Brief Definitive Report

Cytomegalovirus Misleads Its Host by Priming of CD8 T Cells Specific for an Epitope Not Presented in Infected Tissues

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Abstract

Cytomegaloviruses (CMVs) code for several proteins that inhibit the presentation of antigenic peptides to CD8 T cells. Although the molecular mechanisms of CMV interference with the major histocompatibility complex class I pathway are long understood, surprisingly little evidence exists to support a role in vivo. Here we document the first example of the presentation of an antigenic peptide being blocked by a CMV immune evasion protein in organs relevant to CMV disease. Although this D^b-restricted peptide, which is derived from the antiapoptotic protein M45 of murine CMV (mCMV), is classified as an immunodominant peptide based on response magnitude and long-term memory, adoptive transfer of M45 epitope-specific CD8 T cells did not protect against infection with wild-type mCMV. Notably, the same cells protected C57BL/6 mice infected with an mCMV mutant in which immune evasion protein m152/gp40 is deleted. These data indicate that direct presentation or cross-presentation of an antigenic peptide by professional antigen-presenting cells can efficiently prime CD8 T cells that fail in protection against CMV organ disease because m152/gp40 prevents presentation of this peptide in pathogenetically relevant tissue cells.

Key words: immunodominance • immune evasion • immune control • antigen presentation • cross-priming

Introduction

Viruses encode many potentially antigenic peptides, but only a few are actually involved in the antiviral immune response in any given MHC haplotype, a phenomenon known as immunodominance. Understanding the principles of how certain peptides are selected to participate in host defense is of importance for vaccine design (1). CMVs add a further dimension of complexity to the presentation of peptides in that they code for specialized immune evasion proteins, referred to as "immunoevasins" (2) or "viral proteins interfering with antigen presentation" (3). A set of these proteins is committed to inhibit the MHC class I–restricted presentation of peptides to CD8 T cells. In murine CMV (mCMV) infection, three immunoevasins are operative at different stages of the MHC class I pathway and cooperate in diminishing the display of peptides at the cell surface: m152/gp40 retains peptide-loaded MHC class I complexes in a cis-Golgi compartment, m06/gp48 reroutes the complexes for lysosomal degradation, and m04/gp34 escorts MHC class I molecules to the cell surface but precludes recognition (for review see reference 2).

Yet, these mechanisms in concert do not eventually prevent the immune surveillance of CMVs by CD8 T cells in the infected host. Contrary to the consciousness of many, to date there exists no example of a CMV peptide for which presentation in vivo is prevented by immunoevasins. Instead, in the BALB/c model, mCMV-primed polyclonal CD8 T cells as well as CTL lines (CTLL) specific for five different epitopes protected against CMV disease in immunocompromised adoptive transfer recipients (for review see refer-

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ence 2). Thus, in principle, peptide presentation in vivo can occur in productively infected tissue cells despite the expression of immunoevasins.

For the C57BL/6 model, an immunodominant D^b-restricted antigenic peptide with the sequence 985-HGIRNASFI-993 has been identified (4) in the antiapoptotic E phase protein M45 (5). We show here that fully functional M45 epitope-specific CD8 T cells, propagated as a CTLL or sorted ex vivo, fail to resolve an in vivo infection by WT mCMV (mCMV-WT). To the best of our knowledge, efficient priming of a CD8 T cell response to an antigenic peptide for which the presentation in productively infected tissue cells is actively prevented by a viral gene function is unprecedented and represents a subtle new trick of a virus to subvert immune control.

Materials and Methods

Viruses

Bacterial artificial chromosome–derived mCMV MW97.01 (6, 7) is here referred to as mCMV-WT. Based on MW97.01, mutants mCMV-Δm152 and mCMV-Δm04+06+152 have been generated (8). For the construction of revertant virus mCMV-Δm152-rev, gene *m152* was reinserted into the native site in mCMV-Δm152. This was achieved essentially as previously described (8) by homologous recombination in *Escherichia coli* between bacterial artificial chromosome p-Δm152 and a linear PCR fragment containing the *m152* open reading frame flanked upstream and downstream by 400-bp sequences. Progeny virus mCMV-Δm152-rev was reconstituted by transfection of the revertant genome into mouse embryo fibroblasts (MEFs) as previously described (7).

Infection and Adoptive Cell Transfer

For priming, intraplantar infection of immunocompetent C57BL/6 mice was performed with 10^5 PFU mCMV-WT (9). For adoptive transfer experiments, C57BL/6 recipients were immunocompromised by 7.5 Gy γ irradiation. M45-specific CD8 T cells were transferred i.v. 24 h later, followed by intraplantar infection with 10^5 PFU mCMV-WT or mutants. Animal experiments were approved by the Ethics Commission, permission number 177-07/021-28.

Effector Cells

Acutely sensitized lymphocytes were isolated at day 8 from pooled draining popliteal LNs (PLNs). Memory cells were derived after resolution of productive infection from spleens of latently infected mice.

Immunomagnetic Cell Sorting. For subsequent cytofluorimetric cell sorting, CD8 T cells were enriched by one round of positive immunomagnetic cell sorting using the autoMACS system (Miltenyi Biotec). For ELISPOT assays, two sequential runs were performed to reach >95% purity. Separation programs Possel™ and Posseld™ (Miltenyi Biotec) were used for one and two column separation, respectively.

Cytofluorimetric Cell Sorting. For the purification of M45 epitope-specific memory cells, preenriched CD8 T cells were labeled with FITC-conjugated anti-CD8a mAb (clone 53-6.7, catalog no. 553031; BD Biosciences) and M45 peptide-loaded Dimer X I H-2D^b:Ig (catalog no. 551323; BD Biosciences) with PE-conjugated rat mAb anti–mouse IgG1 (clone A85-1, catalog. no. 550083; BD Biosciences) as second antibody. EPICS ALTRA HyperSort (Beckman Coulter) was operated with EXPO32 acquisition and analysis software. Sort gates were set on living cells with highly positive FL-1 (FITC) and FL-2 (PE) fluorescence, discarding cells with low and intermediate expression. Sorting was performed in ALTRASort mode 3 with a flow rate of ~5,000 cells/s.

Generation of Peptide-specific CTLL and Cytolytic Assay. Cell line M45-CTLL was generated by repetitive restimulation of memory spleen cells (10) at an M45 peptide concentration of 10^{-10} M. Cytolytic activity was measured in a standard ^{51}Cr release assay using C57BL/6-derived EL4 thymoma cells as target cells.

ELISPOT Assays

IFN-γ–based ELISPOT assays were performed as previously described (10), with graded effector cell numbers and triplicate cultures per titration step. In the CD3ε-redirected ELISPOT assay (10), cells were polyclonally stimulated via the CD3ε molecule by using anti-CD3ε mAb-producing hybridoma 145-2C11. M45 peptide-presenting cells were EL4 pulsed with an optimal dose (10^{-8} M) of synthetic M45 peptide. C57BL/6 MEFs were either left uninfected for control or were infected with mCMV-WT, mCMV-Δm152, or mCMV-Δm04+06+152 at a multiplicity of infection of 4 PFU/cell (9). Cells were harvested at 1 h

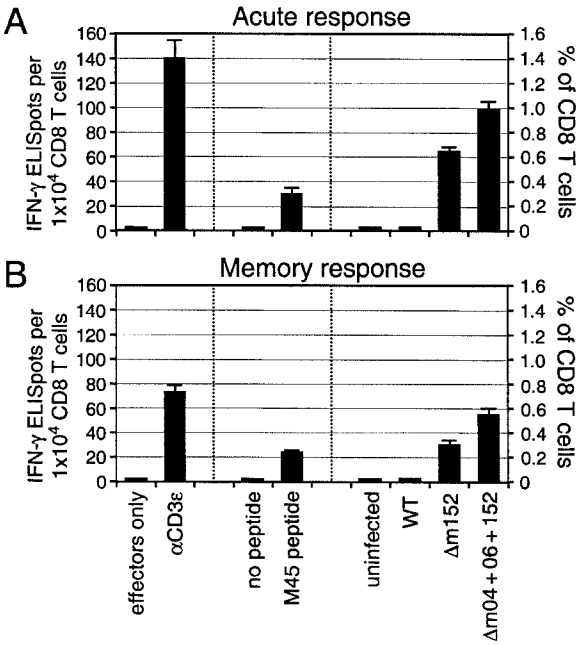

Figure 1. M45 is a dominant antigen. Immunocompetent C57BL/6 mice were infected with mCMV-WT. (A) The acute immune response in the draining PLN was measured on day 8. (B) Immunological memory in the spleen was measured at 3 mo. The recognition of target cells by purified CD8 T cells was tested in the ELISPOT assay. The total response was determined by polyclonal stimulation via CD3ε. The M45 peptide-specific response was determined by stimulation with EL4 thymoma cells pulsed with 10^{-8} M synthetic M45 peptide. C57BL/6 MEFs were used either uninfected or after infection with the indicated viruses. Frequencies are given as numbers of IFN-γ–secreting cells (left) as well as in percent of all CD8 T cells (right). Bars represent most probable numbers determined by linear regression analysis. The upper 95% confidence limits are indicated. P-values for random, nonlinear distribution were <0.001 throughout.

after infection and they continued viral gene expression throughout the assay period. Frequencies of IFN-γ–secreting, spot-forming cells were calculated by intercept-free linear regression analysis using Mathematica V4.2.1 Statistics software ("LinearRegression"; Wolfram Research Inc.).

Quantitation of Infection in Host Organs

Infectious virus was quantitated in organ homogenates by a virus plaque assay on MEFs (9). The number of infected cells in tissue sections of host organs was determined by IE1 protein-specific immunohistology or by gene-specific DNA-DNA two-color in situ hybridization (2C-ISH) (9).

Results

Immunodominance of M45 in Acute Response and Memory. The numerical participation of M45 epitope-specific CD8 T cells in the immune response of C57BL/6 mice to mCMV was measured in the draining PLNs at day 8 after infection, representing the acute primary immune response, as well as in the spleen after resolution of productive infection, representing immunological memory during viral latency (Fig. 1).

In the acute response (Fig. 1 A), 1.4% of all CD8 T cells responded to polyclonal signaling via CD3ε and 0.3% of all CD8 T cells, which is ~20% of the CD3ε-responsive cells, were specific for the M45 peptide. Fibroblasts infected with mCMV-WT were not recognized. This implies that immunoevasins affect the presentation of all H-2^b–restricted mCMV peptides, not just of the M45 peptide, in this cell type. Accordingly, antigenic peptides are presented in fibroblasts infected with mutants mCMV-Δm152 and mCMV-Δm04+06+152 (8), which led to the recognition by 0.6 and 1% of all CD8 T cells, respectively. The overall pattern was similar for the memory cells (Fig. 1 B), except that M45-specific cells were somewhat enriched relative to the declined numbers of the CD3ε-responsive and the total virus-specific cells.

M45-specific Effector Cells Fail to Control In Vivo Infection. The T cell line M45-CTLL was generated by repetitive restimulation of spleen-derived memory cells with M45 peptide. After the fifth round of stimulation, M45-specific cytolytic activity (Fig. 2 A) and induction of IFN-γ secretion (Fig. 2 B) were tested. Half-maximal lysis was observed with target cells pulsed with M45 peptide at a concentration of 10^{-12} M (Fig. 2 A), which indicates an affinity that is ~100-fold higher than that of the in vivo protective IE1-CTLL and m164-CTLL specific for the two immunodominant mCMV peptides in the H-2^d haplotype (11). A high and comparable proportion of the cells secreted IFN-γ after CD3ε-mediated signaling and after stimulation with the cognate peptide, which indicates monospecificity (Fig. 2 B). Despite the high sensitivity of M45-CTLs, fibroblasts infected with mCMV-WT were not recognized. In contrast, peptide presentation occurred

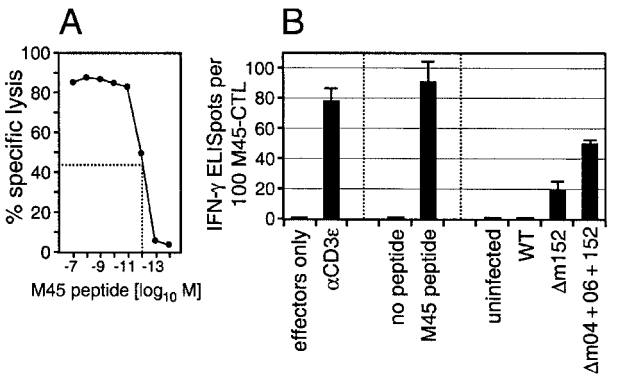

Figure 2. M45 peptide is only presented in cells infected with immune evasion gene deletion mutants. An M45 epitope-specific, polyclonal CTLL was derived from mCMV-WT–infected C57BL/6 mice. (A) Cytolytic effector function and affinity of M45-CTLL. EL4 target cells were pulsed with the indicated molar concentrations of M45 peptide. Lysis of target cells was measured at an E/T ratio of 15. Dot symbols represent mean values of triplicate assay cultures. (B) Presentation of M45 peptide measured by the number of M45-CTLs that respond in the ELISPOT assay. For stimulator cells and statistical analysis, see the legend of Fig. 1.

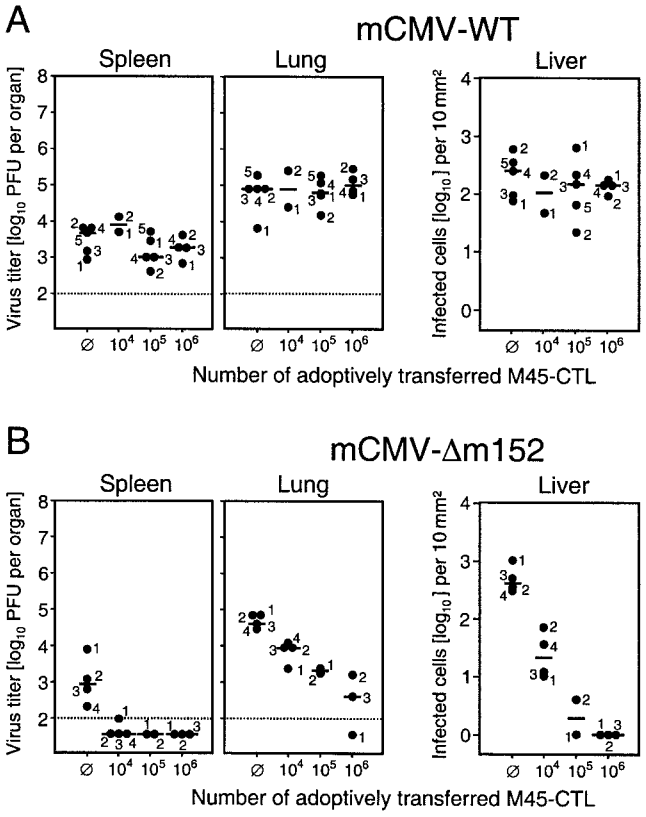

Figure 3. M45-CTLs fail to control in vivo infection with mCMV-WT but resolve infection with mutant mCMV-Δm152. Immunocompromised C57BL/6 mice were infected (A) with mCMV-WT or (B) mCMV-Δm152. The in vivo antiviral function of M45-CTLs was tested by adoptive transfer. ∅, no cell transfer. Virus replication in organs was measured on day 12. For spleen and lung (left), virus titers were determined in organ homogenates by a virus plaque assay. The dotted line indicates the detection level. For the liver, the number of infected hepatocytes was determined by immunohistology specific for the intranuclear IE1 protein. Dot symbols represent data for individually numbered transfer recipients. Median values are marked.

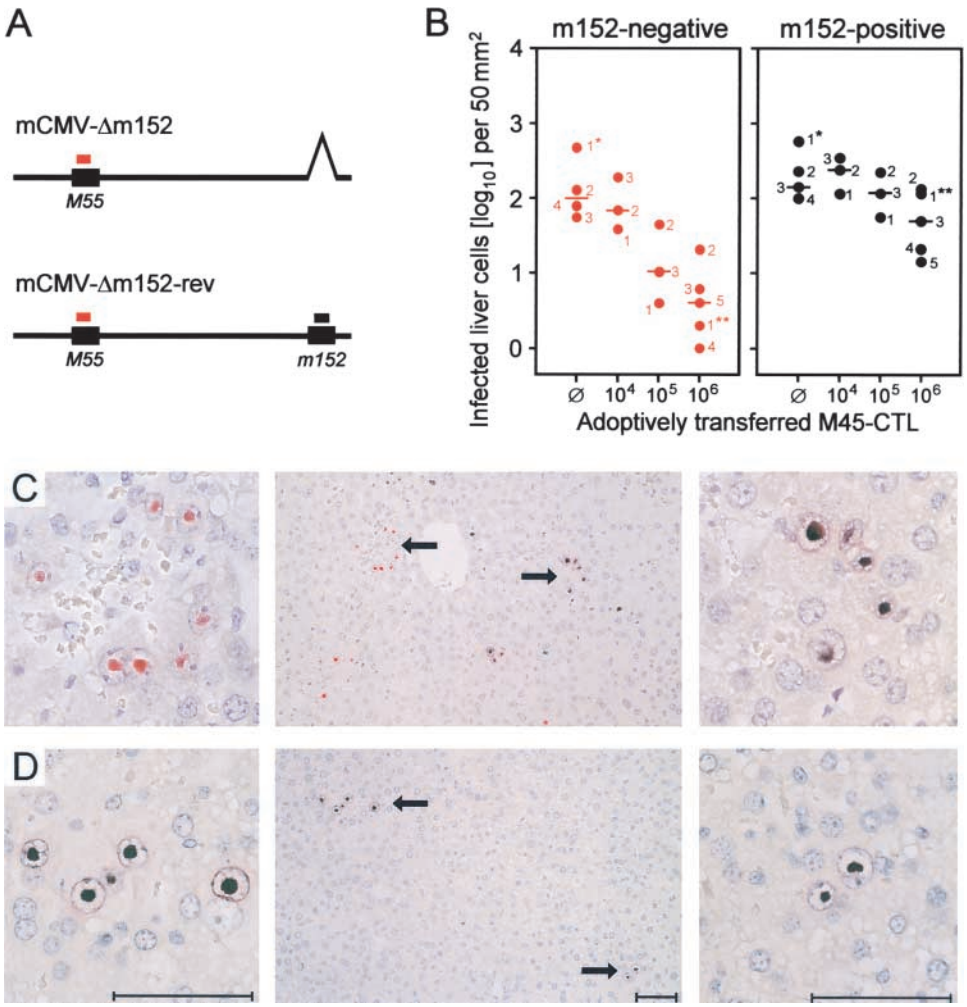

Figure 4. Increasing selective force by M45-CTLs leads to preferential loss of mutant virus in coinfected recipients. Immunocompromised C57BL/6 mice were infected with a mixture of viruses mCMV-Δm152 and mCMV-Δm152-rev, 10^5 PFU each. A selective force was exerted by adoptive transfer of increasing numbers of M45-CTLs. ∅, no cell transfer. On day 12, virus replication in the liver was compared by quantitation of infected cells using 2C-ISH. (A) Maps (not drawn to scale) illustrate the design of DNA probes for discriminating between mCMV-Δm152 (clean red staining) and mCMV-Δm152-rev (mixed black and red staining). (B) Comparative quantitation of hepatocytes infected with either of the two viruses. Dot symbols represent data for individually numbered transfer recipients. Median values are marked. (C) 2C-ISH–stained and hematoxylin counterstained liver section of individual mouse number 1* (no cell transfer), showing separate red and black foci of infection. Arrows in the middle panel point to areas resolved to greater detail in the flanking higher resolution images. Note the stained inclusion bodies, which represent the site of viral DNA packaging in the nuclei of infected hepatocytes. (D) 2C-ISH–stained and hematoxylin counterstained liver section of individual mouse number 1** (10^6 CTLs transferred). Note the absence of red-stained foci. Bar, 50 μm.

in fibroblasts infected with the mutant viruses mCMV-Δm152 and mCMV-Δm04+06+152, but did not engage all M45-specific cells. This finding shows that stimulation with infected fibroblasts, even after deletion of the three immunoevasins, underestimates the number of epitope-specific effector cells.

These in vitro highly functional M45-CTLs were then tested for their antiviral in vivo efficacy by adoptive transfer into immunocompromised C57BL/6 mice infected with mCMV-WT (Fig. 3 A). Notably, the transferred cells were completely ineffectual in controlling virus replication in spleen, lung, and liver.

Immunoevasin Deletion Restores the Function of M45-CTLs. Although M45-CTLs were functional in terms of cytolytic activity and IFN-γ secretion, unknown deficiencies in other parameters could possibly account for their failure in controlling the in vivo infection. Therefore, we tested whether M45-CTLs control virus replication in recipients infected with mCMV-Δm152 (Fig. 3 B). Although 10^6 of these cells were ineffectual in controlling mCMV-WT (Fig. 3 A), as few as 10^4 cells significantly reduced the replication of the mutant virus and 10^6 cells almost resolved the infection in all organs tested.

For ultimate proof, immunocompromised recipients were coinfected with mutant virus mCMV-Δm152 and revertant virus mCMV-Δm152-rev (Fig. 4). Gene probes specific for a shared gene, namely *M55* (gB), and for gene *m152* (Fig. 4 A) allowed for a visual discrimination between cells infected with the mutant (clean red staining) and cells infected with the revertant (mixed red and black staining) in the same tissue sections by 2C-ISH. The two viruses were subjected to an increasing selective pressure exerted by increasing doses of M45-CTLs, and control of infection in the liver was determined by counting the numbers of infected hepatocytes (Fig. 4, B–D). The number of red-stained hepatocytes infected with mCMV-Δm152 declined with increasing selective pressure, whereas the number of black-stained hepatocytes infected with mCMV-Δm152-rev was not significantly reduced. In conclusion, the failure of M45-CTLs to control infection with mCMV-WT or mCMV-Δm152-rev was not caused by a functional deficiency of the effector cells but by an immunoevasin-mediated "invisibility" of infected tissue cells.

Nonprotective Memory. Although the data have shown so far that M45-CTLs fail to control productive infection with mCMV-WT, M45-specific memory cells are main-

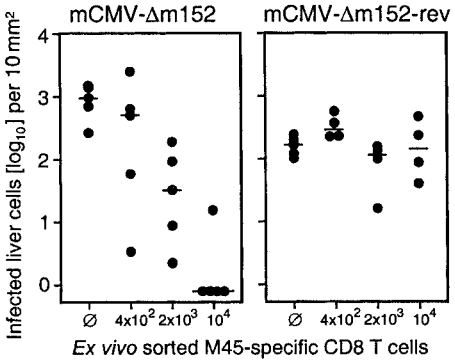

Figure 5. M45-specific memory cells maintain antiviral function but are unable to prevent productive infection by the priming virus genotype. M45-TCR$^+$ CD8$^+$ memory cells were purified by cell sorting from a pool of spleen cells derived from 15 C57BL/6 mice at 4 mo after infection with mCMV-WT. The antiviral function was tested by adoptive transfer into immunocompromised C57BL/6 recipients infected with mutant virus mCMV-Δm152 or revertant virus mCMV-Δm152-rev. ∅, no cell transfer. On day 11, virus replication in the liver was quantitated by immunohistological detection of IE1 protein in the nuclei of infected hepatocytes. Dot symbols represent data for individual recipients. Median values are marked.

tained long-term in the host (Fig. 1). A critical question is whether the findings obtained with an in vitro–selected CTLL also apply to the memory CD8 T cell population present in the host. To answer this question, an adoptive transfer experiment was performed with M45-TCR$^+$ CD8$^+$ ex vivo memory cells sorted by using MHC-Ig hybrid molecules loaded with M45 peptide (Fig. 5).

The sorted M45-specific memory cells were highly efficient in controlling the replication of mutant virus mCMV-Δm152. As few as 2,000 transferred cells gave significant protection against liver infection in all recipients and 10^4 cells prevented liver infection in most. It is worth noting that TCR staining with the dimeric peptide-MHC-Ig hybrid did not interfere with T cell function in vivo. This observation contrasts with the experience made previously with peptide-folded MHC class I tetramers (12).

Despite this remarkable protective capacity of the M45-specific memory cells, they failed in protecting against productive infection with mCMV-Δm152-rev. In conclusion, fully functional M45-specific memory cells are present in the infected host but cannot protect against a productive infection by the virus to which they have originally been primed.

Discussion

The M45 peptide represents a highly antigenic epitope in that it efficiently binds to the presenting MHC class I molecule, namely D^b, primes a quantitatively prominent acute CD8 T cell response, and induces long-lasting immunological memory. According to established terminology, one would name it an immunodominant peptide (1). Currently it is implicit understanding that immunodominance also means being dominant in contributing to immunity. Yet, as we have shown here, the M45 peptide does not contribute to the control of cytopathogenic mCMV-WT infection. Hence, it is not involved in immunity that prevents CMV disease. Therefore, we propose to distinguish between "antigenicity dominance" for epitopes that elicit a quantitatively prominent response and "immunodominance" for epitopes that contribute significantly to protective immunity.

Previous work by Gold et al. and LoPiccolo et al. (4, 13) has shown that the M45 peptide is not presented in cell culture by infected fibroblasts, macrophages, or a DC line if immunoevasin m152/gp40 is expressed. In vivo, disease manifestations of CMV, such as interstitial pneumonia, hepatitis, adrenalitis, ventriculitis, and gastrointestinal disease involve a much broader range of cell types (14). The lack of protection by M45-CTL in vivo indicates that M45 peptide presentation is prevented in the microenvironmental context of infected tissues and, most importantly, in the stromal and parenchymal cell types that are relevant to CMV disease.

This finding was not expected. Although m152/gp40 reduced the efficacy of polyclonal antiviral CD8 T cells in the genetically susceptible BALB/c strain (15), these cells were still protective against mCMV-WT (16). Accordingly, with no exception known so far, CTLL specific for dominant as well as subdominant K^d-, D^d-, and L^d-restricted mCMV epitopes were found to control infection (10, 11).

M45 is an interesting protein. Its critical role in endothelial cell tropism of mCMV was attributed to an antiapoptotic function (5). However, any speculation on a link between inhibition of apoptosis and a selective, M45 protein-specific immune evasion has to be refuted in view of the finding that CD8 T cells specific for the subdominant, D^d-restricted M45 peptide 507-VGPALGRGL-515 (2) protect against mCMV-WT in the BALB/c model (unpublished data). Rather, as suggested by data on ER retention (17) and downmodulation of cell surface expression (8), D^b is more susceptible to the effect of m152/gp40 than other class I allelic products.

The fact that M45-specific CD8 T cells were generated in high numbers in response to mCMV infection implies that precursor cells were efficiently primed by professional APCs presenting the M45 peptide. Notably, the frequencies were found to be about the same in C57BL/6 mice infected with mCMV-Δm152 and mCMV-Δm152-rev (4), which also applies to all known mCMV epitopes in BALB/c (unpublished data). This finding suggests that immunological priming is not influenced by the expression of immunoevasin m152/gp40 in infected cells. For an explanation, Gold et al. (4) discussed the possibility of priming through uninfected DCs presenting M45 peptide derived by uptake of the M45 protein from infected cells, a still-debated concept known as cross-presentation (18, 19). Along the same line, Basta and Bennink (20) recently speculated on cross-presentation as being an adaptive host response to the viral inhibition of direct presentation.

The data presented here reveal an important functional limitation of peptide cross-presentation, at least for CMV infection. Although cross-presentation may indeed be a

way to avoid CMV immunoevasins in the priming of an immune response, the cross-primed effector cells will not find the respective antigen on infected stromal and parenchymal non-APC tissue cells in which immunoevasins are operative (4). Hence, they will not protect against CMV disease. This hypothesis is now substantiated.

Our data contribute to a better understanding of CMV disease. In addition, we see a general importance in the notion that antigenicity dominance and immunodominance are not necessarily congruous properties of an antigenic peptide. The choosing of epitopes for use in a vaccine is largely based on the magnitude of the epitope-specific response. D^b-M45 is a paradigm for a nonprotective dominant epitope. Our findings underscore the importance of in vivo protection studies for decision making in vaccine design and may help to explain, and avoid, vaccine failures.

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