Murine Cytomegalovirus Inhibits Interferon-γ-induced Antigen Presentation to CD4 T Cells by Macrophages Via Regulation of Expression of Major Histocompatibility Complex Class II-associated Genes

By Mark T. Heise, Megan Connick, and Herbert W. Virgin IV

From the Center for Immunology and Departments of Pathology and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110

Summary

CD4 T cells and interferon-γ (IFN-γ) are required for clearance of murine cytomegalovirus (MCMV) infection from the salivary gland in a process taking weeks to months. To explain the inefficiency of salivary gland clearance we hypothesized that MCMV interferes with IFN-γ-induced antigen presentation to CD4 T cells. MCMV infection inhibited IFN-γ-induced presentation of major histocompatibility complex (MHC) class II associated peptide antigen by differentiated bone marrow macrophages (BMMΦs) to a T cell hybridoma via impairment of MHC class II cell surface expression. This effect was independent of IFN-α/β induction by MCMV infection, and required direct infection of the BMMΦs with live virus. Inhibition of MHC class II cell surface expression was associated with a six- to eightfold reduction in IFN-γ-induced IAβ mRNA levels, and comparable decreases in IFN-γ-induced expression of invariant chain (Ii), H-2Ma, and H-2Mb mRNAs. Steady state levels of several constitutive host mRNAs, including β-actin, cyclophilin, and CD45 were not significantly decreased by MCMV infection, ruling out a general effect of MCMV infection on mRNA levels. MCMV effects were specific to certain MHC genes since IFN-γ-induced transporter associated with antigen presentation (TAP)2 mRNA levels were minimally altered in infected cells. Analysis of early upstream events in the IFN-γ signaling pathway revealed that MCMV did not affect activation and nuclear translocation of STAT1α, and had minor effects on the early induction of IRF-1 mRNA and protein. We conclude that MCMV infection interferes with IFN-γ-mediated induction of specific MHC genes and the Ii at a stage subsequent to STAT1α activation and nuclear translocation. This impairs antigen presentation to CD4 T cells, and may contribute to the capacity of MCMV to spread and persist within the infected host.

Human cytomegalovirus (HCMV) is a major cause of morbidity and mortality in immunocompromised individuals. Studies using murine cytomegalovirus (MCMV), which serves as a useful animal model for HCMV, have demonstrated that MCMV infection provokes strong responses by both innate and specific arms of the immune system. However, even in the immunocompetent host, MCMV; (a) causes disseminated acute infection; (b) persistently produces infectious virus within the salivary gland for weeks to months after induction of specific immunity; and (c) establishes a life-long latent state. This suggests that the virus is able to evade or modify responses by the immune system.

Multiple components of the innate and specific immune responses are active during acute MCMV infection. IFN-α/β, TNF-α, IL-12, and IFN-γ contribute to the control of MCMV during initial stages of infection (1-5). NK cells contribute to control of MCMV infection (6) through production of IFN-γ (3, 4) and cytotoxicity (7). Specific immune function is required for protection from virus-induced mortality (8). CD8 T cells mediate clearance of infectious virus from most peripheral organs and confer protective immunity (9). However, CD4 T cells can effectively clear MCMV infection from peripheral organs in the absence of CD8 T cells (10). Clearance of MCMV from the salivary gland involves CD4 T cells (11) and IFN-γ (1).

Both MCMV and HCMV have evolved mechanisms for evading CD8 T cells and NK cells. Both viruses inhibit...
MHC class I expression on infected cells (12–16). Similarly, both MCMV and HCMV interfere with NK cell activity through the actions of virally encoded MHC class I homologs (17, 18). Since CD4 T cells are also essential for control of MCMV infection from salivary gland, it is not surprising that MCMV inhibits priming of CD4 T cells in vivo (19). However, mechanisms underlying CMV-mediated inhibition of CD4 T cell activation have not been completely defined. HCMV-induced IFN-γ, as well as direct infection, inhibits IFN-γ- induced MHC class II expression in endothelial cells (20, 21). HCMV also alters MHC class II expression in cultured human peripheral blood macrophages (Mφs; reference 22). Similarly, MCMV-induced IFN-α/β inhibits MHC class II expression on Mφs during the innate immune response (23).

Mφs are important in the pathogenesis of both HCMV and MCMV. Key functions of Mφs include presentation of antigen to CD4 T cells via H M C class II and secretion of cytokines. Mφs are a site of MCMV replication in multiple sites (24–26). Dissemination of MCMV (and likely HCMV) to secondary sites of infection is mediated by Mφs or Mφ-like cells within the blood (25–27). Mφs are a site of long-term latency for HCMV and MCMV (28–31).

In these studies, MCMV impaired IFN-γ-induced MHC class II–dependent antigen presentation by bone marrow (BM) Mφs. This effect was due to the failure of IFN-γ to efficiently induce mRNA levels for IAβ, invariant chain (II), H-2Mα, and H-2Mβ in infected cells, whereas quantities of transporter associated with antigen presentation (TAP) mRNA were unaltered by infection. This effect on MHC class II–mediated antigen presentation may contribute to the persistence of MCMV in the host, particularly in the salivary gland, where CD4 T cells play a critical role.

Materials and Methods

Animals, Media, and BM Mφ Culture. 4–12-wk-old 129Ev/Sv and IFN-α/β receptor-deficient (IFN R-α/βR-/-) mice (32) were housed in a Biosafety Level 2 facility at Washington University, in accordance with all Federal and University policies. Sentinel animals were negative for adventitious mouse pathogens by serology. Virus stocks were grown and diluted in low endotoxin (≤0.025 ng/ml) DME containing 10% FCS (DME 10%; reference 3). BM Mφs were prepared as previously described (23) and were at least 95% F4/80 positive (data not shown). BM Mφs were mock infected or infected with MCMV or UV-inactivated MCMV, at a multiplicity of infection (MOI) of 5.0 unless otherwise stated, for 1 h at 37°C in 5% CO2 with rocking every 5 min. After infection, cells were treated with IFN-γ (100 IU/ml; Genentech, San Francisco, CA) or medium alone for various periods of time while incubating at 37°C in 5% CO2. Cells were harvested by scraping, then fixed, and analyzed for IAβ expression by flow cytometry. BM Mφ viability was determined by trypan blue exclusion.

Viruses and Viral Assays. MCMV (American Type Culture Collection [ATCC] No. VR-194, Lot 10) was grown, inactivated by UV irradiation, and titrated by plaque assay in BALB/3T12-3 fibroblasts (ATCC, CCL 164; reference 3). Recombinant MCMV expressing bacterial β-galactosidase (R M 427; reference 25) was a gift of Dr. Edward Mocarski (Stanford University, Stanford, CA). 3T12 cells as well as MCMV stocks were negative for mycoplasma using the Mycoplasma TC test kit (Gen-Probe, San Diego, CA).

Analysis of Cell Surface Protein Expression. Flow cytometry was performed as previously described (3, 23) on an Epics flow cytometer (Coulter, Miami, FL) using XL analysis software (Coulter) or WinMDI 2.0 (Joseph Trotter, San Diego, CA). IAβ high and low BM Mφs were separated by fluorescent activated cell sorting on a FACScan Vantage fluorescent activated cell sorter (Becton Dickinson, San Jose, CA). β-galactosidase expression in sorted cells was detected by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining (33).

Supernatant Transfer Studies. Wild-type 129 BM Mφs were either mock infected or infected with MCMV for 48 h. Supernatants were ultracenrifuged for 30 min at 100,000 g to remove free virus as confirmed by plaque assay, and then placed on naive cultures of wild-type or IFN-α/βR-/- BM Mφs at 2 ml plate in 60-mm dishes (Sarted, N ewton, N C) for 1 h. In additional groups, MCMV was added to the cultures at an MOI of 5.0. After 1 h of incubation, IFN-γ (100 IU/ml) was added and culture volumes were raised to 3 ml with fresh medium. Cultures were incubated at 37°C for 48 h and were assayed for IAβ expression.

Electrophoretic Mobility Shift Assays. BM Mφs were either mock infected or infected with MCMV for 1 h at 37°C. For STAT1 activation, cells were harvested at 1, 24, and 48 h after infection. 106 cells were suspended in endotoxin-free PBS (Sigma Chemical Co., St. Louis, MO) containing 10% FCS, and were treated with IFN-γ at concentrations ranging from 0.5 to 1,000 IU/ml for 10 min. Nuclear extracts were prepared and assayed by electrophoretic mobility shift assay (EMSA) against a 32P-labeled oligonucleotide probe derived from the IFN-γ activating sequence of the FcγRII promoter (34). Anti-STAT1 super-shift assays were performed by incubation of 2 μg of STAT1 (p91) specific antisera (Santa Cruz Biotechnology, Santa Cruz, CA) with the nuclear extract/probe mixture for 45 min at 25°C before acrylamide gel electrophoresis. For analysis of IRF-1 DNA-binding activity, BM Mφs were removed from medium containing L cell conditioned medium (23) for 15 h before infection with MCMV for 1 h followed by treatment with IFN-γ (100 IU/ml) for an additional 4 h. nuclear extracts were generated from 106 cells (34) and assayed for IRF-1 DNA-binding activity by EMSA using a 32P-labeled oligonucleotide containing the IFN response factor (IRF)-E of the murine (2′-5′) oligoadenylate synthase promoter (35). Super-shift assays for IRF-1 were performed by adding up 2 μg of anti-murine IRF-1 polyclonal antisera (Santa Cruz Biotechnology) to mixtures of extract and probe and incubating at 25°C for 5 min before analysis.

Northern Blot Analysis. Total cellular RNA was harvested from BM Mφ cultures using RNeZol (Tel-Test Inc., Friendswood, TX) and analyzed by Northern blot hybridization (36). PCR-derived probes used for these studies included a 1000-bp fragment of the murine IAβ beta chain (5’-cgcgcttgcctccgcatgcatgtac; 3’-cgccgatccatccgacagaaaggtctcaggag), a rat β-actin fragment (5’-ttgtaggaaggattggcag; 3’-gtcagccagcaggtgcat; gift of Dr. J. Milbrandt, Washington University) and a 226-bp fragment of mouse IκBα p33 cDNA (5’-aggtggattcagctggtctcgc; 3’-gacagccagcatcaagggcag; gift of Dr. E. Unanue, Washington University, St. Louis, MO). The following cDNAs were also used to generate probes: murine IRF-1 (37), rat cytoplasm (38), murine CD45 (provided by Dr. M. Thomas, Washington University), TAP1 (39), TAP2 (40), H-2Mα, and H-2Mβ (41) (the latter four gifts of Dr. J. Monaco, University of Cincinnati, Cincinnati, OH). All probes were radiolabeled using the
Results

MCMV Inhibition of Antigen Presentation to CD4 T Cells. We examined IFN-γ-stimulated presentation of peptide antigen to CD4 T cells by primary BM M̄̃̄ś̄̄s after infection with MCMV. Primary cells were used because previous studies of MCMV regulation of MHC class I expression had revealed differences between cell lines and primary cells (15). M̄̃̄ś̄̄s were either mock infected or infected with MCMV, treated with IFN-γ for 24 h, fixed, and tested for the ability to present peptide antigen (Fig. 1 A). IFN-γ treatment of M̄̃̄ś̄̄s resulted in effective presentation of β-galactosidase peptide (429–441) to the T cell hybridoma B11, whereas MCMV infection abolished IFN-γ-induced antigen presentation. The antigen presenting cell, rather than the T cell hybridoma, was impaired by virus since (a) M̄̃̄ś̄̄s were fixed before incubation with T cells, and (b) addition of fixed MCMV infected M̄̃̄ś̄̄s did not efficiently inhibit the capacity of uninfected M̄̃̄ś̄̄s to present peptide antigen (Fig. 1 A). Cell surface IAβ levels were evaluated by flow cytometry, since decreased IAβ expression might explain defective antigen presentation. MCMV-infected M̄̃̄ś̄̄s failed to respond to IFN-γ stimulation by upregulation of cell surface IAβ (Fig. 1 B).

MCMV Blockade of IFN-γ Induction of IAβ Cell Surface Expression. MCMV and HCMV infection at a low MOI impair IFN-γ-induced MHC class II expression via induction of IFN-α/β (20, 23). To evaluate MCMV’s effects on M̄̃̄ś̄̄ responsiveness to IFN-γ independently of IFN-α/β, all experiments were performed at a high MOI in M̄̃̄ś̄̄s derived from mice carrying a null mutation in the IFN-α/β receptor (IFN-α/βR−/−; reference 32). These M̄̃̄ś̄̄s do not

Figure 1. MCMV infection impairs IFN-γ-enhanced peptide presentation by MHC class II. 129 mouse BM M̄̃̄ś̄̄s were mock infected or infected with MCMV at an MOI of 5.0 for 1 h, and stimulated with IFN-γ (100 IU/ml) or medium alone. After 24 h BM M̄̃̄ś̄̄s were assayed for ability to present peptide antigen, and analyzed for MHC class II expression by flow cytometry. (A) M̄̃̄ś̄̄s were plated at 105, 5 × 105, 105, and 5 × 105 cells per well, and incubated with the T cell hybridoma B11 (β-galactosidase peptide 429-441, IAβ-restricted) in the presence of β-galactosidase peptide 429-441 control peptide, or medium alone for 24 h. Supernatants were then assayed for IL-2–dependent cell line CTLL2 as measured by 3H thymidine incorporation (43). No IL-2 production above background was observed using a control peptide derived from the MHC class II molecule IAβ in the presence of β-galactosidase peptide 429-441, IAβ-restricted, pro-M (data not shown). (B) Cell surface expression of the MHC class II molecule IAβ on BM M̄̃̄ś̄̄s infected with MCMV and stimulated with IFN-γ as above was measured by flow cytometry.

Figure 2. Infectious MCMV impairs IFN-γ-induced MHC class II expression on M̄̃̄ś̄̄s in a dose-dependent manner. IFN-α/βR−/− BM M̄̃̄ś̄̄s were mock infected or infected with live or UV-inactivated MCMV at an MOI of 1.5, 3.0, or 6.0 for 1 h before addition of 100 IU/ml IFN-γ. M̄̃̄ś̄̄s were harvested 48 h after infection and assayed for MHC class II expression (IAβ) by flow cytometry. Shown is one of three experiments, each of which yielded similar results.
Infection of Mφ raising the possibility that a soluble mediator might contribute to low class II expression in β-galactosidase-negative cells.

To determine whether soluble factors played a role in MCMV’s inhibition of IAβ induction in IFN-α/βR−/− Mφs, supernatant transfer studies were performed. Supernatants from wild-type 129 Mφs infected with MCMV or mock infected for 48 h were ultracentrifuged to remove free virus and placed on naive Mφ cultures derived from either wild-type 129 or IFN-α/βR−/− mice. IFN-γ was added to these secondary cultures, and after 48 h, IAβ expression was evaluated (Fig. 4). As expected from our previous work (23), supernatant from MCMV-infected Mφs inhibited IFN-γ-induced IAβ expression on wild-type Mφs, but not on IFN-α/βR−/− Mφs (Fig. 4), confirming that MCMV-induced IFN-α/β can inhibit IFN-γ-induced MHC class II expression. However, there was no evidence for a soluble mediator affecting IAβ expression in MCMV-infected IFN-α/βR−/− Mφ cultures (Fig. 4). Therefore, direct infection of the Mφ, rather than a soluble mediator, is likely to explain MCMV’s effects on MHC IAβ expression.

MCMV Inhibits IFN-γ-induced MHC Class II and Invariant Chain mRNA Expression. IFN-γ induction of MHC class II expression occurs at the level of gene transcription (45), and studies in HCMV-infected endothelial cells have shown that HCMV infection regulates MHC class II mRNA levels (21). We therefore evaluated MCMV’s ef-
MHC Class II

Figure 4. Supernatants from MCMV-infected Mφs inhibit IFN-γ induction of MHC class II on normal but not UV-inactivated MCMV. Wild-type 129 BM Mφs were either mock infected or infected with MCMV at an MOI of 5.0. At 48 h after infection, supernatants were harvested. MHC class II (IAb) expression was determined by flow cytometry. Shown is data from one of two experiments yielding similar results. (US, unstimulated).

Figure 5. MCMV infection reduces IFN-γ-induced MHC class II and Ii mRNA levels. IFN-α/βR−/− BM Mφs were either mock infected or infected with MCMV or UV-inactivated MCMV at an MOI of 5.0. 1 h after infection, cells were treated with medium alone or with IFN-γ (100 IU/ml). Total cellular RNA was harvested at either 24 or 48 h after infection and analyzed by Northern blot hybridization for the IAb beta chain or Ii. β-actin hybridization is also shown. Similar results were obtained in three separate experiments.

Figure 6. STAT1α activation and nuclear translocation after IFN-γ stimulation is normal in MCMV-infected Mφs. IFN-α/βR−/− BM Mφs were infected with MCMV at an MOI of 5.0 or mock infected for 1 or 24 h. Harvested cells were incubated with IFN-γ for 10 min and nuclear extracts were prepared. Extracts were assayed for STAT1α activation by EMSA against a 32P-labeled IFN-γR1 promoter. The presence of STAT1α in the complex was confirmed by super-shift with antibody to IRF-1. The presence of STAT1α in the activated complex was confirmed by super-shift with antibody against STAT1α (Fig. 6), whereas antibody to IRF-1 did not result in retardation of the complex (data not shown). IFN-γ treatment resulted in comparable levels of STAT1α DNA binding activity in both mock- and MCMV-infected Mφs at 1, 24 (Fig. 6), and 48 (data not shown) h after infection.
MCMV Inhibition of IFN-γ-induced MHC Gene Expression

MCMV Inhibits IFN-γ Induction of Specific MHC Genes. To assess the specificity of the block of IFN-γ signaling caused by MCMV infection, we examined levels of several IFN-γ-induced transcripts in addition to IAβ, Ii, and IRF-1. IFN-α/βR−/− Mφs were infected with MCMV or UV-inactivated virus, or mock infected for 24 or 48 h in the presence or absence of IFN-γ, and mRNA levels were analyzed by Northern blot hybridization. IFN-γ induction of H-2Mα and H-2Mβ expression was significantly inhibited by MCMV at 24 h (Fig. 8), whereas IFN-γ-induced TAP1 mRNA levels were decreased to a lesser extent. However, TAP2 mRNA levels were not significantly decreased by MCMV infection (Fig. 8). Northern blot data from 48-h infections were similar to that of 24-h infections (data not shown). The lack of effect on IFN-γ induction of TAP2 transcript levels by MCMV demonstrated that MCMV selectively affects expression of a subset of IFN-γ-inducible genes.

Discussion

CD4 T cells are required for clearance of salivary gland MCMV infection, yet infectious virus is found in the salivary gland for many weeks after acute infection, suggesting that MCMV antagonizes CD4 T cell function in some manner. It has also been shown that MCMV impairs CD4 T cell priming in vivo (19). These observations led us to examine the effects of MCMV on presentation of antigen to CD4 T cells and induction of genes associated with MHC class II cell surface expression.

MCMV inhibited IFN-γ induction of IAβ and Ii gene expression in IFN-γ/βR−/− BM Mφs, resulting in defective antigen presentation to CD4 T cells. Inhibition of IAβ expression required direct infection of the Mφs with live virus. Early IFN-γ signaling events including STAT1α activation and IFN-γ induction remained largely intact in MCMV-infected cells. Further analysis showed that MCMV infection altered the induction of multiple genes encoding proteins important for MHC class II expression and/or presentation of peptide antigen to CD4 T cells.
Although there is a formal possibility that MCMV exerts its effect at the level of mRNA stability, we doubt this for two reasons. First, we saw little evidence of a general effect of MCMV on mRNA stability in the presence or absence of IFN-γ over multiple experiments based on comparisons of cyclophilin, β-actin, and CD45 mRNA levels. Second, the effects of MCMV are specific to certain MHC genes. Thus, if MCMV infection interferes with IFN-γ induction of MHC genes by destabilizing mRNA, one would have to argue that the effects are specific for certain transcripts (e.g., IAb and II but not TAP2, β-actin, or cyclophilin).

We have documented a novel mechanism of immune avoidance by CMV, selective blockade of IFN-γ induction of genes involved in antigen presentation to CD4 T cells. Further characterization of MCMV’s block on IFN-γ signaling, and identification of the viral genes involved will likely enhance understanding of CMV pathogenesis and the antiviral functions of the immune system.
agents for analysis of STAT1 activation; Dr. Paul Allen supplied the T cell hybridoma and peptide antigen used in antigen presentation studies; Dr. John M onaco supplied several mouse cDNAs; and Dr. Edward Mocarski, who supplied MCMV mutant R M 427.

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Address correspondence to Herbert W. Virgin IV, Department of Pathology, Box 8118, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Phone: 314-362-9223; Fax: 314-362-4096; E-mail: virgin@immunology.wustl.edu

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