin to decrease, and by 4–8 h levels 
return to mock levels (data not shown). The time frame of p38 
phosphorylation (15 min to 1 h) correlates with our data presented 
in Fig. 8 in which the rapid viral-mediated induction of IL-1β 
(within 1 h) was blocked by a p38 inhibitor. Interestingly, in hu-
man fibroblasts, p38 is not induced during this early time frame 
(41), suggesting a possible difference in the mechanisms of viral 
signaling in nonpermissive (monocytes) and permissive (fibro-
blasts) cell types.

Discussion

Our data demonstrate that viral binding itself can dramatically in-
fluence the state of the cell. Specifically, we showed that viral 
binding through at least the gB and gH glycoproteins can up-reg-
ulate the signal transduction pathway in a biologically relevant cell 
type—the human peripheral blood monocyte—which ultimately 
results in an altered pattern of cytokine and other immune mediator 
expression. These results correlate with our previous study in which 
we showed that viral binding alone could up-regulate NF-κB and Sp1 levels in human fibroblasts (32). In addition, this 
work is supported by the many studies that have shown that vi-
ruses, including other members of the herpesvirus family, bind to 
known ‘‘activational’’ receptors (66–70). Therefore, our current re-
sults plus the work from these previous studies support our hy-
pothesis that viruses have evolved a common strategy in which 
activational molecules/receptors are used for not only viral entry, 
but also cellular activation and presumably viral survival.

We showed that there was an increase in the expression of a 
number of monocyte mediators (IL-1β, A20, p105/p50, and IκBα) 
as measured by Northern blot analysis following viral binding. 
Interestingly, the results showed that HCMV induced the message 
for both an NF-κB subunit, p105/p50, and its inhibitor, IκBα. Be-
cause both gene products are NF-κB inducible, these results are 
not surprising. However, one should keep in mind that message 
induction does not mean the protein is induced or up-regulated, 
which under our conditions examined seem to be the case, as IκBα 
levels do not return to untreated levels even by 4 h poststimu-
lation, suggesting that a potential viral-mediated translational or 
posttranslational control exists (perhaps as seen with the hepat-
itis B virus HBx protein (71), the human immunodeficiency 
virus Tat protein (72), or the human T lymphotrophic virus I 
Tax1 protein (73)).

We showed that HCMV binding induced A20 expression (ori-
inally described by Dixit et al. (74), although we originally called 
this gene product MAD-6 (60)), an anti-apoptotic factor (75). We 
have previously shown that this gene product was up-regulated in 
monocytes activated via their adhesion receptors (42) or adhesion 
itself (60). A20 can block p53-mediated apoptosis (75) and with 
the extended life cycle of HCMV in human monocytes (7, 8) 
and the reported induction of p53 (76), the induction of an anti-
apoptotic factor might be necessary for viral survival in these cells. 
Interestingly, the EBV latent membrane protein 1 induces A20 
and this protects these cells from p53-mediated apoptosis (77), 
suggesting that herpesviruses in general might use the anti-apoptotic 
properties of this gene product for their own survival.

It was also shown that viral binding could enhance gene expres-
sion in simultaneously adherently activated monocytes. Adherence 
is a known physiologic signal responsible for monocyte and mac-
rophage activation (42, 51, 60, 61, 78, 79). This HCMV-induced 
message expression was mediated by viral binding as neutralizing 
Abs to the major HCMV glycoproteins (gB and gH) blocked this 
expression, even in adherent activated monocytes (data not 
shown). In addition, we also showed that UV-irradiated virus also 
up-regulated message expression, demonstrating that newly syn-
thesized viral gene products were not required for the signal. This 
UV-irradiated virus-mediated signaling could also be blocked by 
neutralizing Abs. To show that viral binding truly was inducing 
mediator mRNA expression, we examined directly the regulation 
of monocyte activation by using purified HCMV gB (described 
previously (32)) as a stimulus. The results of these studies dem-
strated that there was indeed a viral glycoprotein-mediated sig-
naling event occurring in monocytes. In addition, a well-charac-
terized αd Ab (32, 38, 47–50), which mimics gH and has 
previously been documented to be involved in fibroblast signaling 
(38, 50), also up-regulated monocyte message expression. These 
combined results again support our hypothesis of a novel viral 
binding-mediated signaling event.

Next, we wanted to examine the effects of our activating con-
ditions on monocyte cytokine secretion, because ultimately mono-
cyte function in many cases is measured by its cytokine secretion. 
As the results showed, HCMV binding, as well as gB- and gH-
mediated signaling, all up-regulated the secretion of IL-1β protein. 
In addition, HCMV binding also induced IL-1β secretion in ad-
herently activated monocytes when added at the initial time of 
adhherence. The lack of IL-1β secretion in only adherently activated 
monocytes (in the absence of virus or other activating stimuli) is 
not surprising as it has previously been shown by Haskill et al. (51) 
that adherent monocytes need two signals to generate the secretion 
of IL-1β protein. Adherence only up-regulates message expres-
sion, while a second signal such as that provided by a mitogen 
or cytokine is needed to get the translational up-regulation of IL-1β 
protein. These results suggest the possibility that the virus might 
provide the second signal, much as a cytokine normally would do,
and thus might point to at least one of the reasons behind the immune dysregulation seen in HCMV-infected individuals. Interestingly, we previously showed that integrin engagement in non-adherent monocytes only needed one signal to generate IL-1β secretion (42), a fact that also seems to occur in our system following viral binding.

The gene products described above that were significantly up-regulated during viral binding or gB or gH-mediated signaling are all NF-κB-inducible gene products. Therefore, to continue our investigation of the signaling process we next focused on the regulation of NF-κB in monocytes following the various described treatments. We showed that there was a rapid increase in nuclear NF-κB levels following viral binding or gB- or gH-mediated binding in monocytes, which confirmed the results of previous studies in fibroblasts (26, 27, 29, 30, 33). The data demonstrated that the induction of NF-κB EMSA activity was specific, because ATF/CREB EMSA activity was not induced supporting the results of Kerry et al. who showed that ATF DNA binding activity was only up-regulated at late times of infection (62). Analysis of IkBa protein levels are consistent with the changes in NF-κB DNA binding levels; that is as NF-κB activity increases, IkBa levels decrease. Interestingly, even at 1–4 h poststimulation (either “live” or UV-inactivated virus or purified ligands), IkBa levels were still significantly reduced, suggesting that in our system there are viral mechanisms operating to inhibit IkBa protein levels. In other reported systems, IkBa levels usually return to unstimulated levels within 1 h (39). There were no changes detected in IκBα levels under any of the conditions used in our experiments. It is possible that there might be changes in IκBβ levels at later times of infection or that IκBβ could be acting as a chaperone for NF-κB, protecting it from IkBa, as Suyang et al. have shown for some forms of IκBβ (63). These results thus suggest that the virus is acting like an acute stimuli (39, 63, 64), at least under our laboratory conditions.

Lastly, we showed that there was a rapid increase in p38 phosphorylation (within 15 min), which has been well documented to be correlated to p38 function (65). Furthermore, we showed that viral-mediated signaling (at least IL-1β production) was dependent on p38 activity as a specific inhibitor of p38 (SB203580) nearly completely blocked IL-1β message expression (>95% decrease) in all the doses we tested (1–50 μM). These findings thus begin to map the signaling events associated with viral glycoprotein-cellular receptor engagement in monocytes. Excitingly, this event appears to be unique to monocytes because in permissively infected human fibroblasts p38 activity has no function at IE or early times after infection (15 min to 8 h postinfection; (41)), suggesting that in fibroblasts the signaling cascade through these same glycoproteins (gB and gH) diverges following viral binding. These differences may point to the unique and important biological signaling consequences of viral infection in nonpermissive vs permissive cell types. Our on-going studies are now addressing these potential differences in more detail.

It is important to point out that it is unlikely in any of the cases presented above that the monocyte activation seen was due to some mitogenic factor in the viral supernatant or in the soluble activating agents (gB or the gH anti-idiotypic Ab) because 1) in the viral binding studies we used density gradient-purified virus; 2) in the gB-mediated studies we used a control protein lysate isolated in the same manner as the purified gB; and 3) in the gH-mediated studies we used the appropriate control Abs. In addition, the Ab blocking studies from these various experiments supported this fact. If a soluble mitogenic factor was copurified with the virus or the gB, its effect would not have been specifically inhibited by the anti-HCMV Abs. The UV-inactivated virus studies, which also resulted in activated gene expression and transcription factors, further supports these points because the studies are similar to the work by Boldogh et al. in which they showed that density-purified virus and inactivated virus had identical effects on protooncogene mRNA levels (31).

In conclusion, we showed that there is a bona fide signal transduction event that takes place following HCMV binding to monocytes through the interaction of the surface glycoproteins, gB and gH, on the viral membrane. We have documented a rapid increase in the levels of nuclear NF-κB DNA binding following the incubation of HCMV with monocytes that is regulated by the degradation of IkBa. This transcription factor induction is then probably responsible for the observed increase in monocyte mediators (IL-1β, A20, p105/p50, and IkBa) and then ultimately in the increased cytokine production and secretion (as shown for IL-1β). This dysregulation of monocyte function by HCMV would have serious consequences for the infected host because at the very least an increased cytokine production would profoundly affect the way cells behave in their natural environment. This aberrant HCMV-mediated activation of monocytes could be some of the molecular events behind HCMV’s link to chronic inflammatory diseases such as coronary restenosis, atherosclerosis, arthritis, and other HCMV-associated diseases.

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