An MHC class I immune evasion gene of Marek's disease virus

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ABSTRACT

Marek's disease virus (MDV) is a widespread α-herpesvirus of chickens that causes T cell tumors. Acute, but not latent, MDV infection has previously been shown to lead to downregulation of cell-surface MHC class I (Virology 282:198–205 (2001)), but the gene(s) involved have not been identified. Here we demonstrate that an MDV gene, MDV012, is capable of reducing surface expression of MHC class I on chicken cells. Co-expression of an MHC class I-binding peptide targeted to the endoplasmic reticulum (bypassing the requirement for the TAP peptide transporter) partially rescued MHC class I expression in the presence of MDV012, suggesting that MDV012 is a TAP-blocking MHC class I immune evasion protein. This is the first unique non-mammalian MHC class I immune evasion gene identified, and suggests that α-herpesviruses have conserved this function for at least 100 million years.

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Introduction

The immune response to viral infection usually eliminates infection and protects against subsequent infection. However, a number of viruses are able to establish long-term infections in spite of the immune response. In particular, members of the herpesvirus family are able to establish latent infections and reactivate in spite of a functional immune response. The mechanisms underlying this ability are not well understood.

Another general feature of herpesviruses is their ability to block the class I major histocompatibility complex (MHC class I) antigen presentation pathway. MHC class I is expressed on the surface of almost all nucleated cells, and consists of a trimolecular complex composed of the highly polymorphic MHC class I heavy chain, β2-microglobulin (β2-m), and a small peptide, usually 8–10 amino acids long. These peptides are generated by proteolysis in the cytosol, mainly by the proteasome (reviewed in Rock et al. (2004), Shastri et al. (2002)), and a small fraction of cytosolic peptides are translocated into the endoplasmic reticulum (ER) lumen by the transporter associated with antigen presentation (TAP). Newly synthesized heavy chain and β2-m assemble together to form a dimer, a process that is facilitated by chaperones such as BiP, calnexin, and calreticulin (Rufer et al., 2007; reviewed in Hulpke and Tampe (2013)). After a peptide is loaded onto this dimer, the mature MHC class I complex is allowed to exit the ER and reach the cell surface. If the peptide is derived from a non-self protein, CD8 + cytotoxic T lymphocytes (CTL), an important component of the antiviral immune response, can recognize the MHC class I complex, and respond by lysing the infected cell as well as releasing cytokines that confer an antiviral state on local cells.

Since the first identification of a herpesvirus MHC class I immune evasion gene in 1994 (York et al., 1994), genes that interfere with MHC class I have been described in at least a dozen other α-, β-, and γ-herpesviruses that infect humans, non-human primates, mice, cattle, horses, deer, and cats (Fruh et al., 1999, 2002; Hudson et al., 2001; Hislop et al., 2007; Kleijnen et al., 1997; Koppers-Lalic et al., 2008; Powers et al., 2008; Powers and Fruh, 2008; Verweij et al., 2011). As well, some members of several other viral families, including poxviruses, adenoviruses, and lentiviruses, also have MHC class I immune evasion genes.

Marek's disease virus (MDV) is a ubiquitous α-herpesvirus of chickens that causes T-cell lymphomas in infected birds (reviewed in Jarosinski et al. (2006)). First identified as a disease in 1907 (Marek, 1907), the virus was identified in 1967 (Churchill and Biggs, 1967), and a vaccine was first made available in 1970. However (as with several vaccines against herpesviruses) the vaccine prevents clinical symptoms, but does not prevent infection or transmission of the virus. Probably because of selection for increased transmission in the face of widespread non-sterilizing vaccination, MDV strains have continually increased in virulence; the original vaccine has twice had to be replaced by more effective...
vaccines (Gimeno, 2008; Nair, 2005). Selective pressures, including vaccination, early cohort removal, and host genetic selection (Gimeno, 2008; Nair, 2005; Atkins et al., 2013; Hunt and Dunn, 2013), may continue to drive increases in MDV virulence in the future; and new vaccines will probably be required to handle resulting outbreaks (Gimeno, 2008; Nair, 2005).

MDV has been shown to reduce MHC class I surface expression in the acute, but not the latent, phase of infection (Hunt et al., 2001; Levy et al., 2003). Although the UL49.5 gene of MDV was shown to reduce expression of MHC class I expression in chicken cells, this gene was found to be only partially responsible for the MHC class I inhibition seen (Jarosinski et al., 2010), and additional responsible gene(s) have not yet been identified. Homologs of UL49.5 are conserved throughout the herpesvirus lineage, and have been shown to have MHC class I evasion activity in several, though not all, members of the Varicellovirus genus (Koppers-Lalic et al., 2005, 2008; Verweij et al., 2011) and MDV (Jarosinski et al., 2010). The UL49.5 genes of Varicelloviruses have been shown to function through inhibition of TAP by several mechanisms (Koppers-Lalic et al., 2008), while the mechanism of UL49.5 evasion of MHC class I in MDV is not yet known.

Here we identify an additional MDV gene that prevents MHC class I expression, and demonstrate that the gene prevents transport of cytosolic peptides to the ER but does not inhibit peptide loading in the ER. This gene appears to be unique to the non-mammalian Mardivirus clade, and, along with the identification of UL49.5, suggests that MHC class I immune evasion has been functionally preserved in the α-herpesviruses for at least 100 million years.

Results

MDV012 reduces surface MHC class I on chicken cells

We transfected chicken DF1 and LMH cells with each of ten cloned MDV genes, and stained cell-surface MHC class I two days after transfection, gating on GFP-positive cells to limit analysis to transfected cells. Most of the genes did not alter MHC class I surface expression significantly (Fig. 1A). UL49.5 is an MHC class I immune evasion gene in several, but not all, α-herpesviruses, and has been shown to moderately down-regulate MHC class I in chicken O2 cells (Jarosinski et al., 2010) in one report but had no effect in LMH cells or human MJS cells in another report (Verweij et al., 2011). In our experiments MDV UL49.5 did not consistently reduce MHC class I expression on DF1 (Fig. 1A) or LMH cells (data not shown). MDV087 consistently, but only slightly, reduced surface MHC class I on DF1 cells (Fig. 1A) but had no consistent effect in LMH cells (data not shown). In contrast, the viral gene MDV012 significantly reduced MHC class I surface expression on DF1 cells (Fig. 1A).

In the MDV genome, MDV012 is predicted to consist of two exons separated by an 83-bp intron (with the 5’ exon being termed “MDV011” in some annotations), and our original clone of MDV012 from the BAC-encoded MDV genome included the intron (genomic MDV012; “MDV012g”). To confirm that MDV012 gene is spliced as predicted, RT-PCR was performed on total RNA from chick embryo fibroblasts infected with the Md5 strain of MDV, using primers from exon 1 and exon 2. The Md5 version of MDV012 and the surrounding regions are identical to the Md11 version. A single strong PCR product resulted which comprised exon 1 and exon 2 with the intron removed (data not shown), as predicted from the genomic sequence. We cloned this spliced version of MDV012 (MDV012s), fused to GFP at the C-terminus. Expression of this spliced MDV012s fusion construct had an even more dramatic effect on surface expression of MHC class I than did expression of MDV012g (Fig. 1A).

Chicken MHC class I genes, unlike most mammalian MHC class I, includes only two isoforms and are closely linked to the TAP genes (Kaufman et al., 1999). Of the two isoforms of MHC class I, one is expressed more efficiently (major isoform) (Kaufman and Salomonsen, 1997). The reason for this differential expression is unknown, but the minor isoform may act as an NK cell ligand (Ewald and Livant, 2004; O’Neill et al., 2009; Zhang et al., 2012).
We tested the effect of MDV012 on expression of the major and minor isoform of B21 in DF1 cells, using chicken sera specific for each isoform. The spliced version of MDV012 reduced expression of both isoforms to similar extents (Fig. 1B and C). Unspliced MDV012g did not have a consistent effect on the minor isoform of B21 (data not shown), and the originally noted effect in DF1 cells stained for the major isoform of B21 was mild and somewhat variable in repeated experiments; thus splicing might be important for gene function, although the difference in vectors used precludes a direct comparison between spliced and unspliced forms of this gene. MDV012s efficiently reduced cell-surface levels of MHC class I on LMH cells (Fig. 1D).

MDV012 inhibition of MHC class I expression is specific

Cells transiently transfected with MDV012 showed no obvious signs of toxicity. To test whether the effect on MHC class I expression is specific, we co-transfected DF1 cells with MDV012s (or with empty vector as a control) and either influenza HA, or mouse H-2K\(^\beta\) expressed as a single chain fused with β\(_2\)-m ("SC-β\(_2\)-m"). Mammalian MHC class I heavy chain expressed in chicken cells is expressed very inefficiently. However, in preliminary experiments we determined that single-chain fusions of mammalian MHC class I heavy chains fused to mammalian β\(_2\)-m are expressed effectively in chicken cells. Presumably the reason for poor expression of mammalian MHC class I in chicken cells is that chicken β\(_2\)-m, which is only ~45% identical to human and mouse β\(_2\)-m, does not bind efficiently to mammalian heavy chains. Providing an appropriate binding partner in the form of mammalian β\(_2\)-m allows dimer formation and permits surface expression.

MDV012 reduced expression of endogenous chicken MHC, as expected (Fig. 2A). MDV012 also reduced expression of co-transfected SC-Kb (Fig. 2B). However, surface expression of HA was not reduced by co-expression of MDV012 (Fig. 2C). This observation shows that MDV012 does not non-specifically block protein expression or cell-surface transport of glycoproteins. As well, since MDV012 did block expression of mouse MHC class I as well as endogenous chicken MHC class I, the effect of MDV012 on surface expression of MHC class I must not be dependent on chicken-specific MHC class I sequence or conformation.

MHC class I-binding peptide rescues expression of MHC class I

The TAP peptide transporter is a bottleneck in the antigen processing pathway and is probably not required for cellular processes other than MHC class I antigen presentation. Accordingly, many mammalian herpesviruses target TAP for evading MHC class I. To test whether chicken TAP is a potential target for MDV012, we used a peptide that has been shown to bind to the chicken MHC class I B21 allele, REVDEQLLSV (R10V) (Koch et al., 2007). We targeted the peptide to the ER lumen with a signal sequence, or to the cytosol by expressing the peptide as a ubiquitin fusion protein. Peptides fused to the C-terminus of ubiquitin are rapidly cleaved by ubiquitin C-terminal hydrolases, releasing ubiquitin and the C-terminal sequence as a cytosolic peptide with a defined N-terminus (Bachmair et al., 1986; York et al., 2006). R10V targeted to the ER by a signal sequence (and thereby bypassing the requirement for TAP) restored most of the MHC class I surface expression on DF1 cells (which express B21 (Fig. 3A), but did not affect surface expression of MHC class I in LMH cells (whose MHC class I type is not B21, and which are therefore not expected to bind R10V) (Fig. 3B). In contrast, cytosolic expression of R10V did not rescue surface MHC class I expression in DF1 or LMH cells (Fig. 3A, B). These experiments suggest that MDV012 acts by blocking chicken TAP. Importantly, the rescue of surface MHC class I expression in the presence of ss-R10V further demonstrates that MDV012 does not prevent transcription, translation, or surface expression of MHC class I, but reduces surface expression by preventing newly-formed chicken MHC class I in the ER from having access to peptide.

MDV012 orthologues are present in the Mardivirus clade

In MDV, MDV012 is a 489-amino acid protein that is encoded by two exons separated by an 83-bp intron. Related proteins are present in other members of the Mardivirus clade: Gallid Herpesvirus 3 (GaHV-3; also known as Marek's disease virus type 2), Meleagrid herpesvirus 1 (MeHV-1; also known as herpesvirus of turkeys), and Duck Enteritis Virus (DEV) (Fig. 4). The MDV (GenBank: NC_002229) and MeHV-1 (GenBank: NC_002641) genes are annotated as spliced genes in at least some of the genomic sequences. Although the GaHV-3 gene is not annotated as spliced in the genomic sequence (GenBank: NC_002577), analysis with splicing prediction programs (Hebsgaard et al., 1996) indicates that this gene is also very likely to be spliced, extending the similarity with GaHV-1 and MeHV-1 sequences. The DEV (GenBank: NC_013036) gene LORF3 has similarity to the second exon of MDV012. However, we did not identify an obvious upstream exon for this gene. Most of the similarity between these four proteins is in the N-terminus, with the C-terminal half of the proteins being
Discussion

Here we demonstrate that Marek’s disease virus, an α3-herpesvirus of chickens that diverged from mammalian herpesviruses at least 100 million years ago (McGeoch et al., 2006), encodes a gene (MDV012) that specifically blocks surface expression of MHC class I. The ability to block surface expression of MHC class I has been previously shown for MDV (Hunt et al., 2001; Levy et al., 2003), and while one partially responsible gene has been identified, another gene(s) is expected to contribute significantly to this function (Jarosinski et al., 2010).

Our experiments suggest that MDV012 blocks antigen processing at the level of the TAP peptide transporter, a common target of viral MHC class I immune evasion genes. In particular, the MDV012-imposed block on surface expression of MHC class I proteins is overcome when a peptide epitope binding the chicken MHC class I is delivered into the ER, but not when the peptide is delivered to the cytosol. This indicates that MDV012 does not post-translationally destroy MHC class I or prevent its transcription, translation, or trafficking, but rather affects peptide availability within the ER lumen.

Also consistent with a TAP blockade is the observation that MDV012 reduces surface expression of both the major and minor MHC class I isoforms. The function of minor isoforms of MHC class I is not well understood. One possibility is that the minor isofrom of MHC class I acts as an inhibitory NK ligand (Ewald and Livant, 2004; O’Neill et al., 2009; Zhang et al., 2012); if so, the reduction in the minor isofrom by MDV012 may render cells susceptible to NK recognition and lysis, unless, like many other herpesviruses, MDV also has additional genes which block NK recognition of infected cells.

MDV012 is a member of a small family of proteins with unknown functions (the “DUF1509” superfamily) that are found in several avian herpesviruses of the Mardivirus clade (GaHV-3, DEV, and MeHV-1). It is not yet known if the DUF1509 proteins encoded by these other viruses also have an immune-evasion function, but if so MDV012 may be a part of another small cluster of related immune-evasion genes in closely-related viruses. Aside the members of the Mardiviruses, no proteins related to MDV012 have been identified.

The observation that Marek’s disease virus is able to block MHC class I expression suggests that MHC class I immune evasion has been functionally conserved in the α-herpesviruses for at least 100 million years (McGeoch et al., 2006). Although it remains possible that MHC class I immune evasion was independently invented by α-β- and γ-herpesviruses, the widespread presence of MHC class I immune evasion genes in β-, and γ-herpesviruses suggests that the common ancestor of the α-, β-, and γ- herpesviruses, about 400 million years ago (McGeoch and Gatherer, 2005), had MHC class I immune evasion genes. Analysis of αβ-herpesviruses (e.g. ILTV virus of chickens), which diverged from mammalian α-herpesviruses about 200 million years ago (McGeoch et al., 2006), and especially of herpesviruses of reptiles and fish (which diverged over 400 million years ago (McGeoch and Gatherer, 2006), prior to the establishment of distinct α, β, and γ lineages) will be necessary to determine how ancient MHC class I immune evasion is.

Marek’s disease is a major concern for the global poultry industry. MDV strains vary widely in virulence, and new strains have increased in virulence over time. However, the MDV012 gene is identical in very virulent (Md5 strain, GenBank: AF243438.1), virulent (RB-1B, GenBank: EF523390.1, and Md11, GenBank: AY510475.1), mildly virulent (CU-2, GenBank: EU499381.1), and vaccine (CIV988, GenBank: DQ530348.1) strains of MDV, suggesting that MDV012 is unlikely to play a direct role in the differing virulence of different field isolates.

Over 20 billion doses of MD vaccine are given annually, but although the vaccine protects against disease it does not confer sterilizing immunity, leading to concerns that the vaccine itself may be driving increased MDV virulence over time (Gimeno, 2008; Nair, 2005). The presence of MDV012 in the widely-used vaccine strain CIV988 raised the possibility that deleting MDV012 from the virus may attenuate the virus as well as increase its immunogenicity; however, we found that deleting MDV012 from the Md5 strain of MDV by BAC mutagenesis made the virus non-viable in tissue culture, confirming another report that MDV012 is an
essential gene for in vitro replication (Chattoo et al., 2006) and suggesting that it may perform more than one function. A recent report of mild viral attenuation by single-SNP incorporation into non-coding regions of MDV012 without significant loss of in vivo replication suggests that it may be possible to study the function(s) of this gene in vitro and in vivo using small mutations or gene knockdown methods (Hildebrandt et al., 2014).

Resistance to MDV varies widely between different strains of chickens, and resistance is linked to the B21 haplotype (Briles et al., 1977; Hutt and Cole, 1947; Longenecker et al., 1977), likely...

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Fig. 4. MDV012 orthologs. MDV012-related proteins of Marek's disease virus (MDV), gallid herpesvirus 3 (GaHV3), meleagrid herpesvirus 1 (MeHV1), and duck enteritis virus (DEV). Regions of identity are boxed. The junction between exons 1 and 2 in MDV, GaHV3, and DEV is indicated with an arrow.
due at least in part to its broad peptide binding repertoire for MHC class I presentation (Koch et al., 2007; Sherman et al., 2008). Chicken MHC class I heavy chains are tightly linked to TAP genes (Shaw et al., 2007), which are relatively variable between chicken strains (Walker et al., 2005). Could resistance to MDV also be associated with the relative affinity of MDV012 to different TAP alleles? While MDV012 seems to have a somewhat more marked effect on surface expression of MHC class I in LMH (which are not B21 haplotype) than on DF-1 cells (B21 haplotype), further experiments will be necessary to test whether this is a general effect and if it is relevant to MDV pathogenesis in natural infections.

In general, the function of MHC class I immune evasion genes in viral pathogenesis is poorly understood, mainly because of the lack of suitable animal models. Mouse β- and γ-herpesviruses (MCMV and MHV68, respectively) lacking MHC class I immune evasion functions have been analyzed in their natural hosts (Bennett et al., 2005; Stevenson et al., 2002a, 2002b). For MCMV, the lack of MHC class I immune evasion was associated with increased titers of virus in salivary glands (Lu et al., 2006), but had only a modest effect on immune responses, viral titers, viral persistence in other tissues, or virulence (Gold et al., 2004; Krmpotic et al., 1999; Doom and Hill, 2008). MHV68 lacking MHC class I immune evasion was similar to wild-type virus during acute infection of mice, but was more susceptible to immune clearance during the latent phase (Bennett et al., 2005; Stevenson et al., 2002a, 2002b).

Among the α-herpesviruses, the two MHC class I immune evasion genes which have been studied in the natural host are the UL49.5 genes of bovine herpesvirus type 1 (BHV-1) and of MDV. In the case of BHV-1, deleting specific residues in the luminal and cytoplasmic tail regions of UL49.5 allowed abrogation of TAP inhibition without affecting replication in vivo or in vitro (Wei et al., 2011, 2012). In calves, BHV-1 lacking class I immune evasion was equally virulent to the wild-type virus, but elicited an earlier adaptive immune response (Wei et al., 2012). Similarly, deleting the cytoplasmic tail from MDV UL49.5 resulted in a virus with partially reduced immune evasion activity but which retained wild-type replication and virulence in the chicken, although the immune response was not studied in this case (Jarosinski et al., 2010). These studies indicate that MHC class I immune evasion is not an essential function for replication, and deleting this function might be a useful tool to increase the immunogenicity of live vaccines against α-herpesviral diseases of domestic animals or humans.

Although not studied in vivo in the natural host, the ICP47 gene of the human α-herpesviruses HSV-1 and -2 blocks human TAP in vitro (Hill et al., 1995), and an ICP47 knockout showed altered neurovirulence in mice (Orr et al., 2005); however, ICP47 has poor affinity for mouse TAP (Tomazin et al., 1998), so this model is not necessarily relevant to class I immune evasion. The other human α-herpesvirus, VZV, which interferes with MHC class I maturation and trafficking via the ORF66 protein kinase (Eisfeld et al., 2007), lacks a suitable animal model of infection. Thus, evidence for the relative importance of this mechanism in α-herpesvirus infection will likely have to be gained from studying animal diseases in their natural hosts. The chicken is a particularly useful model, as it is small, easily housed, available in inbred lines, and well-characterized. Infection of chickens with MDV lacking functional MHC class I evasion by MDV012 may prove useful for understanding the function of MHC class I immune evasion by α-herpesviruses in pathogenesis.

### Methods and materials

#### Plasmids and cloning

We identified 10 MDV genes that are conserved between MDV strains and whose function has not been determined, and used PCR to clone these genes from a bacterial artificial chromosome (BAC) containing the genome of the virulent Md11 strain of MDV (Niikura et al., 2006). The MDV genes are listed in Table 1; the primers used to amplify each of the genes are listed in Table 2. Genes were amplified using high-fidelity polymerases (either Pfx [Invitrogen, Carlsbad, CA] or Phusion polymerase [Finnzymes,
Woburn, MA]) and subcloned into pTracerCMV2 (Invitrogen). Each gene was completely sequenced to confirm that no point mutations were introduced during the cloning process.

To confirm the spliced nature of the MDV012 gene, total RNA was isolated from chick embryo fibroblasts infected with the Md5 strain of MDV using the RNaseasy kit (Qiagen, Valencia, CA). The RNA was subjected to reverse transcriptase PCR using the OneStep RT-PCR kit (Qiagen) with the following primers: 5′-ATGACTAGCCAGAGATCGCTTCGCTTATCC-3′ and 5′-TCATCAGGCATGCCATGAGCCTG-3′, and the product was completely sequenced.

The spliced MDV012-EGFP fusion construct (MDV012s) was made by PCR amplification of Md5 DNA. The 5′ primer comprises the entire first MDV012 exon (109 bps) and the first 19 base pairs of the second exon. This primer also incorporates a Hind III site at its 5′ end to facilitate cloning. The 3′ primer incorporates a BamHI site, allowing cloning of the PCR product into pEGFP-N1 (Clontech, Mountain View, CA), to construct a GFP fusion protein. Primer sequences are shown in Table 1. The resulting MDV012-pEGFP was sequenced to confirm that no errors were introduced during construction.

To construct plasmids expressing cytosolic and ER-targeted B21-binding peptide REVDEQLLSV (Koch et al., 2007), we annealed oligonucleotides and cloned them into a ubiquitin fusion vector, pUG1 (York et al., 2006), or into a signal-sequence containing vector (York et al., 2002), as previously described (York et al., 2006). The oligonucleotides are listed in Table 3.

Influenza hemagglutinin (HA) was subcloned into pTracerCMV2 (pTracerCMV2-HA). A single-chain MHC class I dimer comprising mouse β2-m and the mouse MHC class I allele H-2Kb, joined by a flexible linker, and cloned into pTracerCMV2 (pTracerCMV2-β2m-SC-Kb), has been previously described (York et al., 2005). Plasmids expressing SLNFEKL (the H-2Kb-restricted immunodominant peptide from ovalbumin) as cytosolic ubiquitin fusion proteins (pUG-S8L) or targeted to the ER (ss-AS8L) have been cloned by a single-chain MHC class I dimer comprising mouse β2-m and the mouse MHC class I allele H-2Kb, joined by a flexible linker, and cloned into pTracerCMV2 (pTracerCMV2-β2m-SC-Kb), has been previously described (York et al., 2005). Plasmids expressing SLNFEKL (the H-2Kb-restricted immunodominant peptide from ovalbumin) as cytosolic ubiquitin fusion proteins (pUG-S8L) or targeted to the ER (ss-AS8L) have been previously described (York et al., 2006; Hearn et al., 2009). MDV UL49.5 cloned into pcDNA3 (Invitrogen) was kindly provided by K. Osterreider (Cornell University) and has been previously described (Jarosinski et al., 2010).

**Cell lines and transfection**

DF1 cells (chicken fibroblasts, B21 haplotype [Himly et al., 1998]) were provided by D. Foster (University of Minnesota). LMH (chicken hepatocarcinoma carcinoma cells, unknown haplotype [Kawaguchi et al., 1987]) was provided by Marcia Miller (Beckman Research Institute of City of Hope, Duarte, CA). Transfections were performed using TransIT LTI (Mirus, Madison, WI) according to the manufacturer’s instructions.

**Antibodies and flow cytometry**

Two days after transfection, cells were trypsinized and stained with the following antibodies: C6B12 (anti-chicken MHC class I [Shamansky et al., 1988], obtained from The Developmental Studies Hybridoma Bank, Iowa City, Iowa); chicken antiserum specific for major or minor B21 (Fulton et al., 2001); anti-H2-Kb (B8.24.3 [Kohler et al., 1981]); or anti-HA (H36.5-4.2; [Caton et al., 1982]). Cells were washed in PBS, and counter-stained with secondary antibodies. Cy5-conjugated second antibodies to mouse or chicken antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Flow cytometry was performed on a BD LSRII or BD FACS Vantage (Becton Dickinson), live cell populations were identified based on forward- and side-scatter characteristics, and transfected cells were identified by gating on GFP-positive cells.

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