seminars in **IMMUNOLOGY**, Vol. 13, 2001: pp. 19–26
doi:10.1006/smim.2001.0292, available online at http://www.idealibrary.com on IDEAL®

Evasion of cytotoxic T lymphocytes by murine cytomegalovirus

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Murine cytomegalovirus causes lifelong infections with little pathology in normal host animals. Control of viral replication and prevention of pathology depend on both innate and adaptive immune mechanisms, and cytolytic T lymphocytes play a key role in this process. The virus encodes a number of genes which alter the normal assembly of class I major histocompatability complex proteins, and thus interfere with the ability of infected cells to present antigen to CD8$^+$ T cells. This review will examine what is known about these viral genes, and present some unanswered questions regarding the role of CTL evasion in the viral infectious cycle.

Key words: murine cytomegalovirus / cytotoxic T lymphocytes / major histocompatability complex class I / immune evasion

Introduction

Herpesviruses, including murine cytomegalovirus (MCMV), are mostly non-pathogenic in normal host animals. When the immune system is compromised, however, MCMV can cause pathological, and sometimes lethal, systemic infections. Both the innate and the adaptive branches of the immune system contribute to normal control of MCMV. In the innate response, monocyte macrophage and natural killer cells have been shown to play especially important roles. In the adaptive response, T cells and B cells both help to control the virus. Class I-restricted CD8$^+$ cytotoxic T lymphocytes (CTLs) in particular play an important role in controlling MCMV infection and preventing disease, and the importance of this subset is reflected in the number of viral genes that alter the assembly and function of classical class I MHC gene products. In this review we will examine the evidence for the role of CTLs in MCMV infection, as well as anti-CTL viral immunoevasive functions, and briefly consider the possible significance of these functions in the viral life cycle.

The MCMV genome

MCMV is a large, enveloped virus with a double-stranded DNA genome that has been entirely sequenced.[1] MCMV encodes 170 open reading frames, numbered from *m01* to *m170* (Figure 1). Much of the MCMV genome is homologous to and colinear with that of human cytomegalovirus (HCMV), and MCMV genes which have an HCMV homolog are labeled with a capital 'M', while those which do not are labeled with a small 'm'. Of particular interest to this review are two MCMV gene families which are defined by internal homology within each family, neither having any known homology to any other genes. As discussed below, the only functions so far ascribed to genes in these two families are modulation of class I MHC assembly or function. It is intriguing that these families occupy positions within the genome homologous to those of HCMV genes which also modulate class I function. However, the mechanisms used by MCMV to affect class I function appear to be quite different from those described for HCMV or any other virus.

Assembly of class I MHC proteins

Classical class I genes are *polygenic*: in mice, there are three loci called H-2 K, D, and L. Each locus

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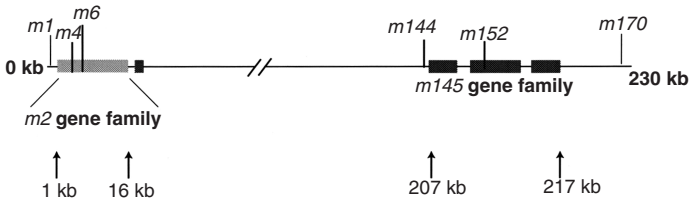

Figure 1. The MCMV genome. Diagram of the MCMV genome showing two gene families. Genes mentioned in this review are marked.

is *polymorphic*, having multiple alleles: for example, BALB/c mice have K^d, D^d, and L^d alleles, while C57-Bl/6 mice have K^b and D^b (but no L gene). Figure 2 shows the assembly pathway of class I molecules in normal and MCMV-infected cells.

CD8$^+$ CTLs and MCMV infection

The importance of CD8$^+$ CTLs in MCMV infection has long been established.[2,3] MCMV-primed CD8$^+$ T cells are sufficient to prevent disease and limit viral replication in most tissues (with the exception of the salivary gland) when transferred to naive animals which are later challenged with virus. Conversely, MCMV-primed CD4$^+$ T cells alone are not sufficient—they can only control virus if transferred in conjunction with MCMV-primed non-T lymphocytes, presumably B cells.[4,5]

Discovery of class I-restricted MCMV antigens

An important first step in the investigation of antiviral CTLs was the identification of a major viral antigen. Herpesvirus gene expression occurs in three stages: first, immediate-early (IE) genes are expressed immediately upon infection; second, the products of immediate-early genes transactivate early (E) viral genes, which carry out a number of functions, especially DNA replication; and finally DNA replication permits the expression of late (L) viral genes, which include a large number of structural genes. The immunological effects of genes from the three stages can be distinguished *in vitro* by treating infected cells with drugs which block progression to the next stage. Using such methods it was determined that in BALB/c mice a significant proportion of anti-MCMV CTLs were specific for an IE antigen,[6,7] which was shown to be a nonameric peptide derived from the IE protein pp89 and presented in the context of L^d.[8–10] CTLs specific for this antigen were shown to protect against viral challenge.[11] This pp89-derived peptide is still the best-defined class I-restricted antigen of MCMV and has served as the basis for much subsequent investigation of antigen presentation in MCMV. In addition to pp89, however, other MCMV antigens, some of which are expressed during the E phase, are recognized by CTLs from MCMV-infected mice. The first E antigen to be identified was recently described by Holtappels *et al.*,[12] who identified an antigenic nonameric peptide derived from the E gene *m4* and presented in the context of D^d. There is no reason to suspect any functional connection between the antigenic properties of this peptide and the immunoevasive effects of *m4* described below, although its antigenicity may be related to the extreme abundance of m4 during the E stages of infection (D. G. Kavanagh, unpublished data). It is likely that more antigens derived from E genes will be identified soon.

CTL evasion and early gene expression in vitro

Using a CTL clone specific for pp89, Reddehase *et al.*[13] demonstrated that infected fibroblasts had a greatly diminished capacity to present pp89 antigen during the E phase of gene expression as compared to the IE and L phases. This led to a remarkable discovery: Del Val and coworkers[14] demonstrated that E gene expression almost completely abrogated presentation of IE antigen to T cell clones without blocking the presentation of (uncharacterized) E antigens. Initially, Del Val *et al.* interpreted these results to represent a pp89-specific block in antigen presentation. As discussed below, it eventually became clear that the effects of multiple MCMV early genes in fact lead to a general inhibition of class I maturation which is not specific for pp89-derived peptide. The intriguing observation that some E antigens still escape these multiple effects and are effectively presented to E-specific CTL clones remains unexplained, and is the subject of current investigation.

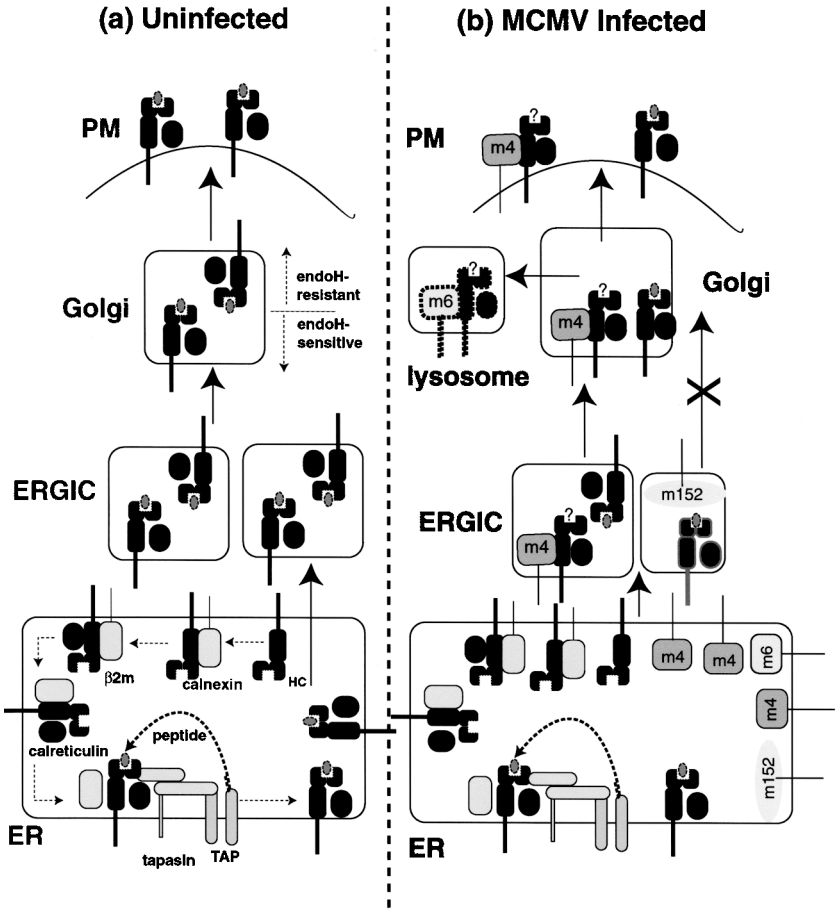

Figure 2. Assembly and export of class I MHC. (a) Normal class I assembly. Nascent heavy chain (HC) is co-translationally translocated into the ER, where it is glycosylated and associates with the lectin-like chaperone calnexin. HC then associates with the light chain, beta-2-microglobulin (β2m). This bimolecular complex is transferred from calnexin to calreticulin and then associates with the peptide transporter TAP via the bridging molecule tapasin. Here it is loaded with antigenic peptide transported from the cytoplasm. The mature trimolecular complex is now released by ER chaperones and exits the ER to be transported through the Golgi and to the cell surface. In the passage through the cis-Golgi, glycan moieties on the HC become modified such that they are resistant to digestion *in vitro* with endoglycosidase H (endoH). As discussed in the text, sensitivity to endoH digestion thus serves as a useful measure of the degree of class I export. (b) Three MCMV gene products alter class I assembly. m152/gp40 blocks class I export to the Golgi, but has not been isolated in association with class I. m6/gp48 binds to class I in the ER and redirects class I transport to the lysosome, where it is destroyed. m4/gp34 also binds class I in the ER, and forms a strong complex which is not degraded but is transported to the cell surface. Nevertheless, some unaffected class I may still be expressed on the surface of infected cells.

So far, three MCMV genes, *m152*, *m6* and *m4*, have been found to alter the assembly and function of class I molecules. A summary of what we currently know about these genes follows.

m152 retains class I in a pre-Golgi compartment

The first step in the biochemical description of CTL evasion by MCMV was the observation that L^d molecules synthesized during E gene expression fail to become endoH resistant, indicating that class I is retained in a pre-medial Golgi compartment [see Figure 2(a)]. In contrast to what is observed in HCMV infections, the retained class I molecules are not rapidly degraded, but in fact have a relatively long half-life and accumulate.[15] Koszinowski and coworkers[16] were able to replicate this effect by micro-injecting fibroblasts with defined fragments of the MCMV genome, and, using a close analysis of restriction sites, identified *m152* as being suffi-

cient to cause class I retention.[17] *m152* encodes a 40 kD type 1 transmembrane glycoprotein, called 'gp40', which is expressed during the E phase of infection with maximal expression between 3 and 6 hours post-infection. Expression of m152/gp40 via transfection caused a marked reduction in surface expression of several classical class I molecules. Expression of m152/gp40 via recombinant vaccinia virus (m152-Vac) was sufficient to protect targets from lysis by vaccinia-specific CTL clones. Recently, Krmpotic *et al.*[18] have published results obtained for mutant viruses specifically knocked-out for *m152* (Δm152 MCMV) as well as control revertant viruses in which the knocked-out gene was restored. Using polyclonal CTL restimulated with pp89 antigenic peptide, Krmpotic *et al.* found that during the E stage of infection, targets infected with Δm152 were highly susceptible to lysis, while targets infected with either wildtype virus or the revertant were completely protected. Thus *m152* serves to protect targets from class I-restricted lysis in the contexts of both MCMV infection and heterologous expression.

The effect of *m152* is specific for mouse class I molecules, since neither murine CD44 (an irrelevant cellular glycoprotein) nor human class I proteins are retained by m152-Vac.[17] Furthermore, murine transferrin receptor is exported with normal kinetics in MCMV-infected cells (D. G. Kavanagh, unpublished data). During infection with both MCMV and m152-Vac, retained class I molecules were not degraded but remained endoH sensitive. Confocal microscopy demonstrated that retained class I molecules co-localize not with the ER marker BiP, but with p58, a marker for the ER-Golgi intermediate compartment (ERGIC).[17]

In order to determine whether retained class I molecules were peptide-loaded, Del Val *et al.*[15] extracted processed peptides from MCMV-infected cells maintained under IE or E conditions. Using a pp89-specific T cell clone, they demonstrated that equal quantities of pp89 peptide were extractable under E and IE conditions. As a control, they showed that the antigenic epitope from pp89 could only be isolated from infected cells that expressed L^d and not from control cells lacking L^d. Del Val *et al.* concluded that retained L^d in MCMV-infected cells is peptide loaded.

In spite of clear evidence for a specific effect of *m152* on class I transport, no direct biochemical interaction between m152/gp40 and class I has ever been found. In fact, it appears that m152/gp40 has a much shorter half-life than the retained class I molecules, and that while class I is retained in a pre-Golgi compartment, m152/gp40 is transported through the Golgi and degraded in the lysosome.[17] Thus m152/gp40 appears to exert its effect on class I by some transient or indirect interaction in the ER or ERGIC.

m152 *alters the CTL response in vivo*

Using knockout and revertant viruses, Krmpotic *et al.*[18] have demonstrated that *m152* has an effect on CTL control of virus *in vivo*. In newborn BALB/c mice infected with 100 plaque forming units (PFU) of virus, mortality was 25% with Δm152 virus, 50% with wildtype virus, and 75% with revertant. Virus titers in both the spleen and the lung of animals infected with Δm152 was reduced tenfold compared to animals infected with wildtype and revertant MCMV, and this reduction was dependent on $CD8^+$ T cells. Finally, either MCMV-primed or naive CTLs were transferred to irradiated BALB/c mice which had been infected with Δm152 or revertant. Both CTL populations controlled replication of Δm152 better than revertant in the lung and liver (except that primed CTLs completely controlled both viruses in the liver). Thus *m152* has a measurable phenotype *in vivo*.

m6/gp48 redirects class I to degradation in the lysosome

Even after the identification of *m152*, it was clear from surface staining using a mutant virus that the effects of *m152* could not account for all of the loss of class I expression in MCMV-infected cells.[16] Reusch *et al.*[19] therefore used an innovative approach to look for viral gene products affecting class I expression. Reasoning that some important viral factor(s) could be found in tight complexes with class I molecules, Reusch and coworkers immunized BALB/c mice with class I molecules isolated from MCMV-infected BALB 3T3 fibroblasts. Since the mice were necessarily tolerant to $H\text{-}2^d$ molecules, the resulting humoral immune response was especially directed against foreign proteins associated with class I. When a panel of B cell hybridomas was screened for activity against MCMV gene products, two hybridomas were found which specifically interacted with the product of *m6*.

m6/gp48 is another type 1 transmembrane glycoprotein, of molecular weight 48 kD. It is a classic E gene, with maximal expression at 3–6 hours post-infection. *m6* carries a functional dileucine motif in the cytoplasmic tail which targets the protein for

destruction in the lysosome. In the ER, m6/gp48 binds tightly to β2m-associated class I molecules and eventually redirects their transport away from the plasma membrane and into the lysosome where both proteins are destroyed, and this destruction is sensitive to specific inhibitors of lysosomal proteases. If the terminal dileucine motif of m6/gp48 is removed, class I complexes appear on the surface of transfected cells with an increased half-life relative to that seen with wildtype m6/gp48. As with *m152*, heterologous expression of *m6* is sufficient to protect target cells from lysis by class I-restricted CTLs. Thus MCMV encodes at least two different proteins which protect infected targets from class I-restricted CTLs by means of a general downregulation of class I.

m4/gp34 forms complexes with class I which are expressed on the cell surface

Shortly before the description of *m6*, another class I-associated MCMV protein was identified using a similar but more direct strategy.[20] In this case, class I molecules were precipitated from metabolically labeled MCMV-infected fibroblasts and directly analyzed by SDS-PAGE. By comparing infected and uninfected cells, it was found that infected cells contained a glycoprotein of 34 kD apparent molecular weight which was in a detergent-stable complex with class I molecules. Using mutant viruses and a serum generated against the predicted peptide sequence of *m4*, it was demonstrated that this protein was m4/gp34, the product of *m4*.

m4/gp34 has proved to be quite a puzzling protein, in that it has not conformed to any simple expectations for viral immune evasion proteins. Like m6/gp48, m4/gp34 forms stable complexes with β2m-associated class I in the ER which are exported through the Golgi. Unlike m6/gp48–class I complexes, however, m4/gp34–class I complexes have a long half-life and travel to the cell surface where they are available for labeling by cell-surface iodination.[20] m4/gp34 itself is completely retained in the ER except when complexed with class I.

One function proposed for *m4*[20] is that it could serve to counteract the 'missing self' reaction by which which some NK cells are activated when target cells fail to express class I at the cell surface. The impetus behind this proposal was the observation that following infection of fibroblasts, the export of newly-synthesized K^b increased over time coincident with increased m4/gp34 expression. However, we find that the rate of K^b export over a 90 minute chase experiment is the same in cells infected with wildtype virus or virus specifically knocked out for *m4* (Δm4)—implying that *m4* does not counteract the effects of *m152* (D.G.K. manuscript in preparation). These results do not rule out the possibility that *m4* may affect NK function, but currently no data support this hypothesis. This is in sharp contrast to the case of the class I homolog *m144*, for which there is ample evidence of an effect on NK function.[21–23]

m4 alters the CTL response in vitro

Using Δm4 viruses our lab has recently produced *in vitro* evidence for an immunoevasive function for *m4* (D.G.K. manuscript in preparation). In accord with the results of Krmpotic *et al.* (see above), we found that CTL clones derived from MCMV-primed mice lyse targets infected with Δm152 but not targets infected with wildtype virus. Additionally, we find that some, but not all, MCMV-specific CTL clones lyse targets infected with Δm4 better than targets infected with wildtype virus. Thus we conclude that *m4* also protects MCMV-infected cells from MCMV-specific CTLs.

Co-operation or redundancy?

It should be clear from the above summary that there is a complex set of MCMV genes which alter the normal processing and function of class I MHC molecules. However, it is still unclear how these various viral genes relate functionally and biochemically to each other. Before these questions can be answered, investigators will have to address several outstanding issues regarding MCMV. These include: the biochemical disposition of class I molecules in MCMV-infected cells; differential effects of MCMV genes on different class I gene products; and differential expression or function of MCMV genes in different tissue types.

What is the disposition of MCMV-altered class I?

Class I molecules in MCMV-infected cells can be grouped into four sets: m152/gp40-retained molecules, m6/gp48-associated molecules, m4/gp34-associated molecules, and 'normal' free molecules apparently unaffected by MCMV immune evasion genes. The relative sizes of these sets and the relationships among them is a matter of active investigation.

In MCMV-infected cells pulse-labeled for 30 minutes at 6 hours post-infection, over half of the newly

synthesized K^b molecules remain endoH sensitive after a 90 minute chase, whereas essentially all of the K^b molecules are exported over the same period in uninfected cells (D. G. Kavanagh, unpublished data). Both *m152* (which blocks export) and *m6* (which degrades exported class I) probably contribute to the absence of endoH-resistant molecules. It is not known whether m6/gp48 and m152/gp40 can have co-operative effects on the same class I molecule, or whether they affect different populations of molecules. Likewise, it is not known to what degree the populations of m4/gp34- and m6/gp48-affected class I molecules overlap.

Newly synthesized class I molecules rapidly enter assembly complexes which contain chaperones such as calnexin, calreticulin, TAP, and tapasin (Figure 2). These complexes are generally stable in a 1% solution of the detergent digitonin, but unstable in a 0.5% solution of NP-40. m6/gp48 is found in digitonin-stable complexes with calnexin as well as with class I in the ER.[19] We have determined that m4/gp34 is found in *digitonin-stable* but *NP-40-unstable* complexes with class I, calnexin, and tapasin—this is in addition to previously described m4/gp34 complexes with class I,[20] which were *NP-40 stable*. Thus m4/gp34 engages class I in two different types of complexes: '*strong*' complexes which are stable in the strong detergent NP-40, and '*weak*' complexes which are stable in the weak detergent digitonin but not in NP-40. Weak complexes may represent direct interactions of m4/gp34 with class I and ER chaperones, or may represent indirect associations via larger complexes. In cells infected with wildtype MCMV, K^b molecules in weak complexes with m4/gp34 remain entirely endoH sensitive for a period of up to 3 hours post-synthesis. In cells infected with Δm152, however, K^b molecules in weak complexes rapidly become endoH resistant, and the half-life of these complexes is greatly decreased (D.G.K., unpublished data). The relative immunological significance of weak and strong complexes of class I with m4/gp34 is entirely unknown; however, the combined effects of *m152* and *m4* on K^b molecules in weak complexes demonstrate for the first time that some MCMV gene products co-operatively affect the same class I proteins.

With regard to strong complexes, we find that at least 50% of endoH-resistant K^b can be co-precipitated by antibody to m4/gp34 after overnight infection. This presumably means that over 50% of the K^b on the surface of infected cells is m4/gp34 associated. Do these molecules represent class I which has escaped the effects of *m152* and *m6*? Are they peptide loaded? If so, is the peptide antigenic? We hope to have answers to these questions soon.

Are different class I molecules targeted by different MCMV genes?

One attractive hypothesis to explain the multiplicity of immune-evasion genes in both MCMV and HCMV is that different viral genes may be required to modulate the products of different class I genes or alleles. Several lines of evidence suggest that these MCMV proteins are promiscuous: *m6* is reported to downregulate K^d, L^d, D^d, K^b, D^b and K^k;[19] *m152* is reported to downregulate H-2^d, H-2^k, H-2^b, and H-2^q proteins;[17] and *m4* is reported to bind K^b, D^b, D^d, and L^d, but not K^d.[20] Currently, there are no published data comparing the relative biochemical and immunological effects of different MCMV genes on different class I molecules. However, we found that the biochemical effects of *m152* are greater on D^b than on K^b, and that, conversely, *m4* is required for complete evasion from K^b-, but not D^b-restricted CTL clones (manuscript submitted). These results suggest that CMVs may carry multiple immune-evasion genes in order to alter the assembly of different class I gene products.

Is immunoevasive gene function tissue specific?

During the course of infection *in vivo,* MCMV infects a number of different tissue types, each of which probably plays a specific role in the virus's infectious cycle. Dendritic cells (DCs) are probably of primary importance in priming the immune response; however, there is currently little information available regarding antigen presentation by DCs in MCMV infection. For other viruses it has been shown that *uninfected* DCs are capable of 'cross-priming' $CD8^+$ T cells by presenting exogenous viral antigens generated in infected cells.[24,25] Therefore, we might expect that genes such as *m152* or *m6* would have no effect on the ability of DCs to prime an immune response. Nevertheless, we have found that mice infected with the viral mutant Δ94.5, which lacks genes *m151–m165,* produce significantly stronger CTL responses than those infected with wildtype virus (Marielle Gold, unpublished data). This suggests that one or more of these genes does affect the priming or boosting of the CTL response.

Monocyte macrophages probably play an extremely important role in systemic infection by MCMV.[26] A recent preliminary report, however, suggests that

neither *m6* nor *m152* affects antigen presentation by macrophages *in vitro*.[27] This raises the possibility that the primary role of these genes may be to protect only certain cell types from CTLs. It is noteworthy that $CD8^+$ CTLs can effectively control virus titers in most tissues, but not in salivary gland,[4] which is presumably an important site of viral replication for transmission to a new host. It will therefore be of interest to analyze expression and function of immune evasion genes in different murine tissue types.

Conclusions

MCMV is a classic example of a virus in evolutionary equilibrium with the host organism: it is vertically transmitted through the host population and is efficient at causing new infections with minimal pathology for the host animal. During the course of its evolution, MCMV has developed an intimate relationship with the murine adaptive immune system, and the findings we have summarized here raise interesting questions about how the virus exploits this relationship to its ultimate advantage. How does MCMV strike a balance between the need to avoid pathology and the need to replicate and cause new infections?

There is no doubt that CTLs will only be a part of this puzzle, which includes both the adaptive and innate immune systems. With regard to the CTL response the most compelling challenge is to explain the individual effects of genes such as *m4*, *m6*, and *m152* in terms of viral replication *in vivo* and transmission throughout natural host populations. The first step will be understanding to what degree these genes co-operate on a cellular level in different tissue types and on different genetic backgrounds. The next step will be to ask how they work *in vivo*, which will require development of new techniques to study antigen presentation in different tissue types. We believe that answers to these questions are important both because of clinical implications for human disease and because of the light they will shed on the basic biology behind the co-evolution viruses and the adaptive immune system.

Acknowledgements

Δm152 and Δm4 viruses were kindly provided by Dr Ulrich Koszinowski. We wish to thank Marielle Gold, Michael Munks, and Diane LoPiccolo for advice in preparing the manuscript.

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