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 IA^b 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)[1] 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

[1] *Abbreviations used in this paper:* BMMϕ, bone marrow macrophage; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay; HCMV, human CMV; IRF, IFN response factor; Mϕ, macrophage; MCMV, murine CMV; MOI, multiplicity of infection; TAP, transporter associated with antigen presentation.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/98/04/1037/10 $2.00
Volume 187, Number 7, April 6, 1998 1037–1046
http://www.jem.org

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-β, 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 MHC 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). Monocytes and 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 mRNAs for IA^b, invariant chain (Ii), H-2Ma, and H-2Mb in infected cells, whereas quantities of transporter associated with antigen presentation (TAP)2 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 BMMϕ Culture. 4–12-wk-old 129Ev/Sv and IFNαβ receptor–deficient (IFNR-$\alpha/\beta R^{-/-}$) 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). BMMϕs were prepared as previously described (23) and were at least 95% F4/80 positive (data not shown). BMMϕ cultures 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% CO_2 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% CO_2. Cells were harvested by scraping, then fixed, and analyzed for IA^b expression by flow cytometry. BMMϕ 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 titered by plaque assay in BALB/3T12-3 fibroblasts (ATCC, CCL 164; reference 3). Recombinant MCMV expressing bacterial β-galactosidase (RM427; 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). IA^b high and low BMMϕ were separated by fluorescent activated cell sorting on a FACS® 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 BMMϕs were either mock infected or infected with MCMV for 48 h. Supernatants were ultracentrifuged 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-$\alpha/\beta R^{-/-}$ BMMϕs at 2 ml/plate in 60-mm dishes (Sarstedt, Newton, NC) 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 IA^b expression.

Electrophoretic Mobility Shift Assays. BMMϕs were either mock infected or infected with MCMV for 1 h at 37°C. For STAT1α activation, cells were harvested at 1, 24, and 48 h after infection. 10^6 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 ^{32}P-labeled oligonucleotide probe derived from the IFN-γ activating sequence of the FcγRI promoter (34). Anti-STAT1 super-shift assays were performed by incubation of 2 μg of STAT1 (p91) specific antiserum (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, BMMϕ were removed from medium containing L cell conditional 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 10^6 cells (34) and assayed for IRF-1 DNA binding activity by EMSA using a ^{32}P-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 2 μg of anti–murine IRF-1 polyclonal antisera (Santa Cruz Biotechnology) to mixtures of extract and probe and incubating at 25°C for 45 min before analysis.

Northern Blot Analysis. Total cellular RNA was harvested from BMMϕ cultures using RNAzol (Tel-Test Inc., Friendswood, TX) and analyzed by Northern blot hybridization (36). PCR-derived probes used for these studies included a 300-bp fragment of the murine IA^b beta chain (5′-ccggaattcgccagtgcctccagaggtgacagtgtatc; 3′-cgcggatccccatgccacagaaacaggtctcaggag), a rat β-actin fragment (5′-tatggagaagatttggcacc; 3′-gtccagacgcaggatggcat; gift of Dr. J. Milbrandt, Washington University) and a 226-bp fragment of mouse Ii p33 cDNA (5′-agctctgtacaccggtgtctctgtcc; 3′-gacattggacgcatcagcaagggag; gift of Dr. E. Unanue, Washington University, St. Louis, MO). The following cDNAs were also used to generate probes: murine IRF-1 (37), rat cyclophilin (38), murine CD45 (provided by Dr. M. Thomas, Washington University), TAP1 (39), TAP2 (40), H-2Ma, and H-2Mb (41) (the latter four gifts of Dr. J. Monaco, University of Cincinnati, Cincinnati, OH). All probes were radiolabeled using the

Megaprime DNA Labeling System (Amersham Corp., Arlington Heights, IL). Loading of mRNA was normalized against 28S ribosomal RNA (42) or against cyclophilin or β-actin mRNA levels. Northern blot hybridizations were quantitated on a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Fold reduction of IFN-γ–induced mRNA levels by MCMV was computed as [(IFN-γ–stimulated signal in uninfected cells) − (unstimulated signal in uninfected cells)]/[(IFN-γ–stimulated signal in infected cells) − (unstimulated signal in infected cells)], after signal was normalized to 28S rRNA or cyclophilin levels.

Antigen Presentation Assays. BMMφs were mock infected or infected with MCMV and treated with IFN-γ (100 IU/ml) for 24 h. Cells were then harvested, counted, and incubated in 1% paraformeldahyde in RPMI 10% FCS for 15 min at 25°C. The fixed cells were washed vigorously, plated at 10^3, 5×10^3, 10^4, or 5 x 10^4 cells/well, and incubated with the T cell hybridoma B11 (IA^b-restricted, β-galactosidase peptide 429-441–specific, provided by Dr. Paul Allen, Washington University) in the presence of β-galactosidase peptide 429-441, control peptide, or medium alone for 24 h. Supernatants were then assayed for IL-2 by proliferation of the IL-2–dependent cell line CTLL2 as measured by [^{3}H]thymidine incorporation (43). No IL-2 production above background was observed using a control peptide derived from the MCMV MCK-1 open reading frame (VVLVVSTVADLREPC; reference 44) at 10, 1, or 0.1 μM (data not shown).

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 BMMφs 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). Mφs 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 Mφs 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*) Mφs were fixed before incubation with T cells, and (*b*) addition of fixed MCMV infected Mφs did not efficiently inhibit the capacity of uninfected Mφs to present peptide antigen (Fig. 1 *A*). Cell surface IA^b levels were evaluated by flow cytometry, since decreased IA^b expression might explain defective antigen presentation. MCMV-infected Mφs failed to respond to IFN-γ stimulation by upregulation of cell surface IA^b (Fig. 1 *B*).

MCMV Blockade of IFN-γ Induction of IA^b 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 Mφ responsiveness to IFN-γ independently of IFN-α/β, all experiments were performed at a high MOI in Mφs derived from mice carrying a null mutation in the IFN-α/β receptor (IFN-α/β$R^{-/-}$; reference 32). These Mφs do not

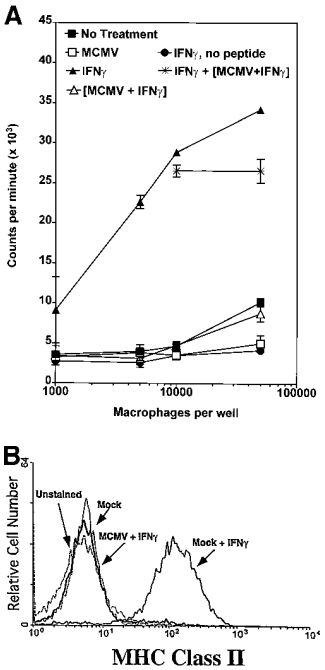

Figure 1. MCMV infection impairs IFN-γ–enhanced peptide presentation by MHC class II. 129 mouse BMMφs were mock infected or infected with MCMV at an MOI of 5.0 for 1 h, and stimulated with IFN-γ (100 IU/ml) or medium alone. After 24 h BMMφs were assayed for ability to present peptide antigen, and analyzed for MHC class II expression by flow cytometry. (*A*) Mφs were plated at 10^3, 5×10^3, 10^4, and 5×10^4 cells per well, and incubated with the T cell hybridoma B11 (β-galactosidase 429-441, IA^b-restricted) in the presence of β-galactosidase peptide (429–441) at a concentration of 10 μM. Supernatants were harvested and assayed for IL-2 content using [^{3}H]thymidine incorporation by CTLL2 cells. Mock + IFN-γ and MCMV + IFN-γ BMMφs were mixed 1:1 and assayed as a control for potential toxic effects of MCMV-infected cells. Similar data was obtained at a peptide concentration of 1.0 μM. Data is from one of two similar experiments. All groups were plated in triplicate and are shown as mean cpm ± SEM. (*B*) Cell surface expression of the MHC class II molecule IA^b on BMMφs infected with MCMV and stimulated with IFN-γ as above was measured by flow cytometry.

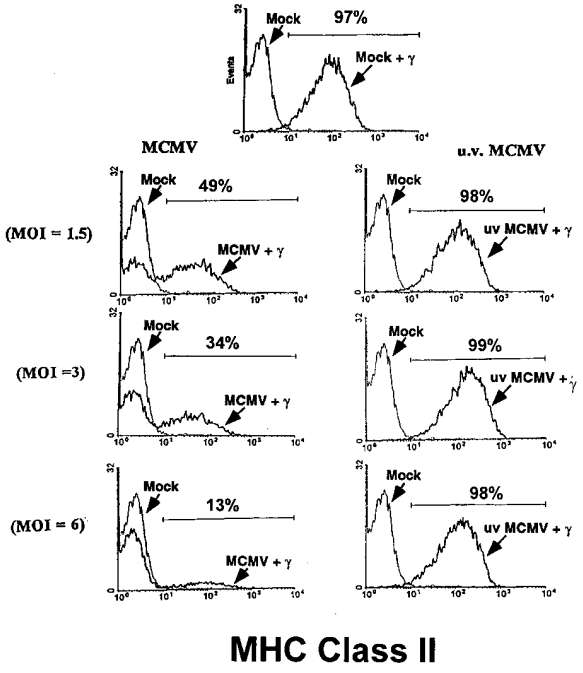

Figure 2. Infectious MCMV impairs IFN-γ–induced MHC class II expression on Mφs in a dose-dependent manner. IFN-α/β$R^{-/-}$ BMMφs were mock infected or infected with live or UV-inactivated MCMV at an MOI of 1.5, 3.0, or 6.0 for 1 h before addition of 100 IU/ml IFN-γ. Mφs were harvested 48 h after infection and assayed for MHC class II expression (IA^b) by flow cytometry. Shown is one of three experiments, each of which yielded similar results.

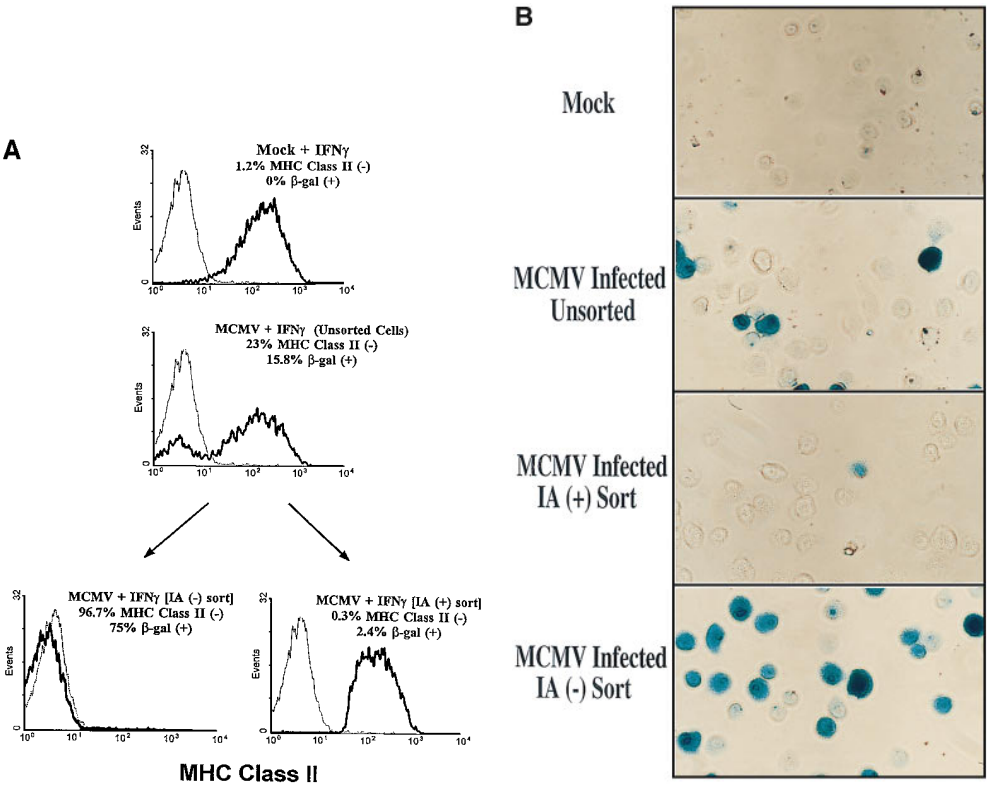

Figure 3. MCMV-infected BMMϕs express low levels of MHC class II. IFN-α/βR$^{-/-}$ BMMϕs were either mock infected or infected with recombinant MCMV expressing β-galactosidase (RM427) at an MOI of 0.6. 1 h after infection, cells were treated with IFN-γ (100 IU/ml) or medium alone for 48 h. Cells were harvested, stained for MHC class II (IA^b) expression, and separated into MHC class II high and low populations by fluorescent activated cell sorting. The numbers of cells expressing β-galactosidase in total and sorted cell populations was determined by 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (x-gal) staining. (*A*) Mock-infected or infected cells (*top panels*), and IA^b high and low populations from infected cultures (*bottom panels*) were stained for IA^b. The dotted line represents staining on unstimulated, mock-infected cells. The solid line depicts staining of IFN-γ–treated groups. (*B*) x-gal staining of mock- or MCMV-infected, and MHC class II high and low, populations is shown from one of three similar experiments.

respond to IFN-α/β stimulation, but do respond to stimulation with IFN-γ as measured by induction of IA^b expression (Fig. 1 *B* and Fig. 2; references 23, 32). Infection of IFN-α/βR$^{-/-}$ Mϕs with increasing doses of MCMV resulted in a progressive decrease in the percentage of cells expressing IFN-γ–induced IA^b (Fig. 2). Cell viability in the MCMV-infected Mϕ cultures was >95% at 72 h after infection. Live virus was required for inhibition of IA^b expression, since UV-inactivated MCMV, even at a MOI of 6.0, had no effect on Mϕ IA^b expression (Fig. 2).

Inhibition of MHC Class II Induction Correlates with Direct Infection of Mϕs. MCMV-mediated inhibition of IFN-γ–induced IA^b expression was dependent on viral dose (Fig. 2), which suggested a role for direct infection. To address this possibility, IFN-α/βR$^{-/-}$ Mϕs were infected with RM427 (25), a recombinant MCMV expressing β-galactosidase under the MCMV ie1/ie2 promoter/enhancer at an MOI of 0.6, and treated with IFN-γ. Cells were fixed 48 h after infection, stained for IA^b expression, and separated into IA^b high and low populations by fluorescent activated cell sorting (Fig. 3 *A*). IA^b low Mϕs were enriched for β-galatosidase positive (infected) cells (Fig. 3, *A* and *B*), whereas the IA^b high cells were only 0.5–2.5% positive for β-galactosidase. This demonstrated that IA^b expression is low in MCMV-infected BMMϕs. However, over several experiments, β-galactosidase was detected in <100% of IA^b low cells, 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 IA^b 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, IA^b expression was evaluated (Fig. 4). As expected from our previous work (23), supernatant from MCMV-infected Mϕs inhibited IFN-γ–induced IA^b 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 IA^b 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 IA^b 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-

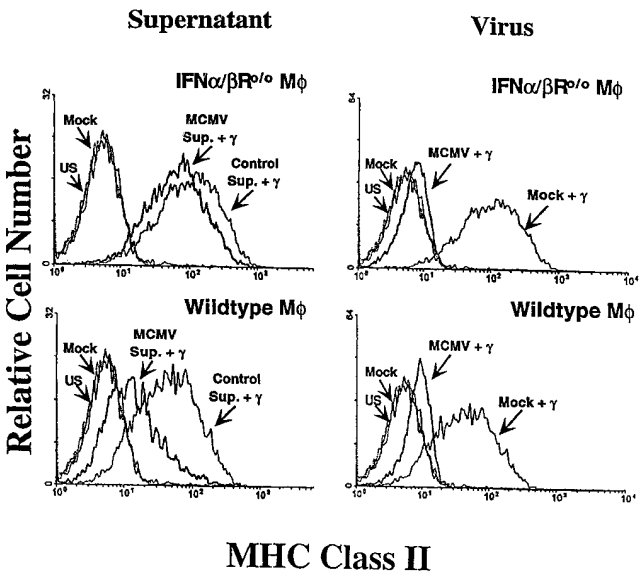

Figure 4. Supernatants from MCMV-infected Mϕs inhibit IFN-γ induction of MHC class II on normal but not IFN-α/βR$^{-/-}$ BMMϕs. Wild-type 129 BMMϕs were either mock infected or infected with MCMV at an MOI of 5.0. 48 h after infection, supernatants were removed, ultracentrifuged to remove free virus, and placed on naive cultures of either 129 or IFN-α/βR$^{-/-}$ BMMϕs. At the time of supernatant transfer, additional cultures were infected with MCMV (MOI = 5.0) as positive controls. After 1 h of incubation with the supernatants or MCMV, cells were treated with IFN-γ (100 IU/ml), incubated for 48 h, and harvested. MHC class II (IA^b) expression was determined by flow cytometry. Shown is data from one of two experiments yielding similar results. (*US*, unstained).

fect on mRNA levels of an IFN-γ–induced MHC class II gene, IA^b. Infection with MCMV, but not UV-inactivated MCMV, decreased IFN-γ–induced accumulation of IA^b mRNA 6–8-fold at 24 h and 5–>10-fold at 48 h after infection (Fig. 5).

Although a 6–8-fold reduction of IFN-γ–induced IA^b mRNA accumulation within MCMV-infected cells explained a portion of the effects of MCMV infection on antigen presentation (Fig. 1 *A*), it did not plausibly account for the at least 30-fold loss of cell surface expression of IA^b (Figs. 1 *B*, 2, 3, 4). Therefore, we examined the effect of MCMV on expression of another IFN-γ–inducible gene, Ii, which is essential for efficient cell surface expression of MHC class II (46, 47). Northern blot hybridization showed that IFN-γ–induced Ii transcript levels are reduced 5–>10-fold at 24 h and >10-fold at 48 h in MCMV-infected BMMϕ cultures. The combined effect of MCMV infection on IFN-γ–induced IA^b and Ii mRNA accumulation plausibly explains most or all of the decreased MHC class II expression on MCMV-infected Mϕs.

To address the possibility that MCMV's reduction of IA^b and Ii mRNA levels was due to a general effect on host mRNA, levels of several constitutive host mRNAs were evaluated. MCMV infection for up to 48 h did not significantly decrease levels of β-actin (Fig. 5, *A* and *B*), cyclophilin (see Fig. 8), or CD45 (data not shown) mRNAs as measured against a 28S RNA loading control. In contrast,

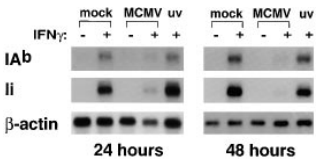

Figure 5. MCMV infection reduces IFN-γ–induced MHC class II and Ii mRNA levels. IFN-α/βR$^{-/-}$ BMMϕs were either mock infected or infected with MCMV or UV-inactivated MCMV at an MOI of 5.0. 1 h after infection, cells were treated with medium alone or with IFN-γ (100 IU/ml). Total cellular RNA was harvested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

treatment of BMMϕs with actinomycin D for 5–10 h revealed diminished β-actin, cyclophilin, and CD45 mRNA levels compared to untreated cells (data not shown), demonstrating that the half lives of these mRNAs were short enough for us to detect significant MCMV induced alteration in their stability. Thus, inhibition of IFN-γ–induced IA^b and Ii mRNA accumulation is not solely due to a general effect on mRNA levels by MCMV.

MCMV Does Not Alter IFN-γ Induction of STAT1α DNA Binding Activity. IFN-γ initiates a cascade of events that leads to MHC class II mRNA accumulation, any one of which could, a priori, be altered by MCMV infection. We examined the effect of MCMV on an essential early event in IFN-γ receptor signaling, activation and nuclear translocation of the latent cytoplasmic transcription factor STAT1α. Activation of STAT1α is essential for IFN-γ induction of MHC class II expression (48). Mϕs were either mock infected or infected with MCMV for 1, 24, or 48 h before stimulation with IFN-γ, preparation of nuclear extracts, and evaluation of STAT1α binding activity by EMSA (Fig. 6). 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 in-

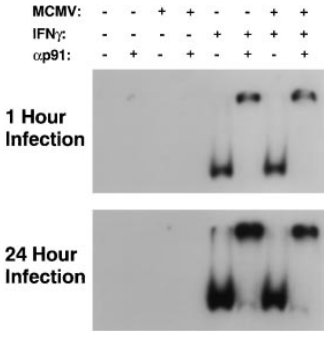

Figure 6. STAT1α activation and nuclear translocation after IFN-γ stimulation is normal in MCMV-infected Mϕs. IFN-α/βR$^{-/-}$ BMMϕs were infected with MCMV at an MOI of 5.0 or mock infected for 1 or 24 h. Harvested cells were incubated with IFN-γ for 10 min and nuclear extracts were prepared. Extracts were assayed for STAT1α activation by EMSA against a ^{32}P-labeled IFN-γ activation sequence containing oligonucleotide derived from the murine FcγR1 promoter. The presence of STAT1α in the complex was determined by antibody super-shift using STAT1α-specific antiserum. Shown are STAT1α activation by IFN-γ at 1 h (100 IU/ml IFN-γ) or 24 h (5 IU/ml IFN-γ) after infection. Similar results were obtained in four separate experiments.

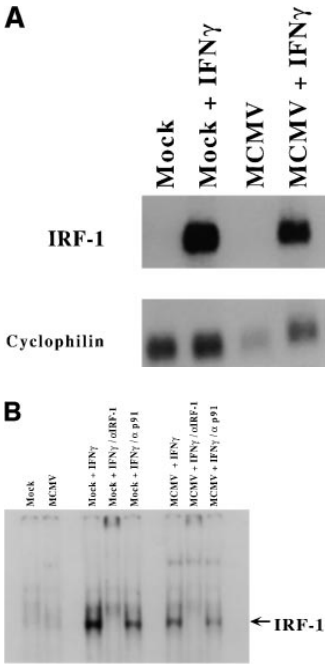

Figure 7. IRF-1 mRNA and DNA binding activity is inducible by IFN-γ in MCMV-infected Mϕs. (*A*) IFN-α/βR$^{-/-}$ were either mock infected or infected with MCMV at an MOI of 5.0 for 3 h. Cultures were treated with IFN-γ for the final 2 h of infection and assayed for IRF-1 mRNA levels by Northern blot hybridization. Shown is one of two experiments yielding similar results. (*B*) IFN-α/βR$^{-/-}$ BM-Mϕs were infected with MCMV at an MOI of 5.0 or mock infected for 5 h. Cells were treated with IFN-γ for the final 4 h of infection and nuclear extracts were prepared. IRF-1 DNA binding activity was assessed by incubating extract with a ^{32}P-labeled IRF-E oligonucleotide probe, which was then analyzed by EMSA. The presence of IRF-1 within the complex was confirmed by incubation with antiserum against murine IRF-1 or STAT1α. Three separate experiments yielded similar results.

fection. STAT1α activation was also equivalent in mock- and MCMV-infected Mϕs after treatment with IFN-γ at doses as low as 0.5 IU/ml (data not shown). We conclude that the IFN-γ signaling pathway remains intact through STAT1α activation in MCMV-infected BMMϕs.

MCMV Effects on IFN-γ Induction of IRF-1. To directly assess the effects of MCMV on the transactivating function of STAT1, we examined IFN-γ induction of IRF-1 mRNA and protein. IRF-1 is rapidly induced at the transcriptional level after IFN-γ stimulation in a STAT1α-dependent manner (48), with maximal mRNA expression by 2 h of cytokine treatment (49). Mϕs were mock infected or infected with MCMV at an MOI of 5.0 for 3 h and treated with IFN-γ (100 IU/ml) for the final 2 h of infection. Northern blot analysis showed that induction of IRF-1 mRNA accumulation by IFN-γ in MCMV-infected Mϕs was 70–90% that of uninfected cells (Fig. 7 *A*). To assess MCMV effects on expression of IRF-1 protein, we assayed nuclear extracts of Mϕs that were mock or MCMV infected for a total of 5 h, with IFN-γ treatment during the final 4 h for binding to the IRF-E element of the murine (2′-5′) oligoadenylate synthase promoter (35) by EMSA. IFN-γ treatment induced IRF-1 DNA binding activity in MCMV-infected cells (Fig. 7 *B*) as confirmed by super-shift of the complex after incubation with anti–IRF-1, but not anti-STAT1α, antiserum. IFN-γ–induced IRF-1 DNA binding activity was decreased by at most 50% in MCMV-infected Mϕs compared to mock-infected Mϕs. IRF-1 DNA binding activity could also be induced by IFN-γ in Mϕs that had been infected with MCMV for 24 h (data not shown).

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 IA^b, 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-2Ma and H-2Mb 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 (11), 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 IA^b and Ii gene expression in IFN-α/βR$^{-/-}$ BMMϕs, resulting in defective antigen presentation to CD4 T cells. Inhibition of IA^b expression required direct infection of the Mϕs with live virus. Early IFN-γ signaling events including STAT1α activation and IRF-1 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,

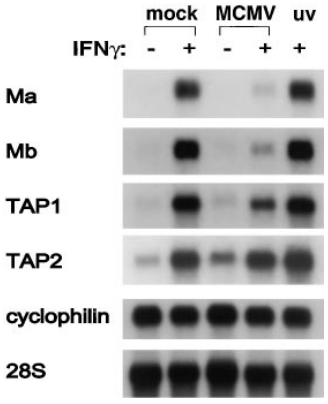

Figure 8. MCMV infection inhibits H-2Ma, H-2Mb, and TAP1 mRNA expression, but not IFN-γ–induced TAP2 mRNA expression. IFN-α/βR$^{-/-}$ BMMϕ were either mock infected or infected with MCMV or UV-inactivated MCMV at an MOI of 5.0 in the presence or absence of IFN-γ (100 IU/ml) for 24 h. Total cellular RNA was harvested and analyzed by Northern blot. Blots were probed using radiolabeled probes for murine TAP1, TAP2, H-2Ma, H-2Mb, cyclophilin, and 28S ribosomal RNA. Shown is one of three experiments which yielded similar results.

while another IFN-γ–inducible MHC gene was largely unaffected.

Effects of MCMV on MHC Class II Antigen Presentation. MCMV impaired the ability of IFN-γ to induce expression of mRNAs encoding multiple genes important for antigen presentation to CD4 T cells. These genes included the MHC class II allele IA^b, as well as the Ii p33 subunit and the peptide-loading protein subunits H-2Ma and H-2Mb. Although the 6–8-fold reduction of IFN-γ–induced IA^b mRNA levels observed in the setting of MCMV infection undoubtedly contributed to loss of cell surface expression of IA^b (e.g., Fig. 1 *B*), it was possible that additional factors accounted for the near complete inhibition of IA^b expression. Ii is required for maximal cell surface expression of MHC class II proteins (46, 47), and induction of Ii by IFN-γ was significantly inhibited in MCMV-infected cells. Decreased expression H-2Ma and H-2Mb might also contribute to impaired MHC class II peptide presentation, since these proteins are necessary for optimal loading of peptide onto MHC class II molecules (50–52). However, loss of H-2Ma and H-2Mb expression probably did not contribute to decreased detection of cell surface IA^b, as the antibody 25-9-17S detects IA^b loaded with the class II Ii-associated peptide, CLIP (50–52).

Implications of β-herpesvirus Impairment of Antigen Presentation to CD4 T Cells. Three lymphocyte classes (CD4 and CD8 T cells, and NK cells) are important for resistance to CMV during acute infection. CD8 T cells promote MCMV clearance and mediate protective immunity (9), and are also important for control of HCMV (53). As a countermeasure, both MCMV and HCMV inhibit antigen presentation to CD8 T cells by limiting MHC class I protein–dependent antigen presentation (12–16). HCMV and MCMV prevent decreased expression of MHC class I on infected cells from triggering NK cells via expression of cell surface MHC class I homologs (17, 18). CD4 T cells and IFN-γ are important for clearance of virus from the salivary gland (1, 11), but priming of CD4 T cells is inhibited in MCMV-infected mice (19). The findings in this paper delineate a potential mechanism by which MCMV might impair CD4 T cell activation and avoid recognition by this third important class of lymphocytes.

We believe that viral impairment of IFN-γ–induced antigen presentation by Mϕs in particular has important implications for acute and chronic MCMV pathogenesis. Mϕs are involved in dissemination of MCMV and HCMV (25–27) and yet they are activated by IFN-γ during acute MCMV infection (3, 23). IFN-γ inhibits MCMV growth in Mϕs and other cells (3, 54, 55). How then does the virus disseminate in a cell activated by an anti-viral cytokine? We propose that signaling by IFN-γ is inhibited in Mϕs if the virus is able to infect them and express protein before activation by IFN-γ. This would allow the virus to take advantage of the mobility of the Mϕs for dissemination, while minimizing the effects of IFN-γ on viral growth and enhancement of immune induction. Our hypothesis is supported by the observation that Mϕ-like cells that disseminate MCMV are MHC class II negative (25), whereas the majority of Mϕs responding to MCMV in inflammatory exudates from immunocompetent mice express high levels of MHC class II and are uninfected (3, 23). Furthermore, we have preliminary data for a role of IFN-γ in controlling chronic MCMV infection (Presti, R., J. Pollock, and H.W. Virgin, unpublished data). Since one site of MCMV and HCMV latency is cells of the monocyte/Mϕ lineage, interference with IFN-γ signaling in latently infected cells may permit viral escape from IFN-γ's antiviral effects, allowing reactivation and subsequent growth.

Mechanism of Inhibition of IFN-γ Induction of MHC Gene Expression. We found that MCMV infection inhibits expression of specific IFN-γ–induced genes. Other viruses such as adenovirus have also been shown to alter IFN-mediated gene induction. Adenovirus protein E1A may block IFN-α signaling by inhibition of STAT2-dependent transactivation involving p300/CBP (56). In our studies, MCMV inhibited IFN-γ signaling distal to STAT1α activation since induction of STAT1α DNA binding activity was normal in infected cells, and mRNA levels of IRF-1, a gene transactivated by STAT1α were also near normal early after IFN-γ treatment. Constitutive and inducible expression of MHC class II, H-2Ma, H-2Mb, and Ii are dependent on the class II transactivator (CIITA) (57–60). MCMV significantly inhibits induced expression of all of these genes. Therefore, one may speculate that a mechanism of viral action is the specific impairment of expression or function of CIITA.

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 mRNAs, one would have to argue that the effects are specific for certain transcripts (e.g., IA^b 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.

We would like to acknowledge helpful discussions that occurred during lab meetings shared with Dr. Sam Speck and Dr. David Leib. We would like to give special thanks to Dr. Robert Schreiber; Dr. Erika Bach; Dr. Keith Pinkard; Scott Rodig; Joan Riley and Dale Campbell, who supplied both advice and critical re-

agents for analysis of STAT1 activation; Dr. Paul Allen who supplied the T cell hybridoma and peptide antigen used in antigen presentation studies; Dr. John Monaco who supplied several mouse cDNAs; and Dr. Edward Mocarski, who supplied MCMV mutant RM427.

This work was supported by a grant to H.W. Virgin IV from the National Institute of Allergy and Infectious Diseases (RO1 AI-39616). H.W. Virgin was additionally supported by the Monsanto/Searle Biomedical Agreement. M. Connick was supported by National Institutes of Health (NIH) training grant AI-01763. M.T. Heise was supported by NIH training grant ST32 AI-07163.

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

Received for publication 12 December 1997 and in revised form 5 February 1998.

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