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Human cytomegalovirus decreases constitutive transcription of MHC class II genes in mature Langerhans cells by reducing CIITA transcript levels

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Abstract

Human cytomegalovirus (HCMV) productively infects CD34⁺ progenitor-derived, mature Langerhans-type dendritic cells (matLC) and reduces surface expression of MHC class II complexes (MHC II) by increasing intracellular retention of these molecules. To determine whether HCMV also inhibits MHC II expression by other mechanisms, we assessed mRNA levels of the class II transcriptional regulator, CIITA, and several of its target genes in infected matLC. Levels of CIITA, HLA-DRA (DRA) and DRB transcripts, and new DR protein synthesis were compared in mock-infected and HCMV-infected cells by quantitative PCR and pulse-chase immunoprecipitation analyses, respectively. CIITA mRNA levels were significantly lower in HCMV-infected matLC as compared to mock-infected cells. When assessed in the presence of Actinomycin D, the stability of CIITA transcripts was not diminished by HCMV. Analysis of promoter-specific CIITA isoforms revealed that types I, III and IV all were decreased by HCMV, a result that differs from changes after incubation of these cells with lipopolysaccharide (LPS). Exposure to UV-inactivated virus failed to reduce CIITA mRNA levels, implicating de novo viral gene expression in this effect. HCMV-infected matLC also expressed lower levels of DR transcripts and reduced DR protein synthesis rates compared to mock-infected matLC. In summary, we demonstrate that HCMV infection of a human dendritic cell subset inhibits constitutive CIITA expression, most likely at the transcriptional level, resulting in reduced MHC II biosynthesis. We suggest this represents a new mechanism of modulation of mature LC by HCMV.

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Keywords

Dendritic cells; viral; MHC

1. Introduction

Human cytomegalovirus (HCMV) causes a persistent, life-long infection in normal hosts and disseminated, life-threatening disease in immunocompromised individuals. CD4⁺ T cell immune responses are required for clearance of CMV from tissues (Lucin et al., 1992) and are crucial for optimal induction of CMV-specific CD8⁺ cytotoxic T cell responses and anti-HCMV humoral immunity (Gamadia et al., 2003; Krmpotic et al., 2003). As CD4⁺ T cells recognize antigen (Ag) as peptide-MHC II complexes, interference with MHC II biosynthesis in antigen-presenting cells (APC) will reduce the antigen-driven T cell response and thus may contribute to immune evasion.

Constitutive expression of MHC class II genes is tightly restricted to cells that express the *MHC2TA* gene, including thymic epithelial cells and professional APC, such as dendritic cells (DCs), macrophages, and B cells. *MHC2TA* encodes the class II transactivator, CIITA, a non-DNA binding transcriptional co-activator of transcription factors bound to MHC class II promoters. CIITA is essential for class II transcription and expression (reviewed in (Wright and Ting, 2006)) and also regulates expression of genes encoding accessory proteins required for MHC class II-restricted antigen presentation: the invariant chain (Ii), HLA-DM, and HLA-DO (LeibundGut-Landmann et al., 2004a).

Several promoters control CIITA transcription, resulting in multiple isoforms of CIITA protein. These isoforms account for cell-type specificity of constitutive MHC II expression by APCs as well as IFN- γ -inducible expression by non-APCs (Muhlethaler-Mottet et al., 1997). Promoter I is predominant in CD11c⁺ DCs (Landmann et al., 2001) and IFN- γ stimulated monocytes/macrophages (Waldburger et al., 2001), whereas promoter III is used by B cells (Lennon et al., 1997), human T cells (Wong et al., 2002), and plasmacytoid DCs (LeibundGut-Landmann et al., 2004b) and is activated in other cell types, including monocytes, by cytokines such as IFN- γ and GM-CSF (Hornell et al., 2003; Landmann et al., 2001; Waldburger et al., 2001). Promoter IV is activated in response to IFN- γ in cells that are inducible for class II expression (Muhlethaler-Mottet et al., 1998; Muhlethaler-Mottet et al., 1997; Piskurich et al., 2006). Promoter IV is also crucial for MHC II expression on rare nonhematopoietic cells that constitutively express class II, such as thymic epithelial cells (Waldburger et al., 2001). Within professional APCs, CIITA transcription is also developmentally regulated. For example, maturation of monocyte-derived DC *in vitro* is associated with a global shut-off of transcription of all CIITA isoforms that is mediated by histone deacetylation (Landmann et al., 2001).

As a master regulator of MHC class II genes, CIITA is an attractive target for modulation by pathogens that are controlled by CD4⁺ T cells (Accolla et al., 2001). Among such pathogens, HCMV has been shown to block induction of class II MHC expression by inhibiting promoter IV-driven inducible CIITA transcription via impairment of IFN- γ mediated signaling (Le Roy et al., 1999; Miller et al., 1998). HCMV has been shown to infect primary APC (Hertel et al., 2003; Odeberg and Soderberg-Naucler, 2001; Raftery et al., 2001), but the effect of HCMV infection on constitutive transcription of CIITA still requires further investigation.

HCMV enters the body through mucosal sites where it may encounter Langerhans-type mucosal DCs (LCs). Using a well-described *in vitro* cell culture system to differentiate

immature LC via cytokine stimulation of CD34⁺ hematopoietic progenitors, we previously showed that productive HCMV infection occurs in CD40L-exposed matLC but not in immature LC, and that infection is associated with substantial changes in the immunostimulatory functions of these cells (Caux et al., 1996; Hertel et al., 2003; Lee et al., 2006; Strobl et al., 1996). Here we find that HCMV infection affects the abundance of *MHC2TA*, *HLA-DRA*, and *HLA-DRB* transcripts in matLC, highlighting another mechanism by which this versatile virus modifies the function of infected APC.

2. Materials and Methods

2.1. Generation of CD34⁺ progenitor-derived cell populations

Immature and mature LC (matLC) were derived from granulocyte-colony stimulating factor (G-CSF) mobilized peripheral blood CD34⁺ progenitors, as previously described (Hertel et al., 2003; Lee et al., 2006; Strobl et al., 1997). Where indicated, an enriched matLC population was isolated from matLC cultures using anti-CD83 and MACS beads (Miltenyi Biotec), per manufacturers' instruction.

2.2. Preparation of virus, infection and LPS treatment of matLC

Virus preparation, infection and *Escherichia coli* LPS treatment was performed as optimized and described previously (Hertel et al., 2003; Lee et al., 2006).

2.3. RNA isolation and reverse transcription

RNA was isolated using Trizol (Life Technologies) or RNeasy kits (Qiagen), according to the manufacturer's instructions, and treated with DNase (Invitrogen and Qiagen, respectively). RNA was quantified by absorbance at 260 nm and 0.5 µg of RNA was reverse transcribed using MMLV reverse transcriptase (Promega).

2.4. Quantitative PCR

Quantitative, reverse-transcriptase PCR was performed as described (Hornell et al., 2003). Briefly, SYBR Green PCR Master Mix (Applied Biosystems) was used in PCR reactions with 200nM of forward and reverse primers, 10–40 ng of the RT product, and RNase/DNase free water to 20 µl. To determine relative quantity, control cDNA generated from total RNA isolated from Raji cells (for GAPDH, total CIITA, and type III CIITA), monocyte-derived dendritic cells (for type I CIITA), or IFN-γ stimulated monocytes (for type IV CIITA) was used to generate a standard curve. Primers and conditions for measuring transcript levels for different CIITA isoforms were described previously (Hornell et al., 2003). Relative quantities of the gene of interest were determined for unknown samples by comparison to this standard curve, and normalized to GAPDH quantities. Fold-changes in expression were determined by dividing the normalized quantity of the gene of interest from HCMV-infected or LPS-treated LC by the normalized quantity of the gene of interest from mock-infected LC.

2.5. mRNA stability assay

To determine stability of CIITA mRNA after HCMV infection, matLC were incubated with/without HCMV at MOI=50 for 16h (Lee et al., 2006). The inhibitor of transcription, actinomycin D (1 µg/ml, Sigma), was then added to the cultures (Gealy et al., 2005). Cells were harvested at 0, 2, 4 and 8h of actinomycin D treatment. RNA extraction, reverse transcription and quantitative PCR were carried out, as described above. Total CIITA mRNA level was normalized to GAPDH mRNA at the corresponding time point, and the ratio in CIITA: GAPDH samples at 0h actinomycin D treatment set to 1.

2.6 Flow cytometry analyses and Abs

MatLC were incubated with/without HCMV (MOI=100) or LPS (0.5 $\mu\text{g/ml}$) for 2 days. Cells were harvested, blocked with 5% human serum and 5% goat serum in FACS buffer (PBS+0.5% BSA+0.1% NaH₃) for 15m on ice, and stained with DR-PerCP 5.5 (L243 (G46-6), BD), CD40-APC (5C3, Biolegend) and CD86-biotin (IT2.2, Biolegend) for 30m. Pacific orange conjugated streptavidin (Invitrogen) was added after CD86-biotin staining for additional 30m. Staining was measured using a BD LSR II flow cytometer, and data were analyzed with Flowjo software (Tree Star, Inc.), gating on only singlet populations.

2.7. Pulse-chase immunoprecipitation

3.4×10^6 mock-infected or HCMV-infected LC were harvested for pulse-chase immunoprecipitation, performed as described (Patil et al., 2001) with the following modifications. Sequential immunoprecipitations were performed with protein A sepharose beads (Amersham Pharmacia Biotech) conjugated with antibodies for 2h at 4°C, in the following order: mAb DA6.147 (anti-DR α), anti-US2 antiserum, mAb L243 (anti-DR dimer), mAb AF8 (anti-calnexin). mAb DA6.147 recognizes the DR α cytoplasmic tail with a preference for early biosynthetic forms of DR, whereas mAb L243 recognizes mature DR dimers (Guy et al., 1982). The complexes were washed; proteins were eluted by boiling the precipitates in reducing SDS sample buffer and then separated by SDS-PAGE. Labeled proteins were visualized by exposing dried gels to radiography films (Kodak).

2.8. Statistics

The statistical significance of differences among results between HCMV-infected or LPS-treated and mock-infected LC were evaluated by the Student's t test (Prism, GraphPad Software). P values were determined using the one-tailed t test.

3. Results

3.1. HCMV infection reduces total CIITA mRNA levels in matLC

We have previously shown that matLC generated from CD34⁺ stem cells become productively infected with TB40/E, an endothelial-tropic strain of HCMV; infected cells express viral proteins and support viral replication (Hertel et al., 2003). As CIITA is a key activator of MHC class II gene expression and a critical regulator of immune responses, we asked whether HCMV infection of matLC affects CIITA transcription. As shown in Fig. 1A, real-time RT-PCR of RNA from matLC infected for 2 days with TB40/E demonstrated a significant decrease in the quantity of CIITA transcript compared to mock-infected cultures (32% of mock, $p < 0.0001$, $n=6$). Time course experiments revealed an initial increase in CIITA transcript levels, followed by a statistically significant decrease by 6 hr which was maintained at 24 hr post-infection ($p < 0.05$ for both time points, Fig. 1B).

HCMV expresses many proteins that could trigger a response in matLC leading to decreased CIITA mRNA levels. Glycoprotein B (gB) plays a major role in virion attachment and entry (Navarro et al., 1993), and activates signaling pathways in host cells (Boehme et al., 2006; Simmen et al., 2001). However, exposure of matLC to purified gB did not recapitulate the reduction in CIITA mRNA levels observed with TB40/E infection ($n=2$, data not shown).

To assess whether exposure to viral particles was sufficient to induce changes in CIITA, we next compared the ability of live versus UV-inactivated HCMV to decrease CIITA mRNA levels in matLC. As shown in Fig. 1C, exposure of matLC to UV-inactivated TB40/E failed to decrease CIITA mRNA levels, while incubation with live HCMV resulted in a significant dose-dependent decrease in CIITA mRNA levels ($p < 0.05$). These results indicate that de

novo synthesis of viral proteins is necessary for down-regulation of CIITA mRNA levels in matLC.

3.2. CIITA message stability is unchanged by HCMV infection

To determine if the HCMV-induced reduction in CIITA transcripts was due to diminished RNA stability, infected matLC were treated with actinomycin D, an inhibitor of transcription. Similar to previous results with mouse cytomegalovirus (MCMV) (Popkin et al., 2003), HCMV did not diminish the stability of total CIITA mRNA (Fig. 1D). The residual CIITA at later time points raised the possibility that different isoforms of CIITA may turnover at different rates. Nonetheless, there is no evidence of increased rate of turnover after HCMV infection, arguing that the HCMV-dependent reduction in CIITA transcript abundance is due to inhibition of message synthesis.

3.3. Infection with HCMV decreases types I, III, and IV CIITA mRNA levels in matLC

Previous studies have shown that HCMV blocks IFN- γ induced upregulation of CIITA mRNA in endothelial cells and fibroblasts by interfering with signaling events that lead to activation of CIITA promoter IV (Baron and Davignon, 2008; Le Roy et al., 1999; Miller et al., 1998). As matLC express class II constitutively, we hypothesized that the decrease in total CIITA mRNA in HCMV-infected LC cultures represented effects on transcription of constitutively expressed CIITA isoforms. To determine which CIITA promoters were active in LC, we determined the ratio of expression by real-time RT-PCR for types I, III, and IV CIITA in immature and mature LC (Fig. 2). Immature LC expressed levels of type I and type III CIITA that were similar to those seen in immature monocyte-derived DC and B cells, respectively, and low but reproducibly detectable levels of type IV CIITA. After maturation of LC with CD40L, we observed a decrease in types I and III CIITA mRNA, as seen in other DC subsets (Pai et al., 2002), but a slight increase in type IV CIITA mRNA. We confirmed that the type IV CIITA mRNA derived from LC, rather than from other cells in CD34⁺ cell-derived cultures, by testing MACS-bead enriched matLC (>80% CD83⁺, n=2, not shown).

We next asked whether HCMV infection leads to a selective decrease in transcription from specific CIITA promoters. We noted that an additional 2 days of culture of mock-infected matLC resulted in an increase in all types of CIITA, although type I CIITA mRNA levels were still well below those observed in immature LC (not shown). In contrast, 48-hour HCMV infection resulted in a marked reduction of levels of types I, III and IV CIITA RNA to 8%, 20% and 26% of mock infection samples respectively (Fig. 3A), suggesting that infection results in a decrease in all isoforms of CIITA transcripts to levels below those seen after maturation of LC with CD40L. Reduction of type IV CIITA transcripts was also observed in HCMV-infected, enriched matLC, isolated from matLC cultures using anti-CD83-coated, magnetic beads (not shown).

In order to compare the effects of HCMV infection to another microbial stimulus, we determined the ratio of CIITA mRNA isoforms in LPS-treated matLC compared to untreated cells. LPS treatment (0.5ug/ml for 2 days) also reduced type I and III CIITA levels in matLC, although less profoundly than after HCMV-infection, but did not affect type IV CIITA levels (Fig. 3A). These data indicate that HCMV infection of matLC results in a more robust reduction of all isoforms of CIITA mRNA than LPS exposure.

We also compared the effect of HCMV with LPS on MHC class II and costimulatory molecule expression in matLC, using surface staining followed by cytometry analysis. Similar to our previous studies (Hertel et al., 2003; Lee et al., 2006), HCMV significantly reduced class II and CD86 levels without changing CD40 expression. LPS, on the other

hand, increased expression of all the tested molecules (Fig. 3B). Thus, HCMV infection and LPS treatment of matLC also result in different surface phenotypes.

3.4. DRA and DRB mRNA levels and new synthesis of DR protein are decreased following HCMV infection of matLC

The down-regulation of CIITA mRNA following HCMV infection of matLC would be predicted to result in decreased transcription of genes controlled by CIITA. As DRA/DRB gene expression depends on CIITA, we tested the effect of HCMV infection on levels of DRA/DRB mRNA. As shown in Fig. 4A, we found that HCMV infection resulted in down-regulation of both DRA and DRB mRNA. On average, HCMV-infected matLC expressed 40% of the level of DRA mRNA and 60% of the level of DRB mRNA compared to mock-infected matLC ($p < 0.0001$, for both). This decrease in mRNA levels was detected by 24 h post-infection for DRA and more marked by 48 h (Fig. 4B). DRB was not significantly decreased until 48 h post-infection (Fig. 4C). The kinetics of change in DRA/DRB transcript levels were delayed as compared to changes in CIITA levels, as would be expected if DRA/DRB transcription was regulated by CIITA and DRA/DRB transcripts were not immediately turned over after synthesis.

New synthesis of DR α and DR β protein was also decreased following infection with HCMV, as detected by pulse/chase experiments (Fig. 5). After a 5 minute pulse to exclusively label newly synthesized molecules, precipitation of DR $\alpha\beta$ dimer/invariant chain complexes with DA6.147, an antibody against the DR α cytoplasmic tail, revealed that levels of all three component proteins were substantially decreased in HCMV-infected (at 2 days p.i.) compared to mock-infected matLC (top row, Fig. 5). Newly synthesized, HCMV-encoded US2 protein was detectable only in the infected cells (3rd row, Fig. 5), indicating the presence of active viral protein synthesis. Synthesis of calnexin (4th row, Fig. 5) was not affected by HCMV, indicating that overall protein synthesis was similar in HCMV- and mock-infected cultures.

Immunoprecipitation with L243, an antibody to mature DR dimers (2nd row, Fig. 5), showed that such dimers were generated with apparently similar kinetics in infected and mock-infected cells, even though the level of dimer is reduced consistent with reduced synthesis of the component chains. The relative amounts of DR in infected versus mock-treated cells were similar for early biosynthetic forms (top row, see 60 minute chase point) and for mature forms (2nd row, see 360 minute chase point), implying that the rate of degradation of DR is not significantly different in the infected and mock-infected matLC during the time period we studied. Taken altogether, our data indicate that HCMV significantly and specifically reduces CIITA and DRA/DRB mRNA, resulting in reduced synthesis of new DR protein in infected matLC.

4. Discussion

HCMV has evolved numerous mechanisms to block innate and adaptive immunity (Jenkins et al., 2008; Kane and Golovkina, 2010; Miller-Kittrell and Sparer, 2009; Wiertz et al., 2007). T cell-mediated cellular immunity is a key target for HCMV immune evasion strategies. Both CD8⁺ and CD4⁺ T cells are important in host defense against HCMV, and various viral products inhibit the function of these T cells, in part through blockade of MHC class I or MHC class II antigen presentation pathways. Here, we focused on HCMV-mediated, negative regulation of class II synthesis at the transcriptional level. A major finding of our study is that HCMV infection of matLC results in decreased levels of CIITA, the master transactivator of MHC II expression. Although phorbol ester treatment can reduce total CIITA mRNA stability in monocytic cells (De Lerma Barbaro et al., 2005), we observed no obvious alteration of CIITA message stability upon HCMV infection of matLC.

Rather, HCMV-mediated CIITA reduction in these cells appears to be due to inhibition of mRNA synthesis.

CIITA abundance and activity are the net result of transcriptional (Muhlethaler-Mottet et al., 1997) and post-translational regulation, including phosphorylation (Greer et al., 2004; Tosi et al., 2002) and ubiquitination (Greer et al., 2003). In immature human monocyte-derived DC or murine bone marrow-derived and splenic DC, transcription of various isoforms of CIITA is rapidly shut down following DC exposure to a variety of inflammatory or infectious stimuli (Landmann et al., 2001). A recent study showed that binding of PU.1, IRF8, NF- κ B and Sp1 activates the CIITA type I promoter in immature DC, whereas recruitment of PRDM1/Blimp-1 represses this promoter in matDC (Smith et al., 2011). Thus, a key question that arises is the relationship of our findings to modulation of CIITA during maturation of dendritic cells, including LC. CIITA reduction in infected matLC cultures could reflect CIITA changes in a population of residual immature LC, stimulated to mature by cytokines from infected matLC. However, we believe this is unlikely as we do not detect a contaminating immature LC population, which would show lower CD83 and MHC class II levels than matLC in flow cytometric analysis of the matLC cultures (Hertel et al., 2003).

Different maturation stimuli have distinct effects on immature DC, including different alterations in CIITA transcription. In human monocyte-derived DC, CD40L induces less total CIITA reduction than LPS (Landmann et al., 2001). CD40L differentiates LC to a maturation stage sufficient for CD83 expression and HCMV infection, but allows residual CIITA expression. To compare the effects of HCMV infection with a stimulus expected to further mature the “matLC”, we investigated the effects of LPS on these cells. The CIITA effects seen with LPS match those reported after LPS maturation of human monocyte-derived DC, where levels of promoter I and III type CIITA are diminished, but type IV CIITA is not reduced (Landmann et al., 2001). Thus, it appears unlikely that the effects of HCMV infection on CIITA represents “further maturation”. Among Toll-like receptors (TLR) expressed on matLC, HCMV activates TLR2, TLR7 and TLR9, whereas LPS binds TLR4 (Compton et al., 2003; Varani et al., 2007). The corresponding signaling pathways for these different TLRs are different (Saitoh and Akira, 2010). This might contribute to the distinct effects of HCMV and LPS on CIITA expression, although the full effects we have observed likely require more than TLR engagement alone (see below).

A related and important question is whether the further reduction in CIITA after HCMV infection has functional consequences for antigen presentation. We have shown that infected matLC are reduced in their ability to stimulate allo-reactive T cells compared to mock-infected matLC, whereas as LPS-stimulated matLC are increased in allo-stimulatory capacity compared to mock-infected matLC (Hertel et al., 2003). However, in addition to effects on CIITA, LPS redirects class II molecules to plasma membrane, whereas HCMV sequesters class II within cells (Inaba et al., 2000; Lee et al., 2006). Consequently, surface class II levels differ after exposure to LPS and infection with HCMV (Fig. 3B). Impaired allo-stimulatory activity is observed in monocyte-derived DCs from patients with HCMV mononucleosis. These *in vivo*-infected DCs also have lower surface class II expression compared to those from healthy controls (Frascaroli et al., 2006). In addition to altering class II levels by several mechanisms, HCMV infection has multiple other consequences for antigen presentation to CD4⁺ T cells (Benedict et al., 2008; Senechal et al., 2004; Varani et al., 2007). Further work to assess the unique contribution of HCMV-mediated CIITA reduction on altered antigen presentation by infected cells will require elucidation of the specific mechanism that modulates CIITA.

Characterization of the kinetics of HCMV-mediated inhibition of CIITA transcript revealed an initial increase in CIITA following infection, prior to a significant down-regulation by hours. This early activation in CIITA expression appears to differ from the response of immature monocyte-derived DC to microbial stimuli. Landmann et al. observed a marked decrease in CIITA within 3 h exposure to LPS (Landmann et al., 2001). However, it is possible that transient activation of CIITA transcription occurs even earlier, as Landmann et al. saw a temporary increase in DRA mRNA levels at early time points after LPS exposure, similar to our findings with HCMV. A rapid but transient enhancement of class II synthesis was seen upon exposure of human blood monocytes-derived DC to inflammatory stimuli, LPS and TNF α (Cella et al., 1997). An early increase followed by reduction in MHC II synthesis parallels the observation that, upon receipt of a TLR stimulus, immature DC initially upregulate and then downregulate endocytosis (West et al., 2004). The early CIITA increase in our system might require HCMV contact only, as opposed to infection, and might reflect the same process that leads to increased CIITA abundance after exposure of matLC to UV-inactivated virus. UV-inactivated virus has been shown to activate myeloid DC through interaction with TLR2 (Compton et al., 2003). Similarly, TB40/E viral particles interact with TLR7 and/or TLR9 and increase MHC class II expression in plasmacytoid DC (pDC), although pDC do not support TB40/E replication (Varani et al., 2007).

An initial burst of MHC II synthesis and endocytosis in response to TLR (or other pattern-recognition receptor) stimuli may allow even mature DC to load nascent class II with HCMV-derived antigens after initial encounter. The subsequent reduction in CIITA may protect against dilution of viral antigen/class II complexes, allowing mature DC to maintain presentation of these antigens as surface-displayed peptide-class II complexes. The subsequent reduction in CIITA reduces class II synthesis, but the increased half-life of class II molecules as well as the redistribution of class II from late endosomal compartments to the plasma membrane ultimately upregulates surface class II level significantly compared to immature DC (Cella et al., 1997; Inaba et al., 2000).

Why would live virus be necessary for reduction of constitutive CIITA mRNA expression? An attractive possibility is that endogenous expression of HCMV products is needed. Evidence from MCMV shows that infection blocks the recruitment of IFN response factor 1 (IRF-1) and RNA polymerase II to the promoter of CIITA (Popkin et al., 2003). Another herpes virus, Epstein-Barr, expresses a lytic transactivator Zta, which suppresses CIITA constitutive expression in B cells, by binding to a site in the type III CIITA promoter (Li et al., 2009). HCMV may use similar approach(es) to reduce CIITA expression in matLC. The fact that CIITA mRNA levels begin to drop within 6 hours of infection suggests that the responsible protein(s) is/are likely to be an immediate early (IE) or early (E) HCMV gene product(s). Early gene products mediate the majority of known HCMV immunoregulatory effects (Gilbert et al., 1996; Miller et al., 1998). IE1/IE2 protein was detected in HCMV-treated matLC within 6 h post-infection by flow cytometry (A. Lee, data not shown). Notably, both CMV interleukin 10 (cmvIL-10), a viral homolog of cellular IL-10 expressed by HCMV, and its splicing alternative-latency associated cmvIL-10 (LAcmvIL-10) inhibit CIITA transcription in granulocyte macrophage progenitor cells and monocytes (Jenkins et al., 2008). However, these proteins are encoded by the late viral gene UL111A (Chambers et al., 1999).

We observed associations between HCMV-mediated reductions in steady state CIITA message levels and reductions in DRA and DRB transcripts and DMA and DMB mRNA (J. Harding, not shown). In U373 astrocytoma cells transfected with CIITA driven by a heterologous promoter, neither levels of CIITA nor of DR transcripts were altered by HCMV infection (Cebulla et al., 2002). That HCMV did not increase turnover of CIITA or its target transcripts in this system argues that the reduction in DRA/DRB transcripts we

observe is secondary to diminished CIITA mRNA. However, HCMV proteins also may compete directly with residual CIITA for interaction with DNA-binding class II transcription factors, similar to effects of HIV-1 proteins on transcription (Iannello et al., 2006).

Unlike herpes simplex virus and pseudorabies virus, which shut down host protein synthesis globally, the effects of HCMV on host protein synthesis are selective (Stinski, 1977). For example, we observed persistent synthesis of calnexin and unchanged expression of CD40 in infected cells. Previously, we found reduced surface levels of DR at 2 days post infection with only minimal reductions in total cellular DR protein in HCMV-infected matLC (Lee et al., 2006). We believe our current findings of reduced new synthesis of DR can be reconciled with our earlier results because of the long half-life of MHC II molecules in mature DCs, estimated at ~40 h in bone marrow-derived murine myeloid DC (Pierre et al., 1997).

Taken together, our studies of HCMV and MHC II biosynthesis reveal multiple viral strategies targeting transcription, new protein synthesis, and cellular distribution of MHC II. The use of multiple mechanisms to impact class II antigen presentation at earlier time points (reduced egress to the cell surface) and at later time points (reduced synthesis of class II molecules) has been also observed for other pathogens, such as *Mycobacterium tuberculosis* (Chang et al., 2005). Evidence also has been presented that HCMV disrupts MHC class II-mediated antigen presentation at other stages (Cebulla et al., 2002; Hegde et al., 2003; Hertel et al., 2003; Jenkins et al., 2008; Kessler et al., 2008; Li et al., 2009; Slobedman et al., 2009; Wiertz et al., 2007). Thus, HCMV likely evades immune detection by CD4⁺ T cells using approaches that operate in multiple cellular locations and over varied time frames.

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List of abbreviations

Ag	antigen
APC	antigen-presenting cells
cmvIL-10	CMV interleukin 10
DCs	dendritic cells
DRA	HLA-DRA
E	early
gB	glycoprotein B
G-CSF	granulocyte-colony stimulating factor
HCMV	human cytomegalovirus

IE	immediate early
Ii	invariant chain
imm moDC	immature monocyte-derived DC
IRF	IFN response factor
LAcmvIL-10	latency associated cmvIL-10
LCs	Langerhans-type mucosal DCs
LPS	lipopolysaccharide
matLC	mature LCs
MCMV	mouse cytomegalovirus
MHC II	MHC class II complexes
p.i	post-infection
TLR	toll-like receptor

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Fig. 1.

HCMV infection decreases total CIITA mRNA levels in matLC. A) The mean-fold change $\pm$ SEM in expression of CIITA mRNA in TB40/E-infected (MOI = 100) matLC at 2 dpi compared with mock-infected matLC, was determined by real-time RT-PCR. The relative quantity of CIITA mRNA was determined after normalization to the quantity of GAPDH RNA. * denotes a statistically significant decrease in CIITA mRNA levels from mock-infected, ($p < 0.0001$). Viral dose titration showed a reduction in CIITA mRNA abundance with MOI ≥ 20 (not shown). Data are from four experiments and seven donors. B) Time course of changes in CIITA transcript levels in HCMV-infected matLC. The fold change in expression of CIITA mRNA in TB40/E-infected matLC at the indicated time points

compared to mock-infected matLC, was determined by real-time RT-PCR. The relative quantity of CIITA mRNA was determined after normalization to the quantity of GAPDH RNA. * indicates a statistically significant change compared to mock ($p < 0.05$). Data shown are from three experiments and four donors. C) Live HCMV is required to reduce CIITA message abundance. The mean fold-change $\pm$ SEM in expression of CIITA mRNA by matLC infected with live TB40/E or UV-inactivated TB40/E compared to mock-infected matLC, determined by real-time RT-PCR is shown. MatLC were exposed to 100 ng/ml CD40L alone (mock), or CD40L + untreated or UV-inactivated TB40E (MOI = 20, 67) and RNA was harvested 2 dpi. The relative quantity of CIITA mRNA was determined after normalization to the quantity of GAPDH RNA. * denotes a statistically significant difference from mock-infected ($p < 0.05$, $n=3$). D) Stability of CIITA mRNA upon HCMV infection. Mock- or HCMV-infected matLC were treated with the transcription inhibitor actinomycin D (1 μ g/ml) for 0, 2, 4 and 8h. Total CIITA mRNA levels were measured by quantitative PCR at each time point; levels were normalized with corresponding GAPDH levels. Normalized CIITA levels at different time points after actinomycin D treatment were compared with 0h samples, represented as 1. Data shown are from one of three experiments from four donors, with similar results.

Fig. 2.

LC expression of CIITA isotypes is regulated by maturation. Relative expression levels of CIITA mRNA types I, III, and IV in immLC and matLC were determined by real-time RT-PCR and are shown in (A), (B), and (C), respectively. Relative quantities of each CIITA transcript were determined by using control cDNA generated from immature monocyte-derived DC (imm moDC), Raji B cells, and IFN- γ stimulated monocytes, as controls for type I, type III, and type IV CIITA mRNA, respectively. The relative quantity of each CIITA transcript was determined after normalization to the quantity of GAPDH RNA, and is shown relative to control (set to 1). Data shown are from three experiments and three donors.

Fig. 3.

Multiple isoforms of CIITA mRNA, surface class II and costimulatory molecules are markedly reduced following HCMV infection. A) Comparison of promoter-specific CIITA mRNA levels by RT-PCR. MatLC were exposed to 100 ng/ml CD40L alone (mock), CD40L + HCMV (TB40/E, MOI = 100), or CD40L + LPS (0.5 μ g/ml) for 2 days, and the relative quantity of CIITA types I, III, and IV were determined as in Fig. 2. The mean fold-change $\pm$ SEM in expression of each transcript compared to mock-infect matLC is shown. * denotes a statistically significant decrease in CIITA mRNA levels from mock-infected ($p < 0.05$). Data shown are from three experiments and three donors. B) Effects of HCMV and LPS on MHC class II and costimulatory molecules. MatLC were treated with HCMV or LPS as in panel A. Surface levels of CD40, CD86 and HLA-DR were determined by flow cytometry. CD86 and DR were significantly decreased by HCMV while markedly enhanced by LPS. HCMV did not affect CD40 expression. The mean fold-change $\pm$ SEM in expression of each molecule compared to mock-infect matLC (set up as 1) is shown. * denotes a statistically significant decrease/increase in protein surface levels from mock-infected samples at $p < 0.05$.

Fig. 4.

HCMV infection leads to reduced DR message levels in matLC. The fold change in expression of DRA and DRB mRNA by TB40/E infected matLC, compared to mock-infected matLC, was determined by real-time RT-PCR. The relative quantities of DRA and DRB mRNA were determined after normalization to the quantity of GAPDH RNA. A) The mean fold-change $\pm$ SEM of DRA/DRB in matLC infected with TB40/E compared with mock-infected matLC is shown. Cells were exposed to TB40/E (MOI = 100) and RNA was harvested 2 dpi. * denotes a statistically significant difference from mock-infected ($p < 0.0001$, one-tailed t test). B) Time course of HCMV-mediated down-regulation of DRA. MatLC were infected with TB40/E (MOI = 100) and RNA was harvested at the indicated

times post-infection. The mean fold-change in DRA mRNA for each time point is indicated. A dashed outline of a data column indicates a statistically significant change compared to mock-infected ($p < 0.001$, one-tailed t test). C) Time course of HCMV-mediated down-regulation of DRB mRNA. Experiments were performed as described above. Data shown are from six experiments and six donors. A dashed outline of a data column indicates a statistically significant change compared to mock-infected ($p < 0.001$, one-tailed t test).

Fig. 5. HCMV reduces synthesis of new DR chains. Pulse-chase analysis of mock- and TB40E-infected (MOI = 100) matLC at 2 dpi. Cells were pulsed for 5 minutes and chased for the indicated times. Normalized, pre-cleared lysates were immunoprecipitated with the following antibodies, from top to bottom: DA6.147 (DRα/ Ii, DRβ), L243 (DRα/β dimer), US2N (rabbit antisera to US2), and AF8 (calnexin). One representative of three experiments is shown.