IDENTIFICATION AND MUTAGENESIS OF FELINE HERPESVIRUS UL49.5 IMMUNE EVASION GENE

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ABSTRACT

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Cells present peptides on class I major histocompatibility complex (MHC class I) molecules to cytotoxic CD8+ T lymphocytes (CTL), which recognize viral peptides and destroy infected cells. Many viruses encode proteins that inhibit the antigen presentation pathway. Here we demonstrate that feline herpesvirus 1 infection of cat fibroblasts effectively blocks MHC class I surface expression by blocking the TAP peptide transporter. Based on previous publications that varicellovirus UL49.5 is an immune evasion gene, we cloned UL49.5 from FHV-1 and demonstrated that this gene is sufficient to block MHC class I antigen presentation. As well as being a common pathogen of cats, FHV1 is a relatively convenient model for viral pathogenesis. Using a two-step homologous recombination technique, we have constructed a knockout virus lacking UL49.5 expression. Feline cells infected with the mutant virus show similar MHC class I surface expression as the uninfected controls. As well as offering a potential approach to varicellovirus vaccination, these studies give greater understanding of viral immune evasion and pathogenesis, as well as of the mechanism of MHC class I antigen processing and presentation.

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TABLE OF CONTENTS

KEY TO ABBERVIATIONS

BAC	bacterial artificial chromosome
CMV2	cytomegalovirus
CRFK	Crandell Rees feline kidney cells
CTL	cytotoxic T lymphocyte
FHV-1	feline herpesvirus 1
H-2kb	mouse MHC allele
HLA-A3	human MHC allele
MHC-1	major histocompatibility complex
SIINFEKL (S8L)	peptide single letter amino acid abbreviation
TAP	transporter associated with antigen processing
UL	unique long

CHAPTER 1

INTRODUCTION

Feline herpesvirus

Feline herpesvirus1 (FHV-1) is a member of the *Vavicellovirus* genus *Alphaherpes* subfamily(1). FHV-1 has a double stranded DNA genome with an icosahedra capsid and a glycoprotein-lipid envelope. FHV-1 causes the acute respiratory illness feline viral rhinotracheitis (FVR) (2) (3) (8)and infects domestic cats as well as other members of Felidae (9). Infection is worldwide, occurring in cats of all ages and breeds. Cats can often have several upper respiratory infections at the same time, however FHV-1 is the most common. Cats are usually infected via nasal, oral, and conjunctival mucous membranes (10)(11). Infected queens are known to pass infection to their kittens at birth or via nursing(12) (11). Characteristic symptoms of infection include sneezing, nasal discharge, rhinitis (inflammation of the nose), and conjunctivitis (inflammation of the membrane lining of the eyelid) (13). FHV-1 infection is most prevalent in kittens, cats in adoption shelters and multi-cat households, especially if there is overcrowding. Infection is also common in conditions of poor sanitation, ventilation, and nutrition. Cats that are physically stressed, have weakened immune system due to other sicknesses, or are unvaccinated tend to show more clinical signs of infection. Immune response, vaccination and the overall health of cats seem to determine the pathogenecity of FHV-1 infection.

Vaccination reduces or prevents clinical symptoms, yet viral persistence exists. Even in vaccinations where a strong antibody response is induced, there is persistence (6). The frequency of infection and spread increases especially when kittens lose maternally derived antibodies (MDA) (14)and/or when cats are grouped together in high

numbers. Thus, persistence can lead to virus reactivation and shedding causing difficulty in disease control. Parenterally administered modified live virus vaccines (MLV) and inactivated virus vaccines are available. Though both viral shedding and latency load of the wild-type virus is reduced in cats vaccinated prior to FHV-1 exposure as compared to unvaccinated controls, these vaccines do not protect against infection or development of the feline carrier state(15). Intranasal vaccines have been shown to yield better and more rapid protection, but can often have side effects such as sneezing and other mild clinical signs. It is not known whether vaccinations help control reactivation of already latent virus. Also, sarcomas may develop at the site of injection, usually after administration of aluminum-based adjuvants (16). The majority of cats vaccinated with modified live or inactivated FHV-1 are protected against disease, however some cats will continue to show mild signs of infection, particularly if they are exposed within three months of the initial vaccination(5, 17) (18). Of these vaccines, some have been recombinant poxviruses and baculoviruses expressing FHV-1 glycoprotein D(19, 20). There are also several insertion/deletion mutants including thymidine kinase deletion mutants, with some containing the capsid gene from feline calicivirus(21). Other vaccines include an insertion mutant of open reading frame 2(ORF2), which is downstream of glycoprotein C(22).

Maes and colleagues (23) developed a recombinant FHV-1 with deletion of both glycoproteins I and E (FHVβ-galgIgEΔ), which has been shown to be an effective vaccine for FVR. The same group has measured the virus latency load of vaccinated cats after FHV-1 infection. Quantitative PCR was performed on feline genomic DNA isolated

from trigeminal ganglia, olfactory bulbs, and brain stems. The authors determined that reduction of the virus latency load in vaccinated cats was dependent upon glycoproteins gI and gE deletions in FHVβ-galgIgEΔ.

Treatments of FHV-1 infection using various nucleoside analogs have been developed. Analogs for treatment of human herpesvirus, including herpes simplex virus and varicella-zoster virus, have been tested against FHV-1, mainly in vitro. Acyclovir and valacyclovir have been show to be extremely toxic for cats(24, 25). Treatment with IFN-α (26)and feline omega IFN (27)to manage FHV-1 keratitis has been used, however there are no controlled clinical trials. Bacterial infections are common with FHV-1 infection. Broad-spectrum antibiotics are of use to control secondary bacterial infections in the upper respiratory tract.

Even with current vaccines, treatments, and healthy control of environmental conditions, FHV-1 infection continues to be a problem. I hypothesize that persistence may be due to specific evasion proteins, in particular those that inhibit cell-mediated immunity. The immune evasion gene UL49.5, in particular, is a great candidate for targeted mutagenesis for vaccine development given that its protein downregulates MHC class I, inhibiting adaptive immune responses.

MHC class I Antigen Presentation/ Cytotoxic T Lymphocytes

Class I major histocompatibility complex (MHC class I) is expressed on all nucleated cells and is composed of a light chain (β_2-microglobulin), a highly polymorphic heavy chain, and a small peptide produced from intracellular protein degradation. MHC

class I antigen presentation allows the cell to display self and foreign peptides from potentially pathogenic intracellular microorganisms such as viruses. Viruses produce proteins that are ubiqutinated and targeted for degradation. These proteins are degraded by proteasomes in the cytosol, producing short peptides that are further processed and trimmed by aminopeptidases to about 8 or 11 amino acids in length(28). Peptides are transported into the endoplasmic reticulum (ER) via the ATP-dependent heterodimer transporter associated with antigen processing (TAP). Peptides in the ER bind MHC class I, and the peptide-MHC complex progresses out of the ER, through the Golgi, and are eventually expressed on the plasma membrane. It is only when peptides are bound by MHC that the complex is allowed to progress out of the ER to the cellular surface.

Viruses being intercellular pathogens, MHC class I presentation is an essential function to notify the 'outside world' (i.e. the immune system) that there is potential danger. Cytotoxic T lymphocyte killing is the most important immune response in controlling a viral infection. The presentation of viral peptides on MHC class I is the cells' signal to cytotoxic T lymphocytes (CTLs) that the cell is infected, and the CTLs recognize and destroy the infected cell. The T cell receptors of CTLs recognize both the MHC class I and a specific viral peptide. The CTL degranulates releasing perforin and granzyme B. Perforin polymerize to form pores in the infected cell, while granzyme B enters the cell to induce apoptosis. Granzyme B induces cell death using two mechanisms. Granzyme B acts on BID inducing the oligomerzation of BAX in the mitochondria membrane(29). This causes the release of cytochrome c, assembly of the apoptosome, and caspase-9 activation. Also, granzyme B directly targets caspase-3 and caspase-7, leading to a caspase cascade, proteolysis and eventually cell death(29).

Many viruses have a lytic and a latent phase, where in the latter there is no production of virons or any evidence of any viral protein synthesis. This is a hallmark of α-herpesviurses, which show rapid induction of latency in neurons (11). Evidence of latently infected neurons is the presence of latency-associated transcripts (LAT). Though it is believed there is no viral protein synthesis, several reports revealed CD8+ T cells surrounding the latently infected neurons. It is not fully know why they are attracted to the cells. The expression of chemokines, such as RANTES, that are essential for attracting T cell and other antiviral cytokines, such as IFN-γ and TNF-α are detectable in latently infected trigeminal ganglia for at up to 180 days after HSV-1 corneal infection(30, 31). However, despite undetectable expression of viral proteins, antigen specific CD8+ T cells somehow resist reactivation of virus by secretion of IFN-γ; the mechanism of action is undefined. Data supports the theory of cross-regulation between IFN-γ and the HSV-1 immediate early gene alpha gene product, infected cell protein 0 (ICP0) that may control latent and lytic infection(32). ICP0 is required for efficient HSV-1 reactivation from latency, and also aids in HSV-1 replication by targeting IFN-induced antiviral proteins for destruction by the proteasome(32). IFN-γ counters the ICP0 effect in several ways, one mechanism includes inhibiting ICP0 transcripts and upregulating cyclin-dependant kinases inhibitors(33).

Therefore, T cells are essential for the control of viral titers, pathogenesis and virus spread. Elimination of virally infected cells during acute infection has been shown

to reduce reactivation, while non-apoptotic control with INF-γ secretion reduces reactivation as well.

Viral Immune Evasion

Many viruses have developed mechanisms that allow escape from the host's immune response, including MHC class I antigen presentation. These viral immune evasion genes result in the down- regulation of MHC class I on infected cells; thereby, presumably inhibiting CTL recognition and killing.

For example, Nef protein of HIV removes MHC class I from the plasma membrane(34), murine cytomegalovirus (MCMV) proteins *m*4, *m*6 and *m*152 interfere with MHC class I antigen processing (35), ICP47 from HSV inhibits peptide translocation by blocking TAP(36), and E3gp19k from adenoviruses blocks MHC association with TAP (37). Recently, Koppers-Lalic et al. (2005) (38)showed that the UL49.5 gene from several members of the varicellovirus family of α-herpesvirus (equine herpesvirus1, bovine herpesvirus1, and pseudorabies virus) encodes a ~9kDa immune evasion protein that acts by blocking the TAP peptide transporter.

There are only a few cases where the role of immune evasion proteins in viral pathogenesis during authentic infections has been studied. Because most immune evasion genes that have been identified to date are from human or large animal pathogens (cattle, swine, and horses), analysis of the infection in natural hosts has rarely been attempted, and most studies have been in tissue culture. Exceptions have been for two mouse

viruses, murine cytomegalovirus (MCMV) (39)and murine herpesvirus-68 (MHV 68). MHV68, a γ-herpesvirus, apparently requires immune evasion genes for efficient establishment of latency (40).

In the case of MCMV, a β-herpesvirus, deletion of multiple MHC class 1 immune evasion genes made surprisingly little difference, with mutant viruses apparently establishing normal lytic and persistent infection and only showing some changes in chronic infection of salivary glands (35, 41). As stated before, MCMV has three known immune evasion genes that interfere with MHC class 1 presentation: m6 restricts MHC class 1 molecules to the lysosome(42); m152 retains MHC class 1 in the ER-Golgi intermediate compartment(40); m4 forms complexes with MHC class 1 where together they are exported to the cellular surface, and remain associated for many hours(43). Another evasion strategy of m152 is that it downregulates the expression of RAE-1 ligands for the activating receptor, NKG2D. Thereby cells infected with wild type MCMV expressing m152 have reduced destruction via NK lysis (44).

Several studies have shown that MCMV-specific CD8 T cells are able to kill cells infected with virus lacking one or more of the three MHC class 1 immune evasion genes. However, these CD8 T cells are not able to destroy cells infected with wild type MCMV. An experiment by Pinto et al. (2000)(45) verified that 16 different CD8 peptide driven T cell lines can kill cells infected with a mutant MCMV lacking all three immune evasion genes (Δm4+Δm6+ΔM152-MCMV), but can not kill the wild type MCMV infected cells. However, in vivo studies have shown that there is no significant difference in CD8 T cell phenotype and response in chronic infection of B6 mice infected with either a wild type BAC MCMV, Δm152-MCMV or Δm4+Δm6+ΔM152-MCMV(41, 46, 47). BALB/c mice

also shared the same CD8 T cell response in chronic infection. However, disrupting immune evasion genes in MCMV reduced virus titers. Infection of BALB/c mice with Δm4+Δm6+ΔM152-MCMV had reduced virus titers in the salivary glands compared to wild type (35). The role of immune evasion genes in alphaherpesviruses has not been tested in infections of the natural hosts.

Two-Step Recombineering: Construction of ΔUL49.5 FHV-1

The UL49.5 gene shares some of its coding sequence with UL50. Instead of making a complete UL49.5 knockout, several point mutations outside of UL50 were introduced into UL49.5. Dr. Roger Maes and Dr. Sheldon Tai have fully sequenced FHV-1 and constructed a bacterial artificial chromosome (BAC) clone.

I used the two-step recombineering method to successfully construct a FHV-1 mutant lacking the UL49.5 gene (Δ49.5 FHV-1). Forward and reverse primers are used in PCR to make the product used in the homologous recombination with the FHV-1 BAC. The DNA template used is a pEP-KansS plasmid, which contains the kanamycin-resistance gene. Next, the PCR product is electroporated into SW105 *E. coli* that were transformed with FHV1- BAC. Bacteria with recombined BACs were positively selected on agar plates containing kanamycin. PCR was also used to screen BACs. A second recombination step is needed to remove the kanamycin-resistance gene, as well as the duplicated mutation site. Cells are transformed with pBAD-*I-SceI. I-SceI* expression cuts and linearizes the FHV-1 BAC. Heat induction of recombination proteins results in a second recombination event, in which the kanamycin resistance gene and second mutation site are removed. The SW105 cells now contain the FHV-1 BAC with the

UL49.5 mutation. Mutant colonies were screened by antibiotic selection and PCR. Colonies were grown in liquid culture and mutant FHV-1 UL49.5 BAC were isolated and purified. Purified BACs were again screened by PCR and Nae1 restriction enzyme digestion.

CHAPTER 2

FELINE HERPESVIRUS 1 UL49.5 IS AN MHC CLASS I IMMUNE EVASION MOLECULE

Feline Herpesvirus 1 UL49.5 is an MHC class I immune evasion molecule

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Herpesviruses are large DNA viruses that typically establish a long-term latent infection. They are a widespread and ancient family of viruses that infect virtually all vertebrates, and at least one invertebrate {Davison et al., 2005, J Gen Virol, 86, 41-53}. Since herpesviruses generally speciate along with their hosts, this distribution implies that the common ancestor of herpesviruses was present about 1 billion years ago {Davison, 2002, Virus Res, 82, 127-32}.

As well as structural characteristics and the ability to establish latency, another common factor among those herpesviruses that have been examined to date is the ability to block class I major histocompatibility complex (MHC class I) presentation of antigens (reviewed in {York, 1996, Chem. Biol., 3, 331-5}{Hansen and Bouvier, 2009, Nat Rev Immunol, 9, 503-13}{Griffin et al., 2010, Vet Microbiol}). MHC class I is the ligand for cytotoxic T lymphocyte (CTL) binding, and allows CTLs to recognize and eliminate cells containing intracellular pathogens, such as viruses. CTLs specifically recognize a small peptide (usually 8-10 amino acids in length) that is associated with the MHC class I complex at the cell surface.

This peptide is generated by proteolysis within the cytosol, usually by the proteasome (reviewed in {Rock et al., 2002, Adv Immunol, 80, 1-70}{Rock et al., 2010, J Immunol, 184, 9-15}). Peptides produced in the cytosol are usually degraded further, to amino acids, but a small fraction of peptides escape destruction and are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP). In the ER, newly synthesized MHC class I heavy chain and β_2-microglobulin (β_2-

m) interact with chaperones and with TAP, and are retained within the ER until peptide is added to the complex. The mature trimeric complex is then released from the ER and is allowed to reach the cell surface (reviewed in {Scholz and Tampe, 2009, Biol Chem, 390, 783-94}{Rock et al., 2010, J Immunol, 184, 9-15}.

Feline herpesvirus1 (FHV-1), a ubiquitous pathogen of domestic cats {Gaskell et al., 2007, Vet Res, 38, 337-54}, is a member of the *Vavicellovirus* genus within the *Alphaherpvirinae* subfamily {Davison et al., 2009, Arch Virol, 154, 171-7}. Acute infection with FHV-1 can cause an upper respiratory and conjunctival infection (feline viral rhinotracheitis). Acute infection is generally followed by a lifelong latent infection in trigeminal ganglia, from which the virus intermittently reactivates and can be transmitted to naive cats {Gaskell et al., 2007, Vet Res, 38, 337-54}{Gaskell and Willoughby, 1999, Vet Microbiol, 69, 73-88}{Binns et al., 2000, J Feline Med Surg S, 2, 123-33}. Vaccination against FHV-1 is effective in preventing disease {Gore et al., 2006, Vet Ther S, 7, 213-22}, but does not prevent infection, reactivation, or shedding {Coutts et al., 1994, Vet Rec S, 135, 555-6}{Sussman et al., 1997, Virology S, 228, 379-82}{Scott and Geissinger, 1999, Am J Vet Res S, 60, 652-8}{Gaskell et al., 2007, Vet Res, 38, 337-54}{Maggs, 2005, Clin Tech Small Anim Pract S, 20, 94-101}. As with immunity following natural infection, vaccine-induced immunity is relatively short-lived {Coyne et al., 2001, Vet Rec S, 149, 545-8}{Mouzin et al., 2004, J Am Vet Med Assoc S, 224, 61-6}.

UL49.5 is a gene that is present in many members of the herpesvirus family. A subset of herpesviruses, comprising some, but not all, members of the Varicellovirus genus, use UL49.5 as an MHC class I immune evasion gene {Koppers-Lalic et al., 2005, Proc Natl Acad Sci U S A, 102, 5144-9; Koppers-Lalic et al., 2008, PLoS Pathog, 4, e1000080}. Here we demonstrate that the UL49.5 protein from FHV1 is an immune evasion molecule that acts by blocking TAP.

Methods and Materials

DNA and cloning. FHV-1 UL49.5 was cloned from a bacterial artificial chromosome (BAC) containing the full-length FHV-1 genome using the following primers, which also contain a 5' EcoRI and a 3' XbaI site for cloning purposes: 5'-cggggtaccccgTCCACGCCTCTGAAGTCTTT-3' and 5'-gctctagagcCCACTAATCCCGGGTCTTTT-3'. Following amplification the gene was cloned into pTracerCMV2.

For making a mutant version of FHV-1 UL49.5, we used the QuickChange II XL Site-Directed Mutagenesis Kit using primers 5' - CAA AAT CTT CCG CGT TGC GGG CCG GCT AAA TCG CTG TAG TAG AAG ACA T - 3' and 5' - ATG TCT TCT ACT ACA GCG ATT TAG CCG GCC CGC AAC GCG GAA GAT TTT G - 3'. The mutant UL49.5 was cloned into pZero and then into pTracerCMV2.

Plasmids expressing precursors of the immunodominant H2-Kb-restricted peptide SIINFEKL (S8L) have been previously described: Full-length ovalbumin in

pTracerCMV2 {York et al., 2005, J Immunol, 174, 6839-46}, cytosolic S8L expressed as a ubiquitin fusion protein {York et al., 2002, Nat Immunol, 3, 1177-84.}, and S8L targeted to the ER with a signal sequence ({York et al., 2002, Nat Immunol, 3, 1177-84.}. Plasmids expressing H-2Kb (as a single-chain fusion protein, fused to mouse b2-microglobulin by a flexible linker) and HLA-A3 (expressed as a single-chain fusion protein with human b2-m) have been previously described {York et al., 2005, J Immunol, 174, 6839-46}.

FHV-1 mutagenesis. We constructed a mutant FHV-1 virus in which UL49.5 was disrupted. Since UL49.5 partially overlaps UL50, rather than delete the gene, we introduced a stop codon UAG 69 base pairs from the start of UL49.5 in a region that does not overlap other genes (The mutant is 23 amino acids; the wild-type is 95 amino acids). A UL49-5-disrupted bacterial artificial chromosome (BAC) was constructed using a two-step recombineering approach as described {Copeland et al., 2001, Nat Rev Genet, 2, 769-79; Testa et al., 2003, Nat Biotechnol, 21, 443-7}. Briefly, a kanamycin resistance cassette flanked by the mutant sequence was constructed by PCR using the following primers: 5'-TTATCCGTAA CATCAGTGCT GGTTATCCTA TTGGTCGCCA TGTCTTCTAC TACAGCGATT tAGccGgCCC GCAACGCGGA AGATTTTGAG AGTATGG AGGATGACGACGATAAGTAGGG-3 and 5'- ACGAGGCGCT CCAAAATTGC TTTAGCCGCT CCATACTCTC AAAATCTTCC GCGTTGCGGG cCggCTaAAT CGCTGTAGTA GAAGACATGG CGACCAA CAACCAATTAACCAATTCTGATTAG-3 (the introduced mutations that do not match the wild-type sequence are shown in lower-case). This cassette was transfected into

E.coil SW105 containing the FHV1 genome in a BAC, and the bacteria were shifted to the permissive temperature for recombination. Kanamycin-resistant colonies were screened by PCR for the presence of the mutant sequence. Positive colonies were transfected with the plasmid pBAD-*I-SceI* and shifted to the permissive temperature for recombination in order to remove the kanamycin resistance gene and kanamycin-sensitive colonies were screened for the mutation using PCR, sequencing, and restriction digests.

A BAC containing the mutant UL49.5 sequence was transfected into CRFK cells for virus rescue. PCR was performed using the primers 5'-TCTTCTACTACAGCGATTTAGCC-'3 and 3'-GGTAAGCAGCGAACTTCGAC-'5 to amplify UL49.5 and its flanking region, and the products were sequenced to confirm the presence of the mutation.

Cells, virus, and transfections. Hela-Kb (Hela cells, stably expressing the mouse MHC class I allele H-2Kb) have been previously described {York et al., 2002, Nat Immunol, 3, 1177-84.}

FHV-1 was obtained from Roger Maes. It was grown and titrated on CRFK cells. CRFK cells were transfected using FuGene according to the manufacturer's protocol. Hela-Kb cells were transfected using the Hela-MONSTER reagent according to the manufacturer's protocol.

Antibodies and flow cytometry. Anti-feline MHC class I monoclonal antibody CF298 was purchased from Veterinary Medical Research & Development, Inc. Anti-human HLA-A, B, C (PA2.6) {Parham and Bodmer, 1978, Nature, 276, 397-9} and anti-H-2Kb (B8.24.3) {Kohler. G. et al., 1981, Immune Syst., 2, 202} were obtained from the ATCC. Anti-influenza hemagglutinin H36.5-4.2 {Caton et al., 1982, Cell, 31, 417-27} was kindly provided by Dr W. Gerhard (The Wistar Institute, University of Pennsylvania). The anti-H-2Kb/SIINFEKL antibody 25.D1.16 has been previously described {Porgador et al., 1997, Immunity, 6, 715-726}. Secondary antibodies tagged with Cy5 were purchased from Jackson Immunoresearch.

Results

FHV-1 blocks MHC class I antigen presentation. CRFK cells were infected with FHV-1 at a multiplicity of infection (MOI) of 7.5. In some experiments, CRFK cells were transiently transfected with mouse MHC class I (H-2K^b) or human MHC class I (HLA-A3). Infection with FHV-1 led to a marked reduction in MHC class I surface expression, both of endogenous (feline) MHC class I and of exogenous (transiently-transfected mouse and human) MHC class I alleles.

Epitopes for the feline MHC class I alleles present on CRFK cells are not known. H-2K^b is known to bind strongly to the immunodominant epitope from chicken ovalbumin, SIINFEKL (S8L). Co-transfection of H-2K^b with S8L targeted to the ER via a signal sequence (thus bypassing TAP) completely restored surface expression of H-2K^b on FHV-1-infected cells, compared to cells in which empty vector was co-transfected with

H-2K^b. This suggests that FHV-1 does not destroy MHC class I, or prevent its transcription or translation, but rather prevents peptides from entering the ER.

FHV-1 UL49.5 blocks antigen presentation. Because several varicelloviruses closely related to FHV-1 use UL49.5 as an MHC class I immune evasion molecule {Koppers-Lalic et al., 2005, Proc Natl Acad Sci U S A, 102, 5144-9; Koppers-Lalic et al., 2008, PLoS Pathog, 4, e1000080}, we used PCR to clone UL49.5 from the FHV-1 genomic BAC and subcloned the gene into pTracerCMV2. We transfected feline (CRFK) and human (Hela-Kb) cells with the plasmid. Two days after transfection, cells were stained for surface MHC class I and analyzed by flow cytometry, gating on GFP (expressed by the plasmid) to limit analysis to transfected cells. Cells expressing UL49.5 showed a marked reduction in MHC class I.

Hela-Kb cells transfected with full-length chicken ovalbumin normally generate the H-2K^b-binding peptide SIINFEKL in the cytosol and transport this peptide into the ER via TAP, leading to presentation of H2-K^b/SIINFEKL complexes at the cell surface. H2-K^b/SIINFEKL complexes can be quantified with the T cell receptor-like antibody 25.D1.16 {Porgador et al., 1997, Immunity, 6, 715-726}. Hela-Kb cells transfected with full-length ovalbumin, and co-transfected with FHV-1 UL49.5, showed a marked reduction in cell-surface H-2Kb/SIINFEKL complexes. Similarly, generation of H-2Kb/SIINFEKL complexes from cytosolic SIINFEKL (expressed as a ubiquitin fusion protein, from which ubiquitin C-terminal hydrolases efficiently remove the peptide moiety to generate processed, cytosolic SIINFEKL) was markedly reduced by co-

transfection of FHV-1 UL49.5, demonstrating that bypassing processing in the cytosol does not rescue the block in antigen presentation imposed by FHV-1.

In contrast, co-transfection with SIINFEKL targeted to the ER by a signal sequence (bypassing the requirement for TAP) completely restored surface expression of H-2K^b/SIINFEKL complexes even in the presence of FHV-1 UL49.5. (In fact, surface expression of H-2K^b/SIINFEKL complexes were significantly and consistently increased in the presence of FHV-1 UL49.5, probably because UL49.5 eliminated any competing peptides from the ER.)

These observations demonstrate that FHV-1 UL49.5 is a specific MHC class I immune evasion gene, and strongly suggest that (like UL49.5 from related varicelloviruses) FHV-1 UL49.5 acts by blocking TAP-mediated peptide transport.

FHV1 lacking UL49.5 does not inhibit MHC class I expression. We constructed a mutant FHV-1 lacking UL49.5. Since UL49.5 overlaps UL50, we used point mutations to insert stop codons in UL49.5 outside the coding region of UL50. The mutant UL49.5, expressed from a transfected plasmid, had no effect on surface MHC class I in transfected Hela-Kb cells. We used a two-step recombineering approach {Copeland et al., 2001, Nat Rev Genet, 2, 769-79; Testa et al., 2003, Nat Biotechnol, 21, 443-7} to introduce this mutation into the FHV-1 genomic BAC. The presence of the mutation in the BAC was confirmed by PCR screening and by restriction enzyme digestion, which demonstrated that a new NaeI site was introduced as expected. The mutant and wild-type BACs were

transfected into CRFK cells and virus was rescued from the transfected cells. Sequencing of the UL49.5 region from the genome of these viruses confirmed that the mutation was present.

CRFK infected with mutant FHV-1 showed similar plaque size and reached similar titers as wild type virus, but expression of MHC class I at the cell surface was not reduced, demonstrating that UL49.5 is the sole FHV-1 gene that blocks MHC class I antigen presentation.

We conclude that FHV-1 UL49.5 is a specific immune evasion molecule that prevents MHC class I antigen presentation by blocking ER transport of peptides, probably by inhibiting TAP.

Discussion

The ability to block MHC class I antigen presentation is found in many virus families, and is especially common (if not universal) among the herpesviruses. Herpesviruses of the α, β, and γ families all block MHC class I antigen presentation, using a wide range of immune evasion genes and targeting virtually every stage of the antigen processing pathway. However, the functional significance of MHC class I immune evasion in viral pathogenesis is not well understood, mainly because there are few authentic laboratory animal models of herpesvirus infection. The demonstration here that the ubiquitous virus of domestic cats, feline herpesvirus 1, encodes an immune evasion gene, UL49.5, may

offer a small-animal model to examine the role of MHC class I immune evasion in that pathogenesis of an α-herpesvirus.

Herpesviruses tend to be highly species-specific, and while there are β and γ-herpesviruses of mice (MCMV and *Mus musculus* rhadinovirus 1 (MmusRHV1) {Ehlers et al., 2007, J Virol, 81, 8091-100}, respectively), no rodent-specific a-herpesviruses are known {Ehlers et al., 2007, J Virol, 81, 8091-100}. MCMV lacking all three known MHC class I immune evasion genes is able to establish acute and latent infection of mice almost normally {Gold et al., 2004, J Immunol, 172, 6944-53}{Pinto and Hill, 2005, Viral Immunol, 18, 434-44} (with the exception of reaching lower titers in salivary glands {Lu et al., 2006, J Virol, 80, 4200-2}) and the immune response to the mutant virus is apparently identical to that against the wild-type virus {Gold et al., 2002, J Immunol, 169, 359-65}{Gold et al., 2004, J Immunol, 172, 6944-53}{Munks et al., 2007, J Immunol, 178, 7235-41}.

Although the γ-herpesvirus MHV68 is not a natural pathogen of laboratory mice (*Mus musculus*), the infection does seem to be fairly similar to that in the natural host, wood mice (*Apodemus sylvaticus*) {Hughes et al., 2010, J Virol, 84, 3949-61}. In laboratory mice, MHV68 MHC class I immune evasion does not affect acute infection, but is apparently important for maintenance of latent infection {Stevenson et al., 2002, Nat Immunol, 3, 733-40}{Bennett et al., 2005, PLoS Biol, 3, e120}.

These findings, limited though they are, suggest that different herpesvirus families may use MHC class I immune evasion at different stages of their life cycle. The role of MHC class I immune evasion by a-herpesviruses in pathogenesis of a natural infection remains unclear.

Several (though not all) members of the varicellovirus family of α-herpesviruses (pseudorabies virus, bovine herpesvirus 1, and equine herpesviruses 1 and 4) use the molecule UL49.5 to block MHC class I antigen presentation by inhibiting TAP function {Koppers-Lalic et al., 2005, Proc Natl Acad Sci U S A, 102, 5144-9; Koppers-Lalic et al., 2008, PLoS Pathog, 4, e1000080}. However, infection of cattle, horses, or pigs with UL49.5-deleted versions of their respective viruses has not been described.

Here, we have shown that UL49.5 of the feline α-herpesvirus FHV-1 blocks MHC class I expression. This blockade is rescued by ER-targeted peptides, strongly suggesting that FHV1 UL49.5 (like other immune evasion UL49.5 molecules {Koppers-Lalic et al., 2008, PLoS Pathog, 4, e1000080}) acts by blocking feline TAP. UL49.5 is expressed in many herpesviruses, and in most it does not act as an immune evasion molecule {Koppers-Lalic et al., 2005, Proc Natl Acad Sci U S A, 102, 5144-9}. The molecular adaptations that allow some UL49.5 proteins to block TAP are not known. Howerver, it is reported that the N-terminal region has two residues (^{9}RXE^{11}) (BHV-1 RRE; SHV-1 and EHV-1 REE) required for TAP inhibition. The last two C-terminal residues RG in conjunction with the N-terminal residues target TAP for proteasomal degradation. FHV-1 UL49.5 N-terminus has SME residues, and PH as the last two C-terminal residues.

FHV-1 is a ubiquitous varicellovirus of domestic cats. It usually infects kittens {Binns et al., 2000, J Feline Med Surg S, 2, 123-33}, in some cases causing upper respiratory and ocular disease during acute infection {Stiles, 2003, Clin Tech Small Anim Pract S, 18, 178-85}. Like human herpes simplex virus 1, FHV-1 rapidly establishes a lifelong latent infection in trigeminal ganglia, from which the virus reactivates at intervals and is shed into the environment, potentially infecting new hosts {Stiles, 2003, Clin Tech Small Anim Pract S, 18, 178-85} {Gaskell et al., 2007, Vet Res, 38, 337-54}{Gaskell and Willoughby, 1999, Vet Microbiol, 69, 73-88}{Binns et al., 2000, J Feline Med Surg S, 2, 123-33}. Cats are therefore a potential model for understanding the role of MHC class I immune evasion in natural infection.

Domestic cats are usually vaccinated against FHV-1. The vaccine is effective in preventing disease {Gore et al., 2006, Vet Ther S, 7, 213-22}, but does not prevent infection, reactivation, or shedding {Coutts et al., 1994, Vet Rec S, 135, 555-6}{Sussman et al., 1997, Virology S, 228, 379-82}{Scott and Geissinger, 1999, Am J Vet Res S, 60, 652-8}{Gaskell et al., 2007, Vet Res, 38, 337-54}{Maggs, 2005, Clin Tech Small Anim Pract S, 20, 94-101}. As with immunity following natural infection, vaccine-induced immunity is relatively short-lived {Coyne et al., 2001, Vet Rec S, 149, 545-8}{Mouzin et al., 2004, J Am Vet Med Assoc S, 224, 61-6}. It is of interest to learn whether a virus lacking MHC class I immune evasion would induce a more potent immune response, or whether other mechanisms (such as cross-presentation) compensate for immune evasion{Snyder et al., 2010, PLoS One, 5, e9681} and allow the development of an effective immune response even in the presence of such gene products.

CHAPTER 3

FINAL DISCUSSION AND CONCLUSION

There is the possibility that FHV-1 has other immune evasion properties that not only prevent antigen presentation, but also evade other immune responses. Other genes can be identified by measuring the immune response *in vitro* and with *in vivo* experiment. I can observe many cell types and their activation levels (i.e., cytokines chemokines, transcription factors, intracellular and cell surface receptor). Many genes can be observed using microarray, or flow cytometry for analysis of protein levels. The disruption of UL49.5 alone may not be effective as a vaccine if FHV-1 has other genes that evade immune responses. These are potential genes that I can mutate and evaluate their disruption in *in vitro* and *in vivo* experiments. If other evasion genes are found, several FHV-1 mutants can be generated with various combinations of mutant evasion genes.

UL49.5 has not been mutated in feline herpesvirus-1. UL49.5 maybe an essential gene for the virus. Mutations in the gene may inhibit viral replication or cell-to-cell spread. In EHV-1, deletion of gM/gE and gI (Rudolph et al., 2002), and also HΔgK (Neubauer and Osterrieder, 2004) deletions, led to dramatic inhibition of cell-to-cell spread. There is also great reduction in viral spread when UL49.5 and UL10 were deleted from Marek's disease virus (MDV-1) (Tischer et al., 2002). This may not allow the host to mount a strong cytotoxic T cell response along with other immune responses, which may interfer with effective immunity toward the wild-type virus. This can be tested with *in vivo* experiments comparing wild-type and mutant FHV-1 virus titers and cell viability kinetics. UL49.5 may not be a potential candidate target gene if shown to be essential for viral replication.

There is the possibility that the mutant virus will increase disease. Adenovirus encodes the E3 region that contains the immune evasion gene E3gp19k. E3gp19k similarly to UL49.5 reduces MHC class 1 antigen presentation (Burgert and Kvist, 1987). A cotton rat model of adenovirus pneumonia was used to study the function of the E3 region and the evasion gene. Virus with large deletions in E3 (H2d180 1747-268bp) lost their MHC class I immune evasion function and were able to replicate like the wild-type virus (Ginsberg et al., 1989). However, there were substantial lymphocyte and macrophage inflammatory responses in the lung (Ginsberg et al., 1989). Other adenoviruses, E3 mutants, show a correlation to increased lung inflammation and restoration of antigen presentation. It is possible that infected cells are presenting some viral antigen and massively activating inflammatory cells, thereby releasing large amounts of pro-inflammatory cytokine. Even though the E3 region of adenovirus is termed "non-essential", it plays an important role in an evolutionary balance between virus and host.

Though UL49.5 mutants will only have a few point mutations and not a complete knock out, I presume there will be restored antigen presentation. It is possible this mutation could have similar inflammatory effects as adenovirus E3 deletion mutants.

It maybe that FHV-1 has the ability to "out run" CTL control and quickly infect neurons, becoming latent. However, it is not known if IFN-secreting T cells inhibit reactivation in either MCMV or FHV-1 infection. Those subset of CD8+ T cells may be one of many factors for control of viral latency, reactivation and shedding.

In conclusion, these experiments will give greater insight into viral mechanisms of immune evasion by inhibition of antigen presentation. Also, it will provide better

understanding of viral pathology in natural hosts. In addition to being a clinically important virus of cats, FHV-1 is a useful model for studying the role of immune evasion genes in the pathogenesis of α-herpesvirus infections in humans (e.g., varicella-zoster virus) and large animals (e.g., pseudorabies virus). The availability of a small-animal model for studying α-herpesvirus immune evasion genes will contribute to a better understanding of viral pathogenesis. This knowledge will aid in the production of better vaccines for animals, leading toward potential application in humans.

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