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Effective Inhibition of K^b- and D^b-Restricted Antigen Presentation in Primary Macrophages by Murine Cytomegalovirus

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Macrophages play an important role in murine cytomegalovirus (MCMV) infection in vivo, both in disseminating infection and in harboring latent virus. MCMV encodes three immune evasion genes (*m4*, *m6*, and *m152*) that interfere with the ability of cytotoxic T cells (CTL) to detect virus-infected fibroblasts, but the efficacy of immune evasion in macrophages has been controversial. Here we show that MCMV immune evasion genes function in H-2^b primary bone marrow macrophages (BMMϕ) in the same way that they do in fibroblasts. Metabolic labeling experiments showed that class I is retained in the endoplasmic reticulum by MCMV infection and associates with m4/gp34 to a similar extent in fibroblasts and BMMϕ. We tested a series of K^b- and D^b-restricted CTL clones specific for MCMV early genes against a panel of MCMV wild-type virus and mutants lacking *m152*, *m4*, or *m6*. MCMV immune evasion genes effectively inhibited antigen presentation. *m152* appeared sufficient to abolish D^b-restricted presentation in infected macrophages, as has been previously observed in infected fibroblasts. However, for inhibition of recognition of infected macrophages by K^b-restricted CTL, *m4*, *m6*, and *m152* were all required. The contribution of *m4* to inhibition of recognition appeared much more important in macrophages than in fibroblasts. Thus, MCMV immune evasion genes function effectively in primary macrophages to prevent CTL recognition of early antigens and show the same pattern of major histocompatibility complex class I allele discrimination as is seen in fibroblasts. Furthermore, for inhibition of K^b-restricted presentation, a strong synergistic effect was noted among *m152*, *m4*, and *m6*.

Cytomegaloviruses (CMVs) are members of the beta subfamily of herpesviruses. Like human CMV in humans, murine CMV (MCMV) establishes a lifelong infection in its natural host, the mouse. The virus establishes true latency in macrophages (21), and perhaps in other cells, but periodically reactivates and replicates, enabling it to spread to naive hosts. This lifelong infection continues in the presence of a primed host immune response, and in fact a stable balance is established between the host immune response and the virus. CD8, CD4, and NK cells are all important in maintaining control of the virus during latent infection, with CD8 cells playing the most important role (20).

In common with other herpesviruses, MCMV encodes mechanisms to interfere with the major histocompatibility complex (MHC) class I pathway of antigen presentation to CD8 T cells (12, 15). At this point three genes have been identified that interfere with the ability of CD8 cytotoxic T lymphocytes (CTL) to detect infected cells. m4/gp34 binds MHC class I molecules at the cell surface and in the endoplasmic reticulum (ER) (14, 17), m6/gp48 redirects MHC class I molecules to the lysosome (23), and m152/gp40 retains MHC class I molecules in the ER–*cis*-Golgi (5, 29). The role that these genes play in vivo in contributing to virus persistence is currently under investigation.

Most of the studies examining the functional significance of immune evasion genes in MCMV have used infected fibroblasts. Fibroblasts are readily propagated in vitro and permissive infection with MCMV is easily achieved; it is generally assumed that this model mimics infection of nonhematopoietic cells, especially epithelial cells, which are the primary site of permissive infection in vivo. However, MCMV infection in vivo involves many cell types, among which the macrophage is of particular importance (4, 7, 8). Macrophages support the full virus replication cycle (2, 28), they are probably important for disseminating virus (13), and they are a major site of MCMV latency (9, 18, 21). Because of the central role they play in CMV infection, the question of how effectively immune evasion genes function in macrophages is of particular interest. On the one hand, macrophages are professional antigen-presenting cells and are generally very good targets for CTL; thus, the task of preventing antigen presentation would presumably be more difficult for these cells than for fibroblasts. On the other hand, because macrophages harbor latent virus which must reactivate to spread, MCMV's ability to avoid detection in this cell type should be particularly important.

Hartmut Hengel and colleagues have studied CTL recognition of infected macrophages in BALB/c mice (11). They used CTL recognizing the well-characterized immediate-early (IE) antigen pp89 restricted by L^d. In fibroblasts, this epitope was presented if gene expression was limited to IE genes, but when early (E) genes (which include the immune evasion genes) were expressed, recognition of infected cells by pp89-specific CTL was lost. In contrast to fibroblasts, however, infected macrophages were recognized by pp89-specific CTL throughout the infectious cycle. The immune evasion genes were expressed at the RNA level. However, in a macrophage cell line

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(J774), MCMV infection did not lead to a decrease in cell surface class I expression. These results led the authors to conclude that macrophages could overcome the effects of the immune evasion genes and effectively present antigens to CTL.

We felt that the issue of the efficacy of immune evasion genes in macrophages was worthy of further study for several reasons. First, mutant viruses lacking immune evasion genes are now available (14, 19, 27), which enabled the function of the genes to be addressed directly. Second, it is apparent that different MHC class I alleles vary in the extent to which they are affected by different immune evasion genes (14), and we wanted to know how the H-2^b haplotype alleles are affected. Finally, the epitope studied by Hengel and colleagues is from an IE antigen, whereas most immunodominant MCMV antigens are actually encoded by E genes. Overcoming the effect of the immune evasion genes might be easier for a cell to achieve if the epitope is encoded by an IE gene, given that there is time for some unimpeded presentation before the E-encoded immune evasion genes are expressed. This seems likely because of the observation that if fibroblasts are pretreated with gamma interferon (IFN-γ), pp89 is presented throughout the infectious cycle (10); in contrast, treating fibroblasts with IFN-γ does not rescue presentation of the H-2^b-restricted E antigens that we have described (14). IFN-γ makes fibroblasts much more efficient antigen-presenting cells, and we considered that macrophages may behave like IFN-γ-treated fibroblasts in their ability to present MCMV antigens. For these reasons, we studied the effect of MCMV immune evasion genes on the presentation of K^b- and D^b-restricted antigens in infected macrophages.

MATERIALS AND METHODS

Experimental animals. C57BL/6 (B6) mice were purchased from Simonsen Laboratories (Gilroy, Calif.) and maintained according to institutional protocols.

Cell culture. Mouse embryo fibroblasts (MEFs) were grown from trypsin-digested day 12 to 14 mouse embryos and were used between passages 2 and 4. BALB3T3 cells (ATCC CCL-163) and IC21 simian virus 40 (SV40)-transformed peritoneal macrophages (ATCC TIB-186) were obtained from the American Type Culture Collection (ATCC). MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (NCS for BALB3T3 cells) and penicillin-streptomycin-glutamine. IC21 cells were maintained in RPMI supplemented with 10% FCS, penicillin-streptomycin-glutamine, 10 mM HEPES buffer, 1 mM sodium pyruvate (Gibco BRL), and 2.4 mg of glucose/ml.

Bone marrow macrophages (BMMϕ) were isolated by the protocol of Bouwer et al. (1). Bone marrow was flushed from the femurs of 6-week-or-older B6 mice and strained through a 70-μm-pore-size cell strainer. Cells were washed twice in DMEM–10% fetal bovine serum (FBS) and plated out at 10^7 total cells per 150-mm-diameter petri plate (Lab-Tek) in DMEM–10% FBS plus 30% L929 supernatant as a source of granulocyte-macrophage colony-stimulating factor (GM-CSF). (L929 supernatant is collected from L929 cells grown for 10 days after reaching confluence in DMEM–10% FBS.) Six days later BMMϕ were harvested by rinsing with room temperature phosphate-buffered saline (PBS) to remove nonadherent cells and then incubating with cold PBS at 4°C for 5 min to detach adherent cells.

Viruses. Wild-type MCMV, Smith strain, was obtained from the ATCC (1339-VR). The generation and characterization of the following MCMV mutants and revertants have been previously described: ΔMS94.5 (with a deletion of open reading frames *m151* to *165* [24]), ΔMC96.24 (with a deletion of open reading frame *m152*) and rMC96.27 (revertant for ΔMC96.24) (19), MW97.01 wild-type MCMV BAC (26), and recombinant MCMVs Δm4-MW99.03 (14), Δm152-MW99.05 (14), and Δm6 (25). Virus stocks were generated by infecting subconfluent MEFs with low-passage seed stock at a multiplicity of infection (MOI) of 0.01. Once the monolayer became 100% infected stocks were harvested by scraping and sonication of cells. The titers of PFU were determined by serial dilution and agarose overlay on BALB3T3s (ATCC).

T-cell clones. Generation of MCMV-specific CTL clones has been previously described (14). Clones 3 and 55 are D^b restricted and recognize an epitope in M45 (6). Clones 5, 11, and 96 are K^b restricted and recognize at least two as yet unidentified epitopes. The clones were propagated by the addition of irradiated splenocytes from several allogenic mice once a week and were fed with medium supplemented with rat concanavalin A supernatant and interleukin-2.

Antibodies. Serum 8010 (against exon 8 from K^b; anti-p8), and serum 8139 (anti-m4/gp34) are polyclonal rabbit antisera and have been previously described (14). Monoclonal antibody 28.14.8S (ATCC HB-27) was purified from a hybridoma supernatant. Other antibodies were obtained commercially as follows: rat immunoglobulin G (IgG) (Sigma-Aldrich, St. Louis, Mo.), anti-mouse F4/80 (MCAP497; rat IgG2b; Serotec, Raleigh, N.C.), fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Jackson ImmunoResearch), and FITC-conjugated goat anti-rabbit IgG (KPL, Gaithersburg, Md.). For immunofluorescence staining, rabbit sera (normal rabbit serum [NRS] and serum 8139) were precleared by 1:10 dilution in PBS–3% normal goat serum (NGS) and rocking, first over a 150-mm-diameter plate of fixed, permeabilized, and blocked mouse embryo fibroblasts for 24 h at 4°C and then over a 100-mm-diameter plate of fixed, permeabilized, and blocked BMMϕ for 24 h at 4°C.

FACS analysis. Cells were washed at 4°C and then incubated for 15 min in fluorescence-activated cell sorter (FACS) buffer (PBS, 1% FCS, 0.1% sodium azide, 5% normal mouse serum). Cells were washed and incubated with either rat IgG or anti-mouse F4/80 in FACS buffer for 15 min. Cells were washed, incubated with FITC-conjugated goat anti-rat IgG, and washed again. Cells were analyzed by flow cytometry using a FACScan flow cytometer (BD Pharmingen, Franklin Lakes, N.J.) in conjunction with Cell Quest (BD Pharmingen). All further analyses were performed using FlowJo software (Treestar, San Carlos, Calif.).

Immunofluorescence analysis. Cells were plated out in 10% FBS–DMEM in six-well dishes with glass coverslips at 6×10^5 BMMϕ per well or 9×10^4 MEFs (50 U/ml; IFN-γ treated) per well. Twenty-four hours later cells were infected with MCMV-wtMW97.01 at an MOI of 70. Two hours later virus was removed and replaced with 0.3 mg of phosphonoacetic acid (PAA)-treated medium per ml to prevent late gene expression. At 20 h postinfection coverslips were washed twice with PBS and fixed with freshly made 2% paraformaldehyde for 8 min. Cells were permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 2 min and washed in PBS. Fc receptors were blocked using 5% normal mouse serum–5% NGS in PBS for 45 min at 37°C. Blocking agent was removed before the addition of primary antibody (final concentration, 1% serum in PBS–3% NGS) at 37°C for 45 min. After three washes in PBS over 5 min, cells were incubated with FITC-conjugated goat anti-rabbit IgG (12.5 mg/ml in PBS–3% NGS) at 37°C for 45 min in the dark. After rinsing with PBS as described above, cells were treated with 2 μg of Hoechst 2495 (Sigma-Aldrich)/ml in PBS for 5 min at room temperature in the dark. Cells were then rinsed once with PBS and several times in distilled water. Coverslips were dried completely and mounted on glass slides (Fisher) using Prolong Antifade reagent (Molecular Probes, Eugene, Oreg.). Cells were visualized and infected cells were counted on a Bio-Rad 1024UV laser-scanning confocal microscope equipped with an Axiovert-100 (Zeiss) and a Nikon Optitek microscope.

Immunoprecipitations. C57BL/6 MEFs were pretreated with recombinant mouse IFN-γ at 50 U/μl and BMMϕ were plated in the absence of IFN-γ for 24 h before infection in 60-mm-diameter dishes. Virus medium was removed and cells were incubated overnight in 1.5 ml of cysteine- and methionine-free DMEM supplemented with antibiotics, 5% FBS, 0.3 mg of PAA/ml, and 0.33 mCi of [^{35}S]cysteine-methionine (NEN). Lysis and precipitation steps were carried out at 4°C. Cells were washed with PBS and lysed in NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-HCl [pH 7.6], 5 mM $MgCl_2$) supplemented with Complete EDTA-free protease inhibitor cocktail (Boehringer-Mannheim). Lysates were precleared by incubation with 20 μl of NRS and normal mouse serum and 500 μl of a 10% suspension of fixed *Staphylococcus aureus* for 2 h and were centrifuged for 5 min at 15,000 × *g*. Precleared lysates were then subjected to specific immunoprecipitation with ~10 μl of antibody plus 150 μl of 5% protein A-agarose suspension (Sigma-Aldrich). Immunoprecipitates were washed four times in NET buffer (150 mM NaCl, 50 mM Tris [pH 7.5], 5 mM EDTA, and 0.05% NP-40) containing 0.1% sodium dodecyl sulfate (SDS). Samples were digested with endoglycosidase H_f (endo H_f; NEB) according to the manufacturer's protocol, resuspended in reducing sample buffer, and separated by SDS-polyacrylamide gel electrophoresis on a 12.5% gel.

Cytolytic T-cell assays. MEF or IC21 target cells were plated into 96-well plates at 5,000 cells/well and treated with 50 U of recombinant mouse IFN-γ (Sigma-Aldrich) per ml, and BMMϕ were plated at 20,000 cells/well for 24 h,

infected with MCMV at the indicated MOIs in the presence of 0.3 mg of PAA (Sigma-Aldrich) per ml to prevent expression of viral late genes, and labeled with ^{51}Cr (NEN) overnight. CTL clones described here did not kill MEF targets without IFN-γ pretreatment (data not shown). T cells were added at the indicated effector-to-target ratios for 6 h, after which supernatants were harvested and assayed for γ-irradiation with a Topcount scintillation counter (Packard Instruments, Meriden, Conn.). Background Cr release was determined by incubating targets with medium alone, and total Cr release was achieved by lysing targets with medium containing 2% Triton X-100. Percent specific lysis was calculated as follows: (experimental cpm − background cpm)/(total cpm − background cpm). Each data point represents the mean of triplicate wells, and error bars represent the standard errors of the means.

RESULTS

***m152* prevents antigen presentation in SV40-transformed macrophages.** To determine if *m152* interferes with antigen presentation in H-2^b macrophages, we first used the SV40-transformed macrophage cell line, IC-21, which has previously been used to study MCMV infection of macrophages (3, 4, 8). MEFs were included for comparison as positive control cells in which the immune evasion genes are known to function. IC-21 cells and MEFs were infected with wild-type MCMV or a virus lacking *m152* and tested in a Cr release assay for lysis by an MCMV-specific CTL clone that recognizes amino acids 985 to 93 of M45 in the context of D^b (6). In both cell types, wild-type MCMV was not recognized whereas Δm152 was well recognized (Fig. 1). We concluded that *m152* is functional in the IC-21 macrophage cell line, and is capable of preventing recognition of the M45 epitope.

Establishment and characterization of bone marrow-derived macrophages. IC-21 is an SV40-transformed cell line which does not possess all the characteristics of primary macrophages. To determine whether *m152* was also functional in primary macrophages, we used the standard method to differentiate macrophages from bone marrow cells by culture in GM-CSF. Because the immune evasion genes are known to function well in fibroblasts, it was important to ensure that our primary BMMϕ cultures were not contaminated with other cells. To this end, bone marrow cells were initially cultured in GM-CSF on non-tissue-culture-treated plastic, a surface to which other cell types are unable to adhere. After removing nonadherent cells by washing in warm PBS, the adherent macrophages were removed by incubation at 4°C. FACS analysis of the resultant cells revealed that this procedure yielded a pure F4/80-positive population (Fig. 2A). To ascertain that these cells could be infected with MCMV, we infected them with increasing MOIs of wild-type MCMV and stained them with antiserum against the early protein m4/gp34. Figure 2B shows that the percentage of cells infected (and expressing an early gene) at each MOI was similar for MEFs and BMMϕ. An MOI of 50 reliably yielded infection of the majority of cells and was used for the remaining studies. Parallel studies with the Δm152 mutant MW99.05 showed that BMMϕ were equivalently infected with this virus (data not shown). MOIs between 50 and 100 were used for subsequent studies.

We routinely treat MEFs with IFN-γ for CTL and immunoprecipitation assays in order to increase the level of MHC class I expression and antigen presentation (14, 16); we find that IFN-γ increases the ability of CTL to detect Δm152-infected cells without enabling CTL to detect wild type-infected cells (data not shown). To perform a strictly parallel comparison

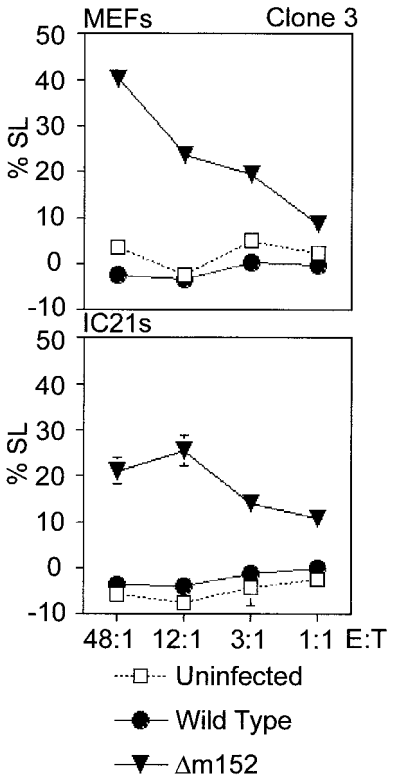

FIG. 1. m152/gp40 prevents CTL recognition in IC21 macrophages. MEFs and IC21 macrophages were treated with IFN-γ and infected at an MOI of 70 in the presence of PAA with wild-type MCMV (MW97.01) or Δm152 (MW99.05). They were then used as targets in a Cr release assay with the D^b-restricted, M45-specific CTL clone 3. Error bars represent the standard errors of the means. E:T, effector-to-target ratio; SL, specific lysis.

between MEFs and macrophages, we therefore would have liked to treat both cell types with IFN-γ. However, IFN-γ has been reported to have a deleterious effect on MCMV gene expression in macrophages over and above its impact on infectivity in fibroblasts (22). We therefore determined whether IFN-γ would impact MCMV gene expression in BMMϕ in our experiments. Figure 2C shows that IFN-γ pretreatment significantly decreased the percentage of BMMϕ that expressed the early protein m4/gp34 (Fig. 2C). In contrast to MEFs, macrophages constitutively express high levels of MHC class I molecules and are efficient antigen-presenting cells in the absence of IFN-γ. For the remaining experiments, IFN-γ was used to pretreat MEFs but not BMMϕ; under these conditions, comparable levels of infection (Fig. 2), synthesis of class I molecules (Fig. 3), and antigen presentation (Fig. 3 and 4) were obtained with both cell types. By optimizing both infectivity and the antigen-presenting ability of both cell types, we were able to compare their susceptibility to the immune evasion genes. We note in addition that in the study by Hengel et al., macrophages were not treated with IFN-γ (11).

m152/gp40 retains K^b and D^b in BMMϕ. The MCMV immune evasion gene m152/gp40 retains class I molecules in the ER–*cis*-Golgi network (29). To study the effect of m152/gp40 on class I export in macrophages, we infected cells with MCMV strains that either contained (Smith, rMC96.27) or

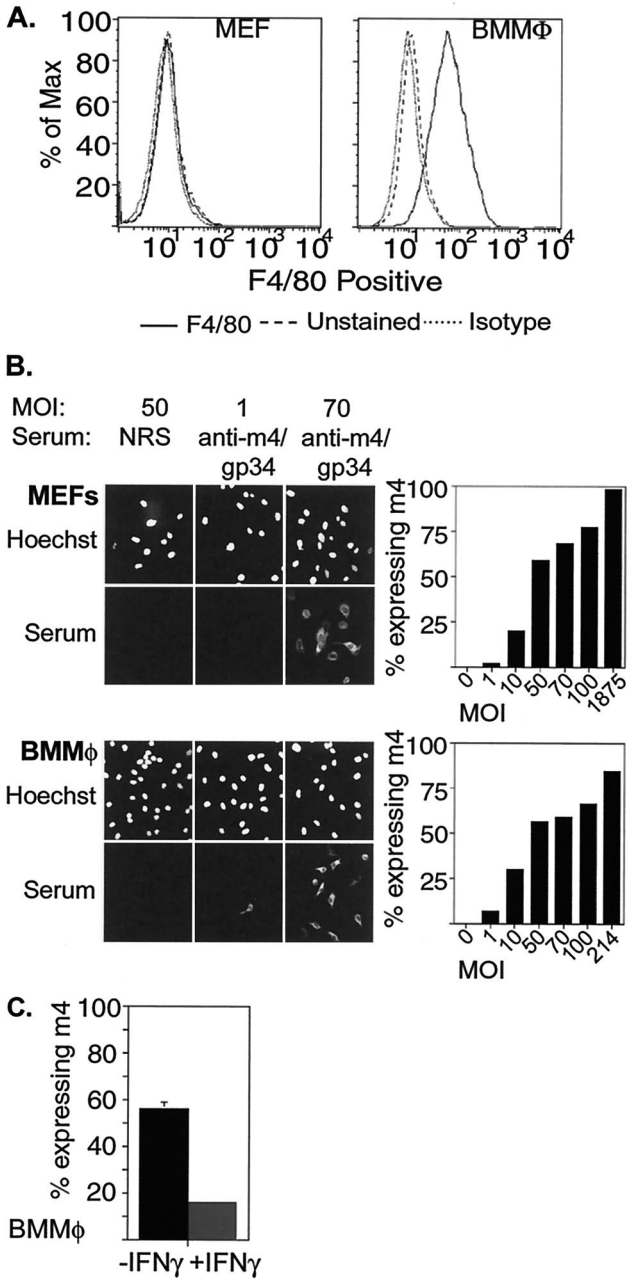

FIG. 2. Isolation and infection of BMMϕ. (A) Isolation of a pure population of macrophages. Bone marrow was flushed from the femurs and cultured for 6 days on non-tissue-culture-treated plastic petri dishes with L929 supernatant as a source of GM-CSF. Adherent cells were removed with cold PBS, stained with anti-F4/80 or isotype control, and analyzed by FACS. (B) BMMϕ and IFN-γ-pretreated MEFs were plated onto glass coverslips and infected overnight with wild-type MCMV (MW97.01) in the presence of PAA at the indicated MOIs. Cells were stained with rabbit antiserum recognizing m4/gp34 or with NRS and were treated with Hoescht DNA stain to detect all cells. Percent cells expressing m4 was determined by the ratio of cells expressing m4/gp34 to Hoechst-staining cells in the same fields. (C) BMMϕ were treated with IFN-γ (50 U/ml) for 24 h or left untreated and then infected overnight as described above at an MOI of 50. Percent cells expressing m4 was determined as described above.

lacked (ΔMC96.24) *m152*, and we continuously metabolically labeled them throughout a 16-h infection. MHC class I molecules were then immunoprecipitated with allele (K^b or D^b)-specific antibodies and treated with endo H, and the extent of class I retention in a pre-Golgi compartment was assessed by the amount of endo H-sensitive class I molecules seen. The results are shown in Fig. 3A. A substantial and equivalent increase in the number of endo H-sensitive class I molecules in infected cells was seen in both MEFs and BMMϕ. This increase in retained class I molecules is attributable to *m152*, because it is observed in wild type-infected cells, not observed in Δm152 infection, and observed again in infection with the revertant virus. We have previously observed that *m152* retains D^b much more effectively than K^b (14); this allelic difference in susceptibility is confirmed in this experiment and is observed equally in fibroblasts and macrophages. Thus, as judged by a biochemical assessment of class I transport, *m152* was functional in both macrophages and fibroblasts and showed the same MHC class I allelic preference.

***m152* prevents CTL recognition of MCMV-infected BMMϕ by CTL clones of two different specificities.** We next tested the ability of *m152* to impair antigen presentation to CTL by testing for lysis of infected cells in a Cr release assay. CTL clones of two specificities were used: clone 3 recognizes M45 restricted by D^b (6), and clone 5 recognizes an unidentified antigen restricted by K^b (14). Figure 3B shows that Δm152 virus was readily detected in both cell types, whereas wild-type MCMV was either undetected or very poorly detected. To determine how robust this impairment of recognition is, we infected cells at a range of MOIs. Although higher MOIs improved the efficiency of CTL recognition, Fig. 3C shows that Δm152 virus was better detected than the wild type across the entire range of MOIs (from 3 to 100).

As described in the introduction, Hengel and colleagues have reported efficient presentation of the L^d-restricted IE epitope pp89 by wild-type MCMV-infected macrophages. Apart from the differences in epitopes and restriction elements, one methodological difference between those studies and the ones we report here is the use of PAA. We have routinely used PAA in our assay to limit viral gene expression to E antigens in order to reduce cytopathic effects (6, 14), whereas Hengel and colleagues did not treat cells with PAA. We wanted to rule out the possibility that PAA treatment might render immune evasion genes more effective in macrophages, accounting for the difference between the two studies. However, as shown in Fig. 3D, the addition of PAA made no difference: in either the presence or absence of PAA, Δm152 virus was recognized whereas wild-type virus was not.

At high effector-to-target ratios and high MOIs, we sometimes see about 10% specific lysis of wild type-infected cells by K^b-restricted CTL clones (see, for example, Fig. 3B, clone 5, and Fig. 4, clone 11). As in the assay for Fig. 3B, there is always very much better lysis of cells infected with Δm152 virus. We have not detected any lysis of wild type-infected BMMϕ by D^b-restricted CTL. We conclude that, while there may be some low level of detection of wild-type virus in infected macrophages by K^b-restricted CTL, *m152* nevertheless functions in these cells to powerfully impair antigen presentation.

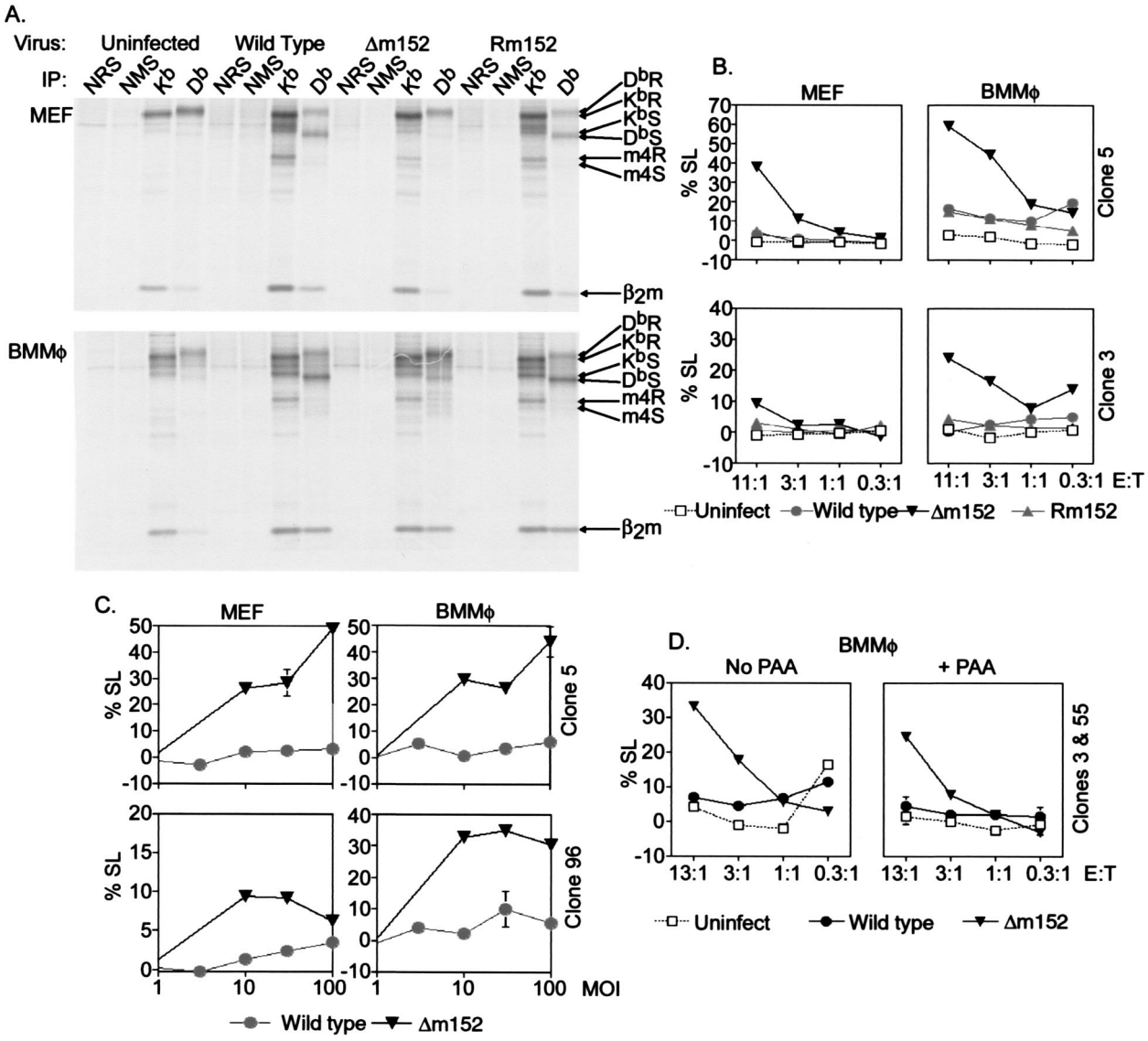

FIG. 3. m152/gp40 retains class I MHC molecules and interferes with antigen presentation in MEFs and BMMϕ. (A) *m152* retains MHC class I molecules in a pre-Golgi compartment in BMMϕ. MEFs were pretreated with IFN-γ for 48 h. MEFs and BMMϕ were infected with wild-type MCMV (Smith), Δm152 (MC96.24), or revertant Δm152 (rMC96.27) or were left uninfected. Cells were ^{35}S labeled overnight in the presence of 0.3 mg of PAA/ml. Cell lysates were subjected to immunoprecipitation (IP), endo H treated, and run on an SDS–12.5% polyacrylamide gel electrophoresis gel. MEF lysates were immunoprecipitated with serum 8010 (rabbit anti-K^b), NRS control for 8010, 28.14.8S (anti-D^b), or normal mouse serum (NMS) control for 28.14.8S. endo H-resistant (R) and -sensitive (S) forms of K^b, D^b, and m4 are indicated. The identification of the m4 bands is based on the reimmunoprecipitation of these bands with anti-m4 serum in previous similar experiments (17) and on the appearance of these bands in cells infected with virus lacking m6 (data not shown). (B) *m152* inhibits CTL recognition of MCMV-infected BMMϕ. BMMϕ and IFN-γ-pretreated MEF targets were ^{51}Cr loaded and infected at an MOI of 45 overnight, in the presence of PAA, with no virus, Smith (wild-type MCMV), Δm152 (MC96.24), or revertant Δm152 (rMC96.27). K^b-restricted CTL clone 5 and D^b-restricted clone 3 were tested for the ability to recognize and lyse targets. SL, specific lysis. (C) Wild-type virus is poorly recognized in BMMϕ regardless of MOI. Targets were infected as described before with Δm152 (MW99.05) or wild-type (MW97.01) virus at the MOIs indicated and were tested for lysis by CTL clones 5 and 96 (both K^b restricted). The effector-to-target ratio (E:T) for both clones was 20:1 for MEFs and 5:1 for BMMϕ. (D) PAA does not affect recognition of wild-type virus in BMMϕ. Targets were infected as described before in the presence or absence of PAA, and a CTL assay was performed as before using a mixture of clones 3 and 55 (both specific for M45 restricted by D^b).

***m4* interferes with K^b-restricted antigen presentation in BMMϕ.** *m4* is an immune evasion gene of MCMV whose product, m4/gp34, associates with MHC class I molecules and cooperates with m152/gp40 to prevent CTL lysis of MCMV-infected fibroblasts (14, 17). We wanted to determine whether *m4* is also functional in macrophages. As already shown in Fig. 2B, m4/gp34 is expressed at high levels in infected macrophages. Furthermore, a protein of the molecular weight of m4/gp34 coprecipitated with class I molecules in infected macrophages and fibroblasts (Fig. 3A). Direct immunoprecipitation of m4/gp34 from metabolically labeled infected cells confirmed that similar amounts of m4/gp34 were synthesized in

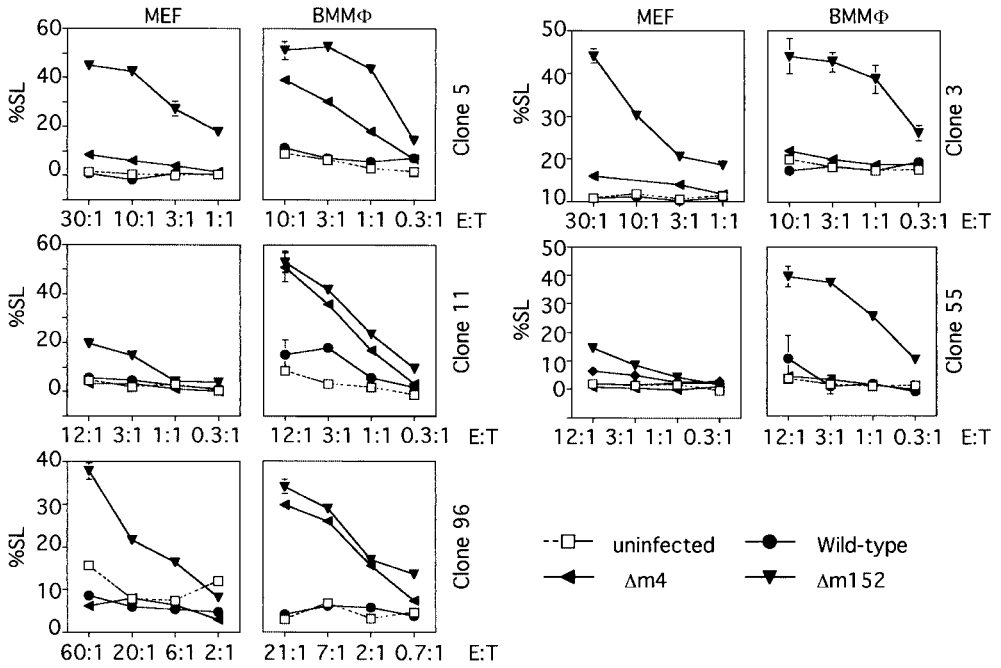

FIG. 4. m4/gp34 prevents antigen presentation in BMMϕ. BMMϕ and IFN-γ-pretreated MEF targets were ^{51}Cr loaded and infected at an MOI of 100 overnight, in the presence of PAA, with no virus, wild-type MCMV (MW97.01), Δm4 (MW99.03), or Δm152 (MW99.05). K^b-restricted CTL clones 5, 11, and 96 and D^b-restricted clones 3 and 55 were tested for their ability to recognize and lyse targets. E:T, effector-to-target ratio; SL, specific lysis.

infected MEFs and macrophages (data not shown). Because the effect of *m152* on K^b is much less effective than it is on D^b, complete abolition of antigen presentation to K^b-restricted clones requires additional contributions from other immune evasion genes, including *m4* (14; M. Gold, unpublished data). To test whether *m4* plays a role in preventing recognition of MCMV-infected macrophages, we infected BMMϕ with wild-type and Δm4 viruses. Note that these viruses both expressed *m152*, hence the assay tests whether *m4* is required in addition to the known requirement for *m152*. These targets were tested for lysis by two D^b-restricted clones and three K^b-restricted clones (Fig. 4). The killing pattern was the same as that previously reported for MEFs: no or little lysis was seen of wild type-infected targets; however, K^b- but not D^b-restricted CTL clones lysed targets infected with the virus lacking *m4*. It is noteworthy that the recognition of Δm4-infected macrophages is far stronger than that of Δm4-infected MEFs, suggesting that *m4* plays a more important role in inhibiting recognition in macrophages than in fibroblasts.

***m6* also interferes with antigen presentation in BMMϕ.** The third MCMV gene known to be able to interfere with antigen presentation to CTL is *m6*, whose product, m6/gp48, binds to class I molecules and directs them to the lysosome for destruction. To test whether *m6* can also affect antigen presentation, we performed CTL assays on BMMϕ infected with either wild-type MCMV or viruses lacking *m4*, *m6*, or *m152*. A representative assay is shown in Fig. 5; results with one K^b-restricted and one D^b-restricted CTL clone are shown. The K^b-restricted clone was able to lyse cells infected with any of the three mutant viruses, but not the wild-type virus, indicating that a unique contribution from each of *m4*, *m6*, and *m152* is essential to prevent K^b-restricted antigen presentation. This indicates that *m6* is functional in macrophages. As before, the D^b-restricted clone lysed targets infected with Δm152 but not with wild-type or Δm4 virus. In this assay weak lysis (beyond the limits of reliable interpretation) of Δm6-infected cells by the D^b-restricted clone was seen. This suggests that while *m6*

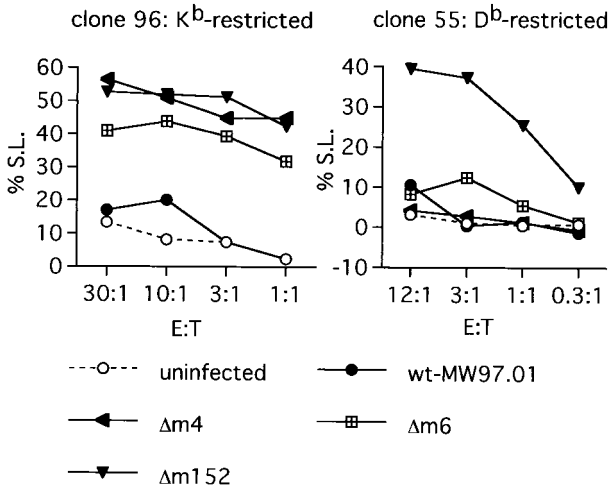

FIG. 5. *m6* also affects antigen presentation in infected BMMϕ. BMMϕ were isolated and infected as described above with wild-type MCMV (MW07.01), Δm152MW99.05, Δm4MW99.03, and Δm6-MCMV and were exposed to MCMV-specific CTL clones 96 (K^b restricted) and 55 (D^b restricted, M45 specific) in a Cr release assay. The results shown are typical of three separate experiments. E:T, effector-to-target ratio; SL, specific lysis.

may play some role in preventing D^b-restricted antigen presentation, *m152* is the dominant immune evasion gene for this MHC class I isoform.

DISCUSSION

It was previously observed that the L^d-restricted IE antigen, pp89, is well presented by macrophages despite expression of the immune evasion genes (11). Presentation was observed in both immortalized cells and in primary BMMϕ. Furthermore, in a macrophage cell line (J774), surface expression of L^d was not downregulated by MCMV. Taken together, these results suggested that the immune evasion genes might not affect antigen presentation in macrophages in the same way that they do in other cell types. In this paper we have taken advantage of viral mutants that lack immune evasion genes and compared them to wild-type viruses in which immune evasion is intact. The results presented here demonstrate that the immune evasion genes do in fact function in macrophages in the same way that they do in fibroblasts. *m152* causes class I molecules to be retained in a pre-Golgi compartment. Both the extent of retention and the greater efficacy of retention for D^b than for K^b were similar in both cell types. Most significantly, the combined effect of the immune evasion genes led to an effective inhibition of recognition of infected macrophages by both K^b- and D^b-restricted CTL clones.

There are several differences between the two systems studied that could account for these apparently conflicting results. L^d, the restriction element for pp89, is an unusual class I molecule. It is inefficiently assembled in the ER, such that a large amount of endo H-sensitive L^d can be found hours after synthesis even in uninfected cells. This makes it difficult to assess any increase in retention due to the effect of *m152*. Furthermore, we have already shown that different class I isoforms are differently affected by MCMV immune evasion genes. Thus, it is possible that L^d is handled in macrophages in some way that is different from that for K^b and D^b, such that it is more resistant to the effects of the immune evasion genes. However, we think that it is more likely that a difference in the source of the epitope is mainly responsible for our different results. pp89 is an IE antigen and is thus subject to some processing and presentation before the E genes are expressed. In the efficient antigen-presenting environment of a macrophage, combined with a slower transition from IE to E gene expression in macrophages, this temporal advantage may be enough to ensure pp89's presentation even after the E genes are expressed. However, M45 and the antigens recognized by our K^b-restricted clones are all E genes (data not shown) and are thus not available for processing until after *m152* is expressed. Therefore, they may be much more vulnerable to inhibition by *m152* and the other immune evasion genes than is pp89. The fact that IFN-γ treatment of fibroblasts is sufficient to enable presentation of pp89 (10) but not our H-2^b-restricted antigens is consistent with the notion that inhibition of pp89 presentation is weaker than that of the H-2^b-restricted antigens that we study. Whatever the explanation, it is clear that there is a marked difference in the ability of macrophages to present different MCMV epitopes.

One interesting feature of these studies was the excellent recognition of Δm4 in primary macrophages, shown in Fig. 4 and 5. In fibroblasts, our K^b-restricted CTL clones are unable to detect wild-type virus. Viruses lacking *m4*, on the other hand, are usually detected, but the efficiency of this detection varies, and fibroblasts infected with Δm4 are never as well lysed as fibroblasts infected with Δm152 (14). In assays in which the overall lytic activity of the CTL clones is low, lysis of Δm4 virus-infected fibroblasts may not exceed lysis of wild type-infected fibroblasts, and an example of such an assay is shown in Fig. 4. On the other hand, we have consistently seen excellent recognition of Δm4 virus in primary macrophages, equal to recognition of the Δm152 virus, even though wild-type virus was not recognized. The reason for this apparently stronger phenotype for *m4* in macrophages is not clear. The mechanism by which *m4* inhibits antigen presentation is not yet known, and it may be that it is able to function much more effectively in macrophages, providing the first example of a preferential cell type for the action of an immune evasion gene, one of the hypotheses for the existence of multiple immune evasion genes in a single virus (15). However, this apparently stronger phenotype could also be explained by the greater antigen presentation ability and higher level of expression of K^b of macrophages. In this cell type, *m152* may struggle even more to effectively inhibit K^b-restricted presentation, resulting in a much greater reliance on auxiliary contributions from *m4* and *m6* than is actually needed in fibroblasts. A better understanding of the mechanism of action of *m4* and *m152* will be needed to resolve this question.

We have clearly shown here that antigen presentation in macrophages can be efficiently inhibited by the combined action of three immune evasion genes. However, taking all the results together, it is clear that macrophages have a greater antigen-presenting ability than fibroblasts and that, at least in some instances, they can present antigen despite the effect of the immune evasion genes. As is always the case in a biological system when quantitative effects are seen, it is difficult to extrapolate from these in vitro studies to the situation in vivo. The key questions are as follows. Are CD8 cells able to detect virus-infected macrophages in vivo? Do the immune evasion genes have an impact on the ability of CD8 cells to control virus in infected macrophages? For the K^b- and D^b-restricted epitopes studied in this paper the effect of the immune evasion genes on CTL recognition of infected macrophages was profound. It seems likely, therefore, that this must have a quantitative impact on the efficacy of CD8 detection of infected cells in vivo, whether or not the inhibition of recognition is absolute. There is still much to be understood about the complex relationship between CMV and the host in chronic infection and about the contribution of viral immune evasion mechanisms to this relationship.

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