A NOVEL IMMUNE EVASION GENE OF MAREK'S DISEASE VIRUS

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ABSTRACT

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Marek's Disease Virus, a lymphotrophic, oncogenic alpha-herpesvirus of chickens, has historically presented a significant vaccine challenge to the poultry industry, and is also a useful model for herpesviral vaccine development. Herpesviruses impact both human and animal health, and the development of improved vaccines is needed to overcome obstacles such as a general lack of sterilizing immunity and the persistence of latent infection (both wild-type and vaccine viruses) in vaccinated hosts. Immune evasion by herpesviruses complicates vaccine development, as vaccine strains may evade multiple arms of immune recognition. Evasion of the major histocompatibility complex (MHC) class I has been recognized among a number of herpesviruses, including Marek's Disease Virus (MDV), and may interfere with CD8+ T cell recognition control of viral infection.

Using plasmid-based gene cloning and *in vitro* transfection methods, the gene MDV012 was identified as an evasin of MHC class I, most likely inhibiting the TAP transporter. Potential knock-out viruses were constructed using BAC recombineering to introduce an A-to-C point mutation in the MDV012 start codon. These viruses grew more slowly and to lower titers than the wild-type virus. The MDV012ctg mutant gene was cloned from these viruses and tested in transfection assays. Surprisingly, the MDV012ctg mutant showed no difference in ability to down-regulate overall MHC class I expression relative to the wild-type MDV012, indicating that this gene is still translated or partially translated despite the mutation. Full knock-outs of MDV012 function will be required to test potentially non-MHC class I-evasive viruses.

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ADCC = antigen-dependent cellular cytotoxicity

AIDS = acquired immunodeficiency syndrome

APC-A = allophycocyanin signal area (flow cytometry)

B2m = beta-2 microglobulin

BAC = bacterial artificial chromosome

BF1*21 = chicken BF locus, minor MHC class I alpha chain, B21 haplotype

BF2*21 = chicken BF locus, major MHC class I alpha chain, B21 haplotype

BHV-1 = bovine herpesvirus virus 1

BuHV-1 = bubaline herpesvirus 1

CCP = complement control protein

CCPH = complement control protein homologue

CDCC = complement dependent cellular cytotoxicity

CMV = cytomegalovirus

CTL = cytotoxic T lymphocyte

CvHV-1 = cervid herpesvirus 1

Cy5 = cyanine-5 fluorophore

DAF = decay accelerating factor

DEF = duck embryo fibroblast

DF-1 = a chicken fibroblast cell line

DH5α = a cloning strain of Escherichia coli

DH10B = a cloning strain of Escherichia coli

DMEM = Dulbecco's modified essential medium (cell culture)

EBV = Epstein-Barr virus

EGFP = enhanced green fluorescent protein

EHV-1 = equine herpesvirus 1

EHV-4 = equine herpesvirus 4

ER = endoplasmic reticulum

F(ab')2 = (immunoglobulin) fragment of antigen binding regions

FBS = fetal bovine serum

Fc = (immunoglobulin) fragment crystalizable region

FcR = Fc receptor

FcγR = Fc-gamma receptor

FHV-1 = feline herpesvirus

FITC-A = fluorescein isothionate signal area (flow cytometry)

FL1 or FL-1, FL5 or FL-5 = fluorescence channels 1 and 5 respectively (flow cytometry)

FSC = forward scatter (flow cytometry)

gC, gD, gE, gI, gJ = glycoproteins C, D, E, I, and J respectively

gp2 = glycoprotein 2

GFP = green fluorescent protein

GMFI = geometric mean fluorescence intensity

GPCMV = guinea pig cytomegalovirus

HA = influenza hemagglutinin

HCMV = human cytomegalovirus

HLA = human leukocyte antigen (human MHC class I)

HHV-7 = human herpesvirus 7

HHV-8 = human herpesvirus 8 (KSHV)

hiFBS = heat-inactivated FBS

HIV = human immunodeficiency virus

HSV = herpes simplex virus

HVS = herpesvirus saimiri

HVT = herpesvirus of turkeys (Meleagrid herpesvirus-1)

ICP = infected cell polypeptide

IgG, IgM = immunoglobulins G and M respectively

IL-8 = interleukin-8

ILTV = infectious laryngeotracheitis virus (Gallid herpesvirus-1)

KCP = KSHV complement control protein

KSHV = Kaposi's sarcoma-associated herpesvirus (HHV-8)

LB = Luria-Bertani broth (bacteriological culture)

LM = 50% Leibowitz medium/50% McCoy's medium (cell culture)

LMH = a chicken hepatocellular carcinoma cell line

mAb = monoclonal antibody

MCMV = mouse cytomegalovirus

MCP = membrane cofactor protein

Md5 = a vvMDV strain

Md5B40BAC = a BAC based on the Md5 MDV strain

Md11 = a vMDV strain

Md11BAC = a BAC based on the Md11 strain

MDV = Marek's disease virus

MDV-1 = Marek's disease virus type 1 (Gallid herpesvirus-2)

MDV-2 = Marek's disease virus type 2 (Gallid herpesvirus-3)

MDV012sp = spliced MDV012 sequence

MHC = major histocompatibility complex

MHV-68 = mouse herpesvirus 68

MICA, MICB = MHC class I polypeptide related sequences A and B, respectively

MIRL = membrane inhibitor of reactive lysis

NEB = New England Biolabs, Inc. (company)

NK = natural killer (cell)

ORF = open reading frame

PASA = PCR amplification of specific alleles

PBS = phosphate-buffered saline

PCR = polymerase chain reaction

PFU = plaque-forming units

PKR = protein kinase R

PRV = pseudorabies virus (SuHV-1)

RCA = regulator of complement activation

RCP = rhesus rhadinovirus complement control protein

RFP = red fluorescent protein

RLORF = repeat long open reading frame

RPMI = Roswell Park Memorial Institute medium (cell culture)

RRV = rhesus rhadinovirus

RT-PCR = reverse transcriptase PCR

SC-Kb = single-chain mouse MHC class I Kb allele fusion protein

SSC = side scatter (flow cytometry)

SNP = single nucleotide polymorphism

ssAR10V = signal-sequence-linked AR10V peptide

SuHV-1 = suid herpesvirus 1 (PRV)

SW105 = a recombineering strain of Escherichia coli

TAP = transporter associated with antigen processing

TAE = tris acetate EDTA buffer

TBE = tris borate EDTA buffer

TE = tris EDTA buffer

Th1 = Helper T cell type 1

UL = unique long (region)

US = unique short (region)

USDA-ADOL = United States Department of Agriculture Agricultural Research Service (USDA-ARS) Avian Disease and Oncology Laboratory, East Lansing, Michigan

vMDV = virulent MDV

vvMDV = very virulent MDV

vv+MDV = very virulent plus MDV

VZV = varicella zoster virus

CHAPTER 1

Problems in herpesvirus vaccination: Marek's Disease Virus as a model for rational vaccine development targeting an immune evasive virus.

1.1. Abstract:

In this introductory chapter, three major topics are covered. Section 1.2.1 describes Marek's Disease Virus (MDV), the causative agent of an economically important herpesviral disease of chickens and describes the disease and its control. Marek's disease is a lymphoproliferative disease causing infiltrative nerve lesions and paralysis and visceral organ tumors in chickens. Costs of prevention and losses due to Marek's disease are estimated at at least $1 billion per year in the United States. Vaccines have been developed to control the disease, however continued emergence of more virulent strains has necessitated introduction of new vaccines approximately every 20 years due to increases in mortality and vaccine breakthrough. MDV is an accessible model herpesvirus due to its animal host and characteristic lesions. Section 1.2.2 provides a literature review on the rational development of recombinant vaccine strains for the control of herpesvirus infections. Problems associated with herpesvirus vaccination include the lack of sterilizing immunity obtained with current vaccines and the establishment of latent, transmissible infections in immunized individuals of both wild viruses and vaccine strains. Significant research has been ongoing in the development of herpesviral vaccines that lack specific virulence factors, genes required for latency, or immune evasion genes. Relevant complications of vaccine design and use are also discussed, including in vivo recombination between wild-type and vaccine strains, and selection pressures toward increased virulence in virus populations where non-sterilizing vaccines are used. Section 1.2.3 introduces

immune evasion as a common feature of large DNA viruses. Three specific immune evasion mechanisms are covered in detail with regard to herpesviruses which target the innate, adaptive humoral, and cell mediated immune system. Several herpesviruses have been found to produce or upregulate membrane-bound and soluble complement inhbitors. These include viral complement control proteins among the gamma-herpesviruses, host complement control proteins among the beta-herpesviruses, and glycoprotein C of some alpha-herpesviruses, which appear to have multiple functions including immune evasion. Herpes simplex viruses and several varicelloviruses have been found to produce viral Fc receptors, which are involved in the binding of IgG as well as in viral spread. HSV-1 FcR has been shown to be involved in evasion of antibody-based immunity through an antibody bridging mechanism. Finally, a number of herpesviruses evade cytotoxic T cell immunity through inhibition of MHC class I expression. TAP blockade is a well-conserved mechanism in alpha-herpesviruses to evade MHC class I immunity. Beta-herpesviruses have been found to inhibit both cytotoxic T cell and NK cell immunity through multiple, complementary mechanisms. Immune evasion is expected to play an important role in herpesviral infection and immunity.

1.2. Literature Review

1.2.1. Marek's Disease Virus: an important herpesvirus of chickens.

Marek's Disease Virus type 1 (MDV-1), also known as gallid herpesvirus 2, is an alphaherpesvirus belonging to the Mardivirus lineage (Churchill 1967, Davison 2010) which is the agent of Marek's disease, an oncogenic disease in chickens, producing lymphoid tumors in the peripheral nerve tissue and visceral organs (Payne 1967) and variable morbidity and mortality depending on strain (Witter 1997). The closest relatives of MDV-1 are the other

Mardiviruses, which include the non-oncogenic herpesvirus of turkeys (HVT) and MDV-2 (gallid herpesvirus 3) strains, and infectious laryngeotracheitis virus (ILTV), a more distantly related Iltovirus also infecting chickens (Davison 2002, Witter 1998). For the remainder of this paper, MDV-1 will be abbreviated MDV, and will refer to the oncogenic (type 1) virus. Marek's disease is an important disease to the global poultry industry; in 2005, it was estimated that Marek's disease and its control cost the industry at least $1 billion annually (1% of the total industry value) (Nair 2005).

Marek's disease has been described histopathologically in Payne et al (1967), and is characterized by the development of lymphoproliferative lesions in the large peripheral nerves (sciatic, brachial and celiac nerves and plexi and abdominal vagus nerve), which vary in severity from scattered infiltration to severe, demyelinating lymphoproliferation, as well as visceral lymphomas, particularly in the gonads but also in other organs such as liver, kidneys and heart, in genetically susceptible chickens. Visceral lesions begin to develop as early as 2 weeks after infection. Paralysis is a late (noted around 28 days or later post infection) sequela to nerve infiltration. (Payne 1967).

Birds are infected through the respiratory system through inhalation of virus shed in feather dust. (Calnek 1970). Within the first week after infection, cytolytic infection of B cells predominates (Shek 1983), resulting in immunosuppressive disease. However, in the second week of infection, latent infection of predominantly T cells develops, and B cell populations recover (Shek 1983, Calnek 1984). Excretion of cell-free virus occurs in the feather follicle epithelium, allowing spread via respiratory contact (Calnek 1970), and is thought to develop after circulating latently infected T cells carry the infection to the feather follicles. Lymphoma development in susceptible birds occurs in T cells, presumably due to transformation of latently

infected cells (Powell 1974). Chicks can be infected experimentally as early as 1 day of age (Churchill 1967).

Genetic susceptibility to Marek's disease has been strongly linked to the chicken B locus, which comprises the classical MHC I and II complexes and associated genes (Briles 1977, see Kaufman 2000 for a review). It has been suggested that differing susceptibilities to MDV are correlated with differing expression levels of MHC class I haplotypes, with the most resistant B21 haplotype expressing less MHC class I intrinsically than the highly susceptible B19 haplotype; proposed mechanisms include peptide specificity of B21 MHC class I for MDV antigens resulting in higher expression in infected cells only, or NK cell-mediated tumor lysis of cells expressing lower MHC class I (Kaufman 1997).

Although early strains of MDV were not isolated, it appears from historical increases in mortality and pathology that several shifts in pathogenicity have occurred since disease caused by MDV was first identified in 1907. Witter describes the emergence of virulent MDV as a phenomenon that occurred in the 1950's, which necessitated the development of vaccines to control what had previously been a milder, sporadic disease. Further increases in virulence occurred in the 1970's and again in the early 1990's; therefore, significant increases in vaccine breakthrough have been occurring about once every two decades (reviewed in Witter 1998, Nair 2005).

Witter (1997) also showed that recently isolated strains of MDV could be organized into several pathotypic groups, including virulent (vMDV), very virulent (vvMDV) and very virulent+ (vv+MDV) pathotypes, based on comparison of virulence measures with the well-characterized virulent and very virulent strains JM and Md5, respectively, in birds of designated vaccination status. Virulent MDV was defined as producing similar mortality to JM in birds

vaccinated with HVT, vvMDV was defined as producing greater mortality in that population than JM but equal mortality to Md5 in birds vaccinated with both HVT and SB-1 (bivalent vaccination), and vv+MDV was defined as producing greater mortality in bivalently vaccinated birds than the Md5 strain. vvMDV strains were first isolated in the 1990's indicating the recent emergence of increasingly virulent MDV viruses. More virulent pathotype correlated positively with increased mortality and the development of visceral or ocular tumors (Witter 1997).

Vaccines have been in use against MDV since 1969 (see Witter 1998 and Baaten 2004 for reviews). An attenuated (tissue-culture-passaged) strain, HPRS-16, was introduced in Europe in the early 1970's. An original study with this strain showed that it could be used as a successful vaccine, reducing mortality from 55% to less than 1% in chicks challenged at 46 days with virulent MDV if vaccinated at 1 day of age. Similar results were found for chicks challenged with infected contact chicks. However, the attenuated HPRS-16 vaccine was non-sterilizing. Vaccinated chicks developed serum antibodies to the vaccine virus, but also to challenge virus, and challenge virus was able to be isolated at 14 weeks post infection in vaccinated birds. Also, vaccine virus was re-isolated from vaccinated birds at 14 weeks post-challenge, indicating that the vaccine virus, while attenuated and protective, was also able to achieve long-term infection (Churchill 1969).

In the United States, a vaccine based on the non-oncogenic related virus HVT, the FC126 strain, has been used as a naturally avirulent but immunogenically related MDV vaccine. In a vaccine study in 1970, this strain of HVT was found to be 100% protective against MDV challenge in 4 trials, with no signs of Marek's disease in chicks challenged as early as 2 weeks, when inoculated at 1 day. Unlike MDV, which is highly cell-associated, the FC126 HVT vaccine could be administered as sonicated (nonviable) cell material, indicating that cell-free

virus could be used with HVT vaccines, simplifying administration. Again, this vaccine was non-sterilizing for either vaccine or challenge strains, but the vaccine virus did not appear to be virulent (Okazaki 1970).

The appearance of very virulent (vvMDV) strains in the 1970's necessitated further vaccine development, and bivalent vaccines were introduced. It was found that combination of the HVT strain with the MDV-2 strain SB-1 produced a synergistic effect in vaccinating against a very virulent isolate, RB1B; the addition of SB-1, which alone was less protective against vMDV infection that HVT, reduced incidence in genetically susceptible P-line birds from 54% to 28% (Schat 1982). However, even more virulent strains emerged in the 1990's, and more distantly related strains such as SB-1 and HVT were not found to be protective enough against the vv+MDV isolates, so vaccines based on the partially-attenuated CVI988/Rispens strain were introduced in the United States and have been more successful at controlling vvMDV and vv+MDV (Witter 1998).

Despite differences in host and life cycle between MDV and other herpesviruses of importance, including human diseases, MDV may be a useful model for studying herpes infection. For example, MDV shares gene homologues with at least 68 genes found in the important human alphaherpesvirus pathogen HSV-1 (Davison 2010), and commonalities such as the development of latent infection exist between even more distantly related herpesviruses. The MDV model is highly accessible given that it can readily produce visible disease (such as tumors and mortality) early in the life of its natural host, the chicken, which is a convenient animal model due to its small size and commercial availability.

1.2.2. Targeted engineering strategies for attenuated herpesvirus vaccines.

1.2.2.a. Problems in herpesvirus vaccination

Herpesviral infections are a significant cause of morbidity and mortality among humans and domestic animals, create economic, health and social costs, and pose challenges for prevention and treatment which have not yet been solved. Herpesviruses are large, double-stranded, enveloped DNA viruses which can be grouped into three sub-families: alpha-herpesviruses, beta-herpesviruses, and gamma-herpesviruses. Herpesviruses are wide-spread across numerous vertebrate hosts, and are highly host-adapted, with many host species infected by more than one herpesvirus species. Despite the large number (about 120) species of herpesviruses known across several distant lineages, significant conservation of at least 43 viral genes exists across all three subfamilies and further homologies within subfamilies allow for comparison between viruses (Davison 2002). Herpesvirus infections are characterized by the development of latency and intermittent reactivation, as well as a variety of immune evasion strategies which protect active infection from efficient immune control until latency can be established. These characteristics have contributed difficulty to the development of effective vaccines for herpesviral diseases (Griffen 2010).

Human herpesviruses which cause significant disease include alpha-herpesviruses HSV-1, HSV-2, and VZV, beta-herpesvirus CMV, and gamma-herpesviruses EBV and HHV-8 (Davison 2010). HSV-1 and HSV-2 are responsible for herpes simplex, with both viruses contributing to genital herpes and neonatal herpes infections. Herpes simplex is associated with recurrent activation and shedding, and is also believed to be linked with HIV transmission. Prophylactic and therapeutic vaccines to reduce transmission and symptoms of HSV are sought (Stanberry 2004, Brans 2009). VZV is responsible for primary infection causing varicella (‘chicken pox’)

and reactivation in the sensory ganglia, known as herpes zoster ('shingles'). Herpes zoster is an important cause of morbidity such as post-herpetic neuralgia in aging populations. Vaccines exist to control both the primary infection (through child-hood vaccinations) and herpes zoster in adults, particularly through cell-mediated immunity. Concerns exist over whether vaccination may lead to lower sub-infectious exposure in the population and thus actually decrease population immunity to zoster, and also whether the vaccine virus might itself cause latency-related side effects (Liesegang 2009, Oxman 2008, Brisson 2002).

Cytomegalovirus (CMV) infection is common and generally asymptomatic in immunocompetent hosts, although it occasionally causes mononucleosis. However, in fetal and neonatal infection it is a significant cause of neurological and sensory impairments such as deafness, as well as serious disease and mortality. In immunocompromised hosts such as AIDS patients CMV is known to cause a variety of inflammatory manifestations. Vaccines which stimulate innate and cell-mediated immunity, particularly to prevent congenital CMV infection, are needed (Crough 2009, Bunde 2005). EBV is the more common cause of mononucleosis, as well as being associated with various tumor events, such as Burkitt's and Hodgkin lymphomas, especially in immunocompromised patients. Subunit vaccines have been studied for acute mononucleosis infection, and might provide incidental resistance to EBV-induced cancers (Kutok 2006, Moutschen 2007). HHV-8 is also a cause of viral-induced cancer, and in particular is associated with the development of Kaposi Sarcoma in immunocompromised individuals. Control strategies have focused on treatment or control of associated immunocompromising diseases such as HIV rather than vaccine development (Schiller 2010).

A number of animal herpesviruses are of economic or companion animal health significance. Among the alpha-herpesviruses, BHV-1 is an important cause of shipping fever and abortion in

cattle (reviewed in Jones 2008) and SuHV-1 causes pseudorabies in swine (Davison 2010, van Oirschot 1991). EHV-1 and EHV-4 cause equine abortion syndrome (with systemic, neurological and respiratory components also), and equine rhinopneumonitis respectively, while the avian diseases gallid herpesvirus-2 and gallid herpesvirus-1 cause MDV-1 (Marek's Disease of chickens) and ILTV (infectious laryngiotracheitis) of turkeys, respectively (Davison 2010, Osterrieder 2010, Minke 2004, Witter 1997, Witter 1998, Han 2003). FHV-1 is an important cause of rhinotracheitis in domestic cats (reviewed in Gaskell 2007). Vaccines exist for all of these diseases and are usually effective at reducing or preventing clinical signs, but are generally ineffective at preventing latent infection or asymptomatic shedding. In some cases, as in BHV-1, vaccine virus itself is known to be a potential cause of clinical symptoms (Jones 2008, van Oirschot 1991, Osterrieder 2010, Minke 2004, Witter 1997, Han 2003, Gaskell 2007). Gammaherpesviruses of significance include several causes of malignant catarrhal fever in ruminants, for which no vaccines exist, although attenuated vaccines for cattle are in progress (Russell 2009, Haig 2008). Koi herpesvirus of commercial and domesticated carp is classified in a separate family, the alloherpesviruses (Davison 2010).

A variety of vaccine types have been used in the design of experimental and commercial herpesvirus vaccines including inactivated virus, attenuated live virus, recombinant subunit vaccines, and DNA-vectored subunit vaccines. This review will focus on recombinant attenuated live viruses rather than inactivated or subunit vaccines because attenuated live viruses are expected to produce the broadest and most potent immune responses, particularly where cell-based immunity is critical (Stanberry 2004, Jones 2008, Gaskell 2007). Lessons learned from the study of recombinant attenuated viruses should be applicable to other types of vaccines, such as subunit vaccines, as knowledge is gained about the importance of specific viral proteins and

epitopes in evoking an immune response or conversely in producing vaccine-associated pathology.

An ideal attenuated live herpesvirus vaccine should be designed to overcome several problems common to herpesvirus infections. First, it should be attenuated with regard to pathology—the vaccine should not cause the disease symptoms, so virulence genes must be removed. Second, it needs to overcome both wild type and vaccine virus latency. Herpesviruses achieve latency in several tissues such as the sensory ganglia (HSV-1, VZV), bone marrow (CMV) and lymphoid tissue (EBV); reactivation allows shedding of infectious virus in either a symptomatic or asymptomatic state. Vaccines that control symptoms but do not control wild-type latency may allow breakthrough reactivation and viral shedding to occur, possibly even aiding transmission of virus from asymptomatic carriers (Stanberry 2004, Liesegang 2009, Crough 2009, Kutok 2006). Vaccine viruses which become latent themselves may have pathogenic affects or lead to the development of revertant strains. Finally, it must be as immunogenic as safely achievable—to achieve this in the case of herpesviruses, not only must the important epitopes for immune stimulation be included but viral immune evasion genes should be inactivated to allow a full immune response.

Ideally a vaccine should provide sterilizing immunity; however, so far this has proven difficult with herpesviruses (Stanberry 2004, Jones 2008, Gaskell 2007). Vaccines which do not achieve sterilizing immunity may exert unwanted evolutionary forces on the wild-type virus population which lead to results contrary to those intended. Ganden, et al. predicted through mathematical modeling that vaccines that decrease pathogen growth rates or virulence without decreasing susceptibility to infection or directly blocking pathogen transmission will lead to the evolution of increased pathogen virulence. This is because pathogen virulence optimizes with

respect to increased transmission rate versus decreased survival of infected hosts. In contrast, vaccines that target infection or transmission tend to leave virulence unaffected or even decreased in cases where superinfection plays a role in pathogen selection pressures (Ganden 2001).

A classic example of this problem in the development of herpesvirus vaccines has been demonstrated with Marek's Disease Virus vaccination. Increasingly virulent MDV isolates have appeared in vaccinated flocks following the institution of vaccination; these isolates have appeared within a decade of each new, more protective (and less attenuated) vaccine strategy. These vaccines do not prevent infection and latency, nor do they prevent shedding of the virus from asymptomatic carriers (Witter 1997). While vaccination has remained highly effective at controlling disease prevalence, it may prove difficult to keep pace with the emergence of increasingly virulent strains in the future, unless vaccines can be developed which block infection (Witter 1998).

1.2.2.b. Comparison to and coordination with traditional attenuation methods

Traditional methods of viral attenuation are nonspecific and rely on randomly accumulated mutations, rather than being rationally designed or targeted. Traditionally attenuated vaccines are created through a series of passages in culture, after which a virus no longer maintains its original virulence in the live host (Stanberry 2004). While highly effective vaccines have been produced this way for some viruses (Witter 1998, Gaskell 2007), a more rational approach is sought to maximize efficacy in the face of emerging virulent strains, to improve safety profiles, or to produce effective vaccines for viruses which have so far proven intractable to traditional vaccine methods, such as HSV-1 and HSV-2 (Stanberry 2004).

The use of modern genetic techniques to characterize traditionally attenuated vaccine strains is yielding insights into the modifications which lead to attenuation as well as the advantages and disadvantages of using traditional attenuation methods. Spatz and Silva compared the repeat-long sequences from a number of known strains of gallid-herpesvirus-2 of differing pathotypes, including virulent, very virulent, and attenuated strains, to identify polymorphisms which might be associated with changes in pathogenicity. They found that while changes, most usefully SNPs, segregated only very roughly between pathotypes, mutations in Meq and RLORF12 were most associated with attenuation (Spatz 2007).

Spatz, et al, has analyzed attenuated the gallid-herpesvirus-2 (Marek's Disease Virus) strain 584Ap80 in comparison to its very virulent parent strain, 584Ap9, as well as to several other known strains. The study showed that traditional attenuation produced an attenuated strain with a complicated variety of large and small differences relative to the wild-type virus, ranging from SNPs to repeats, insertions, deletions and even one missing open reading frame, UL3.5. Based on homologies to other herpesviruses UL3.5 is expected to be a virulence factor for MDV. The MDV Meq oncogene was also extensively mutated, such that it was predicted to produce two truncated proteins lacking functional domains. Other attenuated strains of MDV were found to similarly contain gross mutations in Meq (Spatz 2010).

While characterization of attenuated strains may suggest targets for further research, the large number of changes found presents a complex picture which can only be deciphered through testing each mutation individually and which is further complicated by the variety of differences between even virulent strains. Furthermore, Spatz et al showed that traditional attenuation does not produce a uniform population of viruses, as sequencing a sample of high-passaged MDV 648Ap101 yielded heterogenous populations for most mutations found relative to MDV

648Ap11. Differences within populations of wild-type strains are also expected to occur in vivo, further complicating interpretation of changes within and between strains (Spatz 2008).

On the other hand, strong virulence factors can cause clear differences between wild type and naturally attenuated vaccine viruses. Smith, et al compared wild-type and attenuated (KyA) strains of EHV-1 and found a truncated glycoprotein, gp2 in the KyA strain. Recombination with the full-length gp2 and the KyA strain produced revertant KyA virus which was not attenuated in the BalbC mouse model, thus identifying gp2 as an important virulence factor for further study and targeting in potential rationally designed vaccines (Smith 2005). Similarly, Kamiyoshi et al. identified UL49 as an important virulence factor in BHV-1 through comparison of wild-type and attenuated vaccine virus, correlating with other studies which showed decreased pathogenicity of UL49 mutants (Kamiyoshi 2008, van de Walle 2008).

1.2.2.c. Latency gene deletions

Latency in herpesvirus infections, including vaccination, is a significant complicating factor in the development of effective vaccines, either because latent infections are poorly controlled by the immune system or because latent virus, including vaccine virus, can directly lead to negative effects such as tumorogenesis or neurologic disease depending on the latency tropism of the virus. A highly effective and safe herpesvirus vaccine would be able to prevent wild-type infections from establishing latency (that is, generate sterilizing immunity) as well as being unable to establish latency itself (Cohen 2005, Ambagala 2010, Jia 2010, Fowler 2004).

Varicella zoster virus is the only human herpesvirus for which an approved vaccine exists; however, breakthrough infections and establishment of latency occur despite vaccination. Zoster, the reactivation of latent VZV in sensory ganglia, may occur from break-through wild-type reactivation or occasionally from the vaccine virus itself, particularly in immunosuppressed

patients. Cohen, et al, determined that it was possible to target a latency-associated gene in VZV, ORF63, and create viruses impaired for latency in vivo via mutating serine and threonine phosphorylation sites. However, these mutations decreased replication in vitro and thus were not considered ideal for vaccine development. While mechanism of action was not fully elucidated, it was suggested that decreased replication might have reduced virus available to ganglia for establishment of latency (Cohen 2005).

Subsequently, the same group serendipitously discovered a combination of mutations which produced a virus impaired for latency but not for replication. Ambagala, et al, found that deletion of the carboxy-end nuclear localization sequence of ORF63 and an inserted copy of a truncated ORF62 together produced a virus with a 30% reduction in latency frequency. Suprisingly, neither mutation separately had a statistically significant effect on latency or replication, and the truncated ORF62 was transcribed but not expressed at detectable levels. However, ORFs 62 and 63 are known to interact, and it is possible that these mutations together affect some otherwise redundant activity of one or both. Further characterization of this mutant could lead to improvements in VZV vaccine safety (Ambagala 2010).

Human gammaherpesviruses also have the ability to cause disease directly associated with latency; specifically, lymphoproliferative diseases. Epstein-Barr Virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV; HHV-8) are both human gammaherpesviruses which can lead to oncogenic transformation of latently infected cells. No approved vaccines exist for these diseases; due to the oncogenic potential of these viruses, a safe vaccine would need to be latency deficient. Neither of these viruses can be grown easily in an animal model, so developing and obtaining approval for a vaccine is a more difficult problem with EBV and KSHV. However, MHV-68 has an 80% homology with both these viruses and has proved to be

a useful model for understanding how these closely related gammaherpesviruses function (Jia 2010, Fowler 2004).

Antigen-based vaccines such as subunit vaccines have proven insufficient to protect against latency in MHV-68 infection. Therefore, development of latency-deficient attenuated viruses has been pursued as a more promising candidate for the development of sterilizing immunity to gammaherpesviruses. Tibbetts, et al were able to reduce wild-type replication and latency with reactivation-deficient viruses; however, these viruses were able to establish latency and thus did not meet the ideal safety profile for a gammaherpesvirus vaccine (Tibbetts 2003).

Fowler, et al developed a latency-deficient MHV-68 virus deleted for the latency-associated ORF73 which showed long-term protection (117 days) against wild-type challenge, while no detectable latency developed in either vaccinated or challenged mice. The virus was not impaired for replication, but was cleared more rapidly than wild-type virus. As Fowler et al point out, however, it is possible that undetectably low levels of latent virus could be established and it is not certain that such a vaccine is truly sterilizing (Fowler 2004).

Recently, Qingmei et al combined several latency-reducing mutations into one recombinant attenuated MHV-68 virus and demonstrated long-term immunity to wild-type challenge without detectable latency. The strategy chosen was to delete a region containing ORF73 as well as an anti-apoptotic bcl-2 homologue, ORF72, and an oncogenic viral cyclin, M11; and replace the region with an inserted copy of the viral transcriptional activator RTA, which has shown efficacy against latency. The resulting virus showed a lack of detectable latency as well as increased replication in vitro and increased initial replication at the upper respiratory site of initial infection but decreased replication in the lung tissue. These features are suggestive of a highly effective strategy against gammaherpesvirus infection. However, vaccine

efficacy would need to be further characterized, since this study only used one (comparatively low at 500 PFU, relative to 10^5 PFU in the Fowler et al study) dose of challenge or vaccine virus (Jia 2010, Fowler 2004).

While these results appear promising, it remains to be seen how completely they translate to human gammaherpesvirus vaccine design should the same targeted strategies be used. Since EBV and KSHV do not grow in rodent models, testing human vaccine viruses will pose a significantly greater challenge than the model virus MHV-68 (Jia 2010, Fowler 2004).

1.2.2.d. Replication-deficient viruses

Replication-deficient viruses have been explored as a means of producing a vaccine with decreased potential for pathogenicity or latency reactivation effects. These are viruses which can infect host tissue, providing the basis for a cellular immune response, but are unable to undergo a second round of infection and spread in tissue due to deletion of a gene important for replication or infection. A potential concern with this method is that the immune response might be decreased as the vaccine infection would be truncated. Hoshino et. al explored this method by deleting 2 essential early genes from herpes simplex 2 virus, producing deletion mutant dl5-29 which showed good gD immunogenicity in mouse and guinea pig models. Protective efficacy was demonstrated as well, and a decrease in viral shedding, showing that replication-defective viruses have the potential to provide acceptable immunogenicity (Hoshino 2008).

Lu et al. demonstrated that a replication-defective vaccine virus could inhibit replication of wild-type virus as well. Construction of an HSV-1 virus containing a deletion of replication-essential UL9 and a "dominant negative" copy of a replication-inhibiting construct (UL9-C535C) decreased replication of co-infected wild-type HSV-1 in vitro. It was further demonstrated that the immunogenicity of this replication-defective virus could be modulated

through other techniques to nearly that of the wild type virus. Addition of a second copy of gD to this HSV-1 variant under a highly active immediate-early promoter yielded higher antibody levels than other dominant-negative HSV-1 viruses and similar to wild type in a mouse model, as well as T-cell responses similar to that of wild-type virus (Lu 2009).

An intriguing strategy for combating latency with replication-deficient viruses was suggested by Kayhan, et al, who created a latency-competent, replication-deficient gamma-herpesvirus (MHV-68) which triggered a T-cell response including a response to the latency-associated epitope M2. This virus, ORF31STOP, was impaired for replication in the late stage and was only able to establish latency in certain organs depending on its route of administration, suggesting that latency was dependent on site dynamics and no lytic infection was occurring. Kayhan et. al suggest that such a virus might be able to elicit a latency-specific immunity, especially if combined with immune evasin deletions to allow more effective latency-associated antigen presentation (Kayhan 2007).

1.2.2.e. Virulence factor deletions

A direct approach to vaccine virus attenuation through recombinant techniques involves the identification and deletion of a target gene with subsequent characterization of the pathogenicity and immunogenicity of the resulting virus. This may be done de novo to characterize the effects of a gene of unknown function; or, if a virulence gene has been identified through other methods (such as comparison between traditionally attenuated and wild-type viruses) it may be studied more directly through knock-out; or, a gene may be selected based on homology to known virulence genes in other viruses. Lee et al. showed that Meq, shown through strain comparison to be the primary oncogene of MDV, could be knocked out to create a less oncogenic, attenuated virus with an increased protective index in field trials (Lee 2010). Deletion of Meq through

recombinant, rather than traditional attenuation techniques, should provide a more consistently safe vaccine.

Deletion of putative virulence genes based on known homologies has proven useful in creating attenuated vaccines in related infections. Kruger, et al showed that glycoproteins gI and gE could be deleted to attenuate FHV-1 and produce a vaccine strain that was safer than commercially available strains while still maintaining acceptable protection (albeit with lower neutralizing antibody titers) (Kruger 1996). Subsequently the same glycoproteins were deleted from equine herpesvirus-1 to produce mutants highly attenuated for neurovirulence; they were chosen based on known homology to other alpha-herpesviruses. While these mutants were not characterized relative to the parent strain, they were shown to produce an immune response in murine and hamster models and appeared useful candidates for vaccine development (Tsujimura 2006).

Deletion of virulence genes may have effects on immunogenicity which should be considered in the development of a vaccine virus. Fuchs et al produced an ILTV gJ deletion mutant with decreased pathogenicity, although the mechanism behind the attenuation was not yet understood. The deletion mutant replicated to lower titers in vitro and in vivo but was replication- and shedding-competent. The gJ glycoprotein is immunogenic, so this ILTV deletion mutant produced decreased serum antibody titers. However, these decreased titers did not significantly reduce protectiveness of the virus in vivo, demonstrating that in vivo testing is vital to determine the overall effect of any virulence deletion on immunogenicity (Fuchs 2005).

In some cases it may be important to consider the balance of protection between completely and partially attenuated strains, especially against highly-pathogenic viruses. Deletion of viral IL-8 cytokine from MDV created a partially attenuated virus, presumably through reduction in

its ability to cause B-cell lysis and T-cell activation; it was able to cause histological lesions but was significantly more protective than a classical non-pathogenic vaccine strain. In the case of MDV where increasingly virulent strains have emerged, decreasingly attenuated vaccine viruses may continue to be necessary (Cui 2005, Witter 1998).

1.2.2.f. Immune evasin deletions

Immune evasion genes are a useful target for targeted vaccine design because their deletion should directly increase the efficacy of the immune response to the vaccine virus. While trade-offs may occur between viral replication efficiency and immunogenicity, the results of several experiments show that immune evasion knock-outs can have immunogenicity similar to wild-type virus with decreased pathogenesis in vivo. Crumpler et al. demonstrated this with guinea pig cytomegalovirus (GPCMV, a useful model for studying HCMV) through the deletion of three MHC class I homologues which are putative NK evasins. The resulting virus provided similar antigenicity and protection to wild type virus, with an earlier innate immunity response. The virus was also highly attenuated relative to wild type (Crumpler 2009). In another study, Mohr et al. block-deleted MHC class I and NK evasins from MCMV; as with the GPCMV knockout, this virus showed reduced pathogenicity and immunogenicity similar to wild-type. These studies suggest that deletion of immune evasins could similarly attenuate HCMV (Mohr 2008).

Another, rather different study suggests that immune evasion could be very relevant in HCMV vaccination. Snyder et al. created a spread-defective MCMV mutant and used it to demonstrate the dominance of cross-presentation over direct antigen presentation in cytomegalovirus infection. Introduction of non-infectious viral antigen produced the same immunodominance patterns as wild-type infection, suggesting that cross-presentation is the

primary immune-stimulating pathway in cytomegalovirus infection, most likely due to the variety of immune evasins expressed by CMV viruses. Deletion of these immune evasins may modulate the range of immunodominant epitopes and thus the strength of immunity produced by such a vaccine (Snyder 2010).

Herpesviruses use a variety of methods to down-regulate the immune response; one common method is down-regulation of MHC class I and a subsequently reduced CD8+ T-cell response (Griffen 2010). Hunt, et al. showed that active MDV-1 reduces MHC class I antigen presentation and is associated with a weak cytotoxic T-cell response to this virus; this immune evasion activity is present in strains used for vaccination and is expected to reduce the immunicity of these vaccine strains (Hunt 2001). Although the target immune evasin gene in MDV-1 has not yet been determined, genes with similar functions have been identified in other herpesviruses and should also be considered as possible targets for vaccine design (Griffen 2010).

1.2.2.g. Complications of targeted approaches

While targeted recombinant approaches to attenuation in the development of new vaccine strains offer a more fully characterizable and thus potentially safer or more effective result, there continue to be challenges associated with these methods due to the complex nature of herpesviral systems. It is possible that altering a single viral gene in order to reduce virulence could have unexpected or hard-to-characterize effects depending on the host, the virus strain used, or subsequent recombination events with wild-type viruses. While similar problems also exist with classical attenuation techniques, it is important to bear in mind the potential complications with any method used (van de Walle 2008, Muylkens 2006).

Muylkens, et al, demonstrated this problem clearly by showing that a deletion of a single BHV-1 virulence gene, glycoprotein E, which significantly attenuated the vaccine strain, could

also be found in fully virulent wild-type strains of BHV-1. This implies that other differences within the strain chosen for vaccine development played a role in determining the attenuating ability of this gene deletion. While it is expected that vaccine developers might choose a well-characterized, possibly already attenuated strain for development purposes, the actual choice of vaccine strain could be very significant in determining the success of any given technique (Muylkens 2006).

Host differences could also play an important role in characterization of changes made in recombinant viruses for vaccine production. van de Walle, et al, showed that chemokine-binding glycoprotein gC, which has been deleted as a marker in EHV-1 vaccine strains, may actually be beneficial by allowing the host to better control viral infection in Blk/6 mice, whereas deletion of gC was previously shown to be attenuating in Balb-C mice. It remains to be shown whether the naturally affected host, the horse, is benefited or harmed by gC deletion from EHV-1 vaccines (van de Walle 2008).

Marker deletions, while not necessarily involved in attenuation, are an important part of recombinant vaccine design since it is often useful to be able to differentiate wild-type infections from vaccinated status. However, recombination events between vaccine strains and wild-type strains can occur which introduce marker deletions into fully virulent wild-type viruses, thus confusing strain differentiation for testing purposes, as demonstrated by Muylkens, et al. with BHV-1 glycoprotein E. This could necessitate the use of more sophisticated testing methods or the development of new vaccine strains in situations where testing plays a vital role in disease control (Muylkens 2006).

The greatest difficulty with vaccine design for herpesvirus infections may reside not with the vaccine approach but rather with the inherent efficiency of the virus at establishing a permanent

infection. Herpesviruses establish latency rapidly once infection occurs in order to avoid sterilizing immunity. Viral passage of HSV-1 from inoculated skin tissue to innervating ganglia was shown by Wakim, et al. to occur within 8 hours, which was too rapid to be controlled even with injected activated virus-specific CD8+ T cells. Wakim, et al. suggest that natural CTL responses to this herpesviral infection are unlikely to ever be rapid enough to induce sterilizing immunity even with a highly efficacious vaccine; however, the provided CTL response early in infection was able to reduce latent viral copy number, so even non-sterilizing immunity could have some benefit (Wakim 2008).

1.2.2.h. Conclusion

Targeted vaccine attenuation approaches offer a number of attenuation methods which may be combined in a rational manner, in combination with intelligent testing in in vivo models, to produce viruses with the desired ranges of efficacy, with the understanding that certain trade-offs may be necessary between immunogenicity and pathogenicity to achieve the necessary level of protection. Important targets currently under study include virulence factors, latency-associated factors, replication-essential genes, and immune evasins. While no single approach has yet solved the problem of latency and sterilizing immunity in herpesvirus infections, combined targeted approaches offer hopeful possibilities for reducing the prevalence and spread of these important diseases.

1.2.3. Immune evasion: a complex defense mechanism.

1.2.3.a. Viral immune evasion: introduction.

Evasion of the immune system's various defensive elements is a common activity across viruses which allows for enhanced survival and replication in a hostile host environment. Different virus species have accumulated a wide range of immune evasion functions, including

prevention of antigen-specific recognition by humoral immunity, inhibition of innate immune system elements, production of immune-modulating cytokine and chemokine homologues and inhibitors, and inhibition of cell-mediated immunity (reviewed in Vosson 2002, Alcami 2000, Haig 2001). Maintenance of these functions across viral lineages is evidence of their importance for virus survival, while deletion of immune evasion genes may provide targets for the development of less-pathogenic or more immunogenic vaccines in some cases (Crumpler 2009, Mohr 2008). DNA viruses such as the herpesviruses, poxviruses and adenoviruses, because of their large genomic size, have the capacity to encode large numbers of host-control genes, and provide many examples of genes encoding immune evasins (Haig 2001, Alcami 2000). Instances of viral immune evasins have been found that target each arm of the immune system, including innate and adaptive pathways, and in some cases particularly adept viruses evade multiple pathways through multiple immune evasion gene products.

The innate immune system is an important early target of viral interference. The components of innate immunity provide immediate immune control prior to the development of antigen-specific adaptive immunity, and include a variety of host responses such as inflammatory cytokines, apoptosis, and complement pathways, and NK cell cytotoxicity. Evasion of each of these innate defenses has been shown across a variety of viral species. Inhibition of apoptosis and complement pathways prolong the survival of virally infected cells to allow completion of the viral life cycle. Viral inhibitors of apoptosis affect many areas of the apoptotic pathway, via inhibition of pro-apoptotic factors such as p53 and PKR kinase (Prives 1999, Yew 1992, He 1997), or, in the case of poxviruses, direct inhibition of apoptotic caspase enzymes (Tewari 1995, Kettle 1997). The complement pathways are also responsible for early destruction of both infected cells and enveloped virions; inhibitors of these pathways have been

found among several viruses, such as the vaccinia virus complement control protein which inhibits the classical complement pathway (Kotwal 1990). Numerous viral proteins that inhibit cytokines and chemokines or their receptors, or which mimic chemokines or their receptors, have been found which reduce the immune response or alter it to the benefit of viral spread (reviewed in Haig 2001).

The adaptive cytotoxic immune response of CD8+ T cells, and the innate NK cell cytotoxic response, are complementary in their relationship to MHC class I expression on infected cells (Tizard, pp 199, 396). Because cytotoxic T cells rely on MHC class I presentation of antigen to recognize infected cells, viruses that evade this immune response, including herpesviruses as well as HIV and adenovirus, down-regulate cellular MHC class I surface expression (Collins 1998, Burgert 1985). However, decreased MHC class I expression is a stimulating factor for NK cell cytotoxic activity in response to viral infection. Several of the viruses which down-regulate MHC class I have also been shown to either upregulate non-classical MHC class I isotypes, or down-regulate NK-activating ligands, presumably to inhibit NK activity (Tomasec 2000, Schneider 2011).

The humoral adaptive immune response can be prevented if viral antigens adapt to avoid immune recognition, or if host responses to antigen recognition are blunted. Rapidly evolving RNA viruses rely on antigenic changes due to RNA polymerase copy errors, which decrease antigen specificity to pre-existing antibodies and delay the humoral adaptive immune response. For example, changing antigens in HIV within a single infected host prevent recognition of viral antigens and clearance by neutralizing cytotoxic T cells (Phillips 1991). In the case of segmented-genome influenza viruses, re-assortment of genome segments between viral populations can also introduce novel antigens which are not recognized by the host's pre-existing

antibodies (discussed in Hay 2001). DNA viruses, which do not benefit from the lower fidelity polymerases produced by RNA viruses, can have still other means of evading adaptive immunity. For example, several herpesviruses have been found to produce viral Fc receptors, which can bind the Fc portion of antibodies and prevent their interaction with immune effectors such as NK cells or the complement pathway (discussed below, section 1.2.3.b.).

Herpesviruses, which have some of the largest viral DNA genomes known (Davison 2002), are highly adapted to use a variety of these immune evasion mechanisms to enhance survival through successful establishment of long-term latency and chronic infection, despite an immunocompetent host. The remainder of this review will focus on three of the more well-studied mechanisms of immune evasion in herpesviruses, noting that a variety of other mechanisms are known to exist (including some already mentioned), and more will likely be discovered in the future. The mechanisms that will be covered specifically in relation to herpesviruses include evasion of the complement pathway, evasion of humoral immunity through the production of Fc receptors, and inhibition of the MHC class I pathway. It is to be noted that apparent immune evasion functions may have other roles in viral infection as well, such as in binding to target cells or promoting viral replication; thus they may present a complex picture requiring experimental evidence in vivo to confirm the importance of that immune evasion function to herpesviral infection.

1.2.3.b. Herpesviral immune evasion: regulation of complement

The complement system is a major mechanism of immunity important in early infection, which can be triggered by antibody binding (via the classical complement pathway) against foreign antigens, including those displayed on infected cells or virions; or by innate activity (the alternative pathway) against foreign cells or virions which lack inhibitory signals. Host cells

regulate complement to prevent inappropriate host-cell lysis via the production of complement-inhibitory proteins termed regulators of complement activation (RCA proteins). These typically membrane-bound proteins regulate complement at several important steps (notably C3 convertase activity and the formation of the membrane attack complex) to minimize local complement activity. Important mechanisms of complement evasion include upregulation of these RCA proteins allowing increased incorporation into infected cell membranes and viral envelopes; production of viral RCA protein homologues, to similarly protect infected cells and virions; and, in the alpha-herpesvirus family, expression of a unique non-RCA-homologue, glycoprotein C, which has complement-regulatory activity in addition to other functions.

Viral RCA homologues appear to be important immune evasins among the gamma-herpesviruses. Herpesvirus saimiri (HVS) produces two complement-inhibiting proteins with homology to known host RCA proteins. CCPH, or complement control protein homolog, is structurally and functionally similar to the C3 convertase inhibitors, such as decay accelerating factor (DAF; CD55) and membrane cofactor protein (MCP; CD46) which regulate the complement cascade at the C3 cleavage step (Albrecht 1992, Fodor 1995). The other protein, a homolog of the membrane inhibitor of reactive lysis (MIRL; CD59), regulates the end of the complement cascade by preventing the formation of the membrane attack complex (Albrecht 1992, Rothor 1994). Thus, HVS possesses two independent means of evading the complement pathway's activity against infected cells or virions prior to the development of specific immunity.

The homologous RCA protein to HVS CCPH encoded by ORF4 of murine herpesvirus 68, a gamma-herpesvirus of mice, has been found to produce both membrane-bound and soluble isoforms, of which the soluble form is known to prevent complement activation (Kapadia 1999). The ORF4 protein has been shown in vivo to increase acute virulence in normal mice and to

increase chronic virulence and facilitate persistent infection in immunocompromised mice; these effects were shown to occur through its interaction with complement (Kapadia 2002).

ORF4 of Kaposi's sarcoma-associated herpesvirus (KSHV), a gamma-herpesvirus of humans related to herpesvirus samairi, also encodes a regulator of complement activation (RCA) homologous to that of the HVS CCPH. This RCA protein, KSHV complement control protein (KCP) or kaposica, is composed of four complement control protein domains (CCP) that are expressed during lytic infecton as a membrane-bound protein on both infected cells and viral particles (Spiller 2003a, Mullick 2003). KCP has been shown to inhibit complement via both classical and alternative pathways via inhibition of C3 deposition in erythrocyte-based studies with solublized KCP (Mullick 2003) and to inhibit C3 deposition on transfected CHO cells (Spiller 2003a). KCP was found to accelerate decay of classical C3 convertase with 10-fold less activity than human DAF, and had a small amount of decay-accelerating activity (1000-fold less than DAF) against alternative C3 convertase. KCP was also found to be a cofactor of factor-I-mediated cleavage of both classical pathway convertase subunit C4b and alternative pathway convertase subunit C3b (Spiller 2003b), in addition to having other functions such as a likely role in virion binding through interactions with surface molecules such as heparin (Spiller 2006). This surprisingly compact yet multi-functional RCA protein is able to inhibit the complement pathway through multiple parallel mechanisms. (Spiller 2003a, Mullick 2003, Spiller 2003b).

Rhesus rhadinovirus (RRV), which is closely related to KSHV, has a homologous RCA gene located at ORF4. The RRV complement control protein (RCP) has been shown to similarly inhibit complement deposition through C3 and C5 convertase decay accelation and is a co-factor of factor I in both C3b and C4b cleavage. Interestingly, significant differences in structure and functional ability have been found in the RCP of different RRV strains. Unlike KCP, the RCP of

RRV strain 17577 is notably effective against the alternative complement pathway; the RCP encoded by strain H2695, which is smaller and more similar in structure to KCP, has minimal decay-accelerating activity against alternative-pathway C3 convertase. (Mark 2007, Okroj 2009).

Among the beta-herpesviruses, which have no known virally-encoded RCA proteins, several viruses are known to make use of the host's complement regulatory proteins, by incorporating them into their envelopes as well as upregulating them on the surface of infected cells. For example, human cytomegalovirus (HCMV) incorporates the host complement regulatory proteins DAF, MCP, and MIRL into the virion envelope (Spear 1995, Spiller 1997). This gives the virion reduced susceptibility to complement-mediated lysis in the absence of specific neutralizing antibody, and thus may protect the virus against early destruction by the innate immune system (Spiller 1997).

HCMV has also been shown to up-regulate the expression of both MCP (CD46) and DAF on the surface of infected cells. The increase in DAF was shown to reduce C3 deposition on the cell surface by C3 convertases, as well as to protect against non-antibody dependent CDCC in vitro—an effect which could be seen increasing over time from early to late infection (Spiller 1996). Similarly, murine cytomegalovirus (mCMV) up-regulates murine CD46 (not normally expressed on most cells in mice) through interaction with its promoter. The expressed membrane-bound mCD46 has a protective effect against non-antibody-dependent CDCC in vitro, and its expressed soluble form is hypothesized to help protect circulating virus as well (Nomura 2002).

Human herpesvirus-7 (HHV-7) also up-regulates the transcription and expression of both MCP and MIRL in infected cells, unlike closely-related HHV-6 which is known to down-regulate MCP (as well as use it as a receptor for entry). Cellular infection with HHV-7 was

found to have a partial protective effect against CDCC involving the classical (antibody-driven) complement pathway, which may be due to the up-regulation of these surface proteins (Takemoto 2007).

Another category of complement evasion molecules produced by herpesviruses includes glycoprotein C (gC) on the surface of many of the alpha-herpesviruses. The gC of herpes simplex virus 1 (HSV-1) and 2 (HSV-2) has been found to bind to C3b, an important opsonin in the complement pathway as well as a subunit of the C5 convertase and alternative pathway C3 convertase (Friedman 1984, Eisenberg 1987, McNeary 1987). HSV-1 gC has decay-accelerating activity against the alternative C3 convertase, similarly to the several previously described host and viral complement control proteins, despite being unrelated in structure (Fries 1986). Unexpectedly, HSV-2 gC lacks this function and appears to stabilize the alternative C3 convertase instead (Eisenberg 1987). HSV-1 gC can also inhibit the activity of C5 convertase, although this function was only seen in conditions were C5 was limiting (Fries 1986). HSV-2 gC lacks this function as well (Eisenberg 1987). HSV-1 gC also has been shown to competitively bind to the same site on C3b as the regulatory factors factor H and properdin, which may affect the activity of these regulators on the complement pathway (e.g. it is hypothesized that binding competition with the stabilizing factor properdin could decrease the half-life of C3b) (Huemer 1993).

Both HSV gC proteins block virus neutralization in vitro, primarily by the classical complement pathway (Harris, 1990, Hidaka 1991, Hook 2008), but likely also by the alternative pathway (McNearney, 1987). The mechanism(s) by which both HSV gC proteins prevent virus neutralization has not been fully elucidated, although both complement components and IgM appear to play a role in neutralization of gC-deficient viral mutants (Hook 2008). HSV-1 gC

also protects infected cells from complement-mediated lysis by the alternative pathway (Harris 1990, Hidaka 1991). In vivo, both HSV gC's were found to be effective as virulence factors, and their effects were found to be due at least partially to complement evasion as demonstrated in C3-deficient and C3-knockout animal models (Lubinski 1998).

The gC proteins of several other species of alpha-herpesviruses have been found to have similar functions. In vitro studies of pseudorabies virus (PRV; suid herpesvirus I) showed that PRV gC also has C3 binding capacity and blocks virus neutralization (Huemer 1992; Maeda 2002). Both equine herpesvirus (EHV) 1 and 4 and bovine herpesvirus 1 (BHV-1) produce gC proteins that bind C3 (Huemer 1993, 1995) and EHV-4 gC has been shown to protect the virus from complement-mediated neutralization in vitro (Azab 2010). Simian herpes B virus, an alpha-herpesvirus of old world macaques which can cause severe zoonosis in humans, also encodes a gC protein that binds C3 and protects against complement-mediated lysis of infected cells; unlike HSV-1, it does not interfere with C3-properdin binding (Huemer 2003). Despite differences in sequence and function between the various alpha-herpesviral gC proteins, evasion of the complement system by gC appears well-conserved across species tested to date.

1.2.3.c. Herpesviral immune evasion: Fc receptors block IgG-based immunity

Several herpesviruses evade antibody-based immunity through the production of Fc receptors on the surface of virions or infected cells, which has been found to interfere with host recognition of bound antibodies. Of these viral Fc receptors, the most extensively studied is the Fc-gamma receptor (FcγR) of HSV-1 and 2, composed of glycoproteins E and I (gE/gI). The presence of a viral FcγR in herpes simplex viruses was initially detected based on the ability of virus-infected cells to adsorb IgG-coated red blood cells (Watkins 1964). This receptor was found to efficiently bind the Fc but not F(ab')2 portions of IgG, and its interaction with

aggregated non-immune IgG appeared to interfere with both antibody-dependent complement-mediated cytolysis and cell-based cytotoxicity of infected cells, suggesting a possible role in immune evasion (Adler, 1978). The HSV-1 and HSV-2 Fc receptors were found to be present in both primary and chronically-infected cells, and their expression was reduced by an inhibitor of viral polymerase activity, distinguishing between viral and host origin (Bourkas 1979). The HSV-1 Fc receptor was identified as a complex of the glycoproteins gE and gI (Bauke 1979; Para 1982; Johnson 1987), which efficiently binds monomeric IgG of several species; in contrast, gE itself was found to bind aggregated IgG more effectively and gI has no binding activity of its own (Dubin 1990, Hanke 1990). This suggests that HSV-1 may encode two separate Fc receptors, the gE/gI complex and gE alone respectively, with different binding specificities, which may play different roles.

The glycoprotein E/I complex, while in general non-essential for replication, is conserved across multiple alphaherpesvirus species and therefore can be expected to play an important role in herpesviral infection (Favoreel 1997, Litwin 1992, Whitbeck 1996, Schumacher 2001). Interestingly, the gE/gI complex of HSV-1 was found to have several functions in vitro, which initially complicated the determination of its role in immune evasion. Specifically, gE/gI was found to be necessary for efficient cell-to-cell spread of virus between either fibroblasts or neurons in culture, and between either epithelial cells or neurons in several in vivo models (mouse corneal, retinal, and flank infections), which confounds the interpretation of pathogenesis data from gE knock-out virus (Dingwell 1994; Dingwell 1995; Lubinski 2011). However, evidence for a role in immune evasion has been strengthened by the development of HSV-1 gE mutants lacking the Fc receptor activity but not viral spread functions of glycoprotein E (Weeks, 1997; Nagashunmugan 1998). The related gE/gI complexes of varicella-zoster virus and

pseudorabies virus have been found to have Fc receptor activity as well as facilitating cell-to-cell spread, although the importance of an immune evasion role has been less studied in these viruses (Litwin 1992, Favoreel 1997, Mallory 1997, Mettenleiter 1987). On the other hand, gE/gI complexes of bovine herpesvirus-1 and Marek's disease virus have been found to function in viral spread but not as a viral Fc receptor; therefore conservation of this glycoprotein complex may be primarily due to its importance for cell-to-cell spread rather than playing a role in immune evasion in at least some species (Whitbeck 1996; Schumacher 2001).

The HSV-1 FcγR was found to be present on both virions and infected cells (Para 1980), and multiple potential immune evasion mechanisms have been described for this receptor. Due to its ability to bind both specific and non-specific antibody, possible roles include interfering with Fc-mediated antibody-based immunity, as well as steric effects which might protect specific antigens on virions or infected cells from immune targeting. Frank, et al, suggested a novel mechanism by which the HSV-1 FcγR might interfere with Fc-mediated immunity, which is bipolar bridging of specific IgG (Frank 1989). In this model, the viral Fc receptor binds to the free Fc end of specific antibodies attached by their F(ab')2 ends to viral antigens, preventing interaction with the complement and cell-based antibody-dependent immune pathways. Evidence that this occurs in vitro was developed with the use of gE knock-out virus, and it was shown that the HSV-1 FcγR attenuates antibody-dependent complement-mediated cytotoxicity and virus neutralization relative to knock-out virus only in the presence of specific IgG from species capable of Fc-binding to the viral FcγR (Frank 1989; Dubin 1991). On the other hand, it has also been suggested that gE may act with gC (another immune evasion glycoprotein) to reduce antibody-dependent neutralization through an independent mechanism, possibly by

sterically hindering antibody binding to virion-surface glycoproteins critical for entry (Hook 2008).

HSV-1 FcγR-based immune evasion in vivo has also been demonstrated. In a mouse model using passive immunization with IgG followed by flank inoculation with HSV-1, specific human IgG (capable of both Fc and F(ab')2 binding) significantly reduced disease score only in FcγR-deficient, spread-intact viral infection, relative to FcγR-intact virus or non-immune human IgG. Also, no difference was seen between mice immunized with murine HSV-1-specific IgG (capable of F(ab')2 but not Fc binding) regardless of whether the infecting virus expressed an intact Fc receptor (Nagashunmugam 1998). These results implicate bipolar antibody bridging activity of HSV-1 FcγR in evading antibody-dependent immunity, an effect most likely to occur in natural re-infection or reactivation of latent virus (when antibodies are present). These results were extended in a similar model with normal, C3 knock-out or NK-depleted mice to show that the HSV-1 FcγR blocks both antibody-dependent complement-mediated immunity and ADCC in vivo, resulting in increases in viral titers in ganglion tissue (where latency is established) and more severe zosteriform disease (reactivation of latency) (Lubinski 2011). Thus, the FcγR of HSV-1 appears to have more than one function, including evasion of antibody-mediated immunity which was significant in an in vivo model.

1.2.3.d. Herpesviral immune evasion: down-regulation of MHC class I

The cellular immune pathways are particularly important in the control of viral (intracellular) pathogens, and thus it is not surprising that a number of herpesviruses have developed mechanisms to evade both CD8+ T cell and NK cell pathways. The CD8+ T cell and NK cell pathways both rely on changes in the normal expression of MHC class I on most cell types to recognize abnormal cells, such as virus-infected cells or tumor cells, prior to killing

them. Interestingly, the CD8+ T cell response, which is adaptive, and the NK response, which is constitutive, are triggered by opposite MHC class I effects, and thus offer an added layer of protection against viral evasion. Expression of MHC class I molecules carrying non-self intracellular peptides, such as viral products, are recognized by T cell receptors on activated, epitope-restricted CD8+ T cells and trigger immune destruction of the infected cell, as well as cytokine production; if present on professional antigen-presenting cells, they can also activate naïve CD8+ T cells and stimulate a novel cellular immune response (Tizard, pp. 199). In contrast, NK cells target cells with reduced expression of MHC class I; viruses which attempt to evade CD8+ T cell recognition through down-regulation of MHC class I may inadvertently trigger lysis by NK cells, and vice versa (Tizard, pp 396-397). Therefore, viruses have been found to evade both CD8+ T cell and NK cell recognition by modulating both MHC class I expression and NK-receptor-activating ligands.

The MHC class I pathway functions to provide the antigenic stimulus to CD8+ T cells, and requires production of MHC class I heavy chain and beta-2 microglobulin (b2m) molecules, their association in the endoplasmic reticulum (ER), and stabilization by short peptides (Townsend 1989). These peptides, typically 8-10 amino acids in length, are typically provided to the ER by proteasomal degradation of ubiquitinated proteins, and subsequent transport of the resulting peptides into the ER by the transporter associated with antigen processing (TAP) complex (Powis 1991; see Yewdell 2001 for a review). MHC class I molecules, with associated peptide, are transported to the cell surface by means of the secretory pathway. CD8+ T cells recognize non-self epitopes of peptides (such as viral peptides) complexed with MHC class I molecules, and except in the case of autoimmunity are restricted against self epitopes from cellular peptides, which provide protective NK signaling. Lack of available peptides results in

de-stabilization of the MHC class I molecules and reduced expression of MHC class I on the cell surface (Powis 1991). Inhibitors of the MHC class I pathway might act on any part of the MHC class I pathway, including gene expression or protein stability of class I heavy chain or b2m molecules, peptide availability and proteasomal activity, peptide transport or MHC class I complex stability and transport; the end result will be a decrease in MHC class I-peptide surface expression and loss of CD8+ T cell-based immunity.

Several inhibitors of MHC class I expression have been found in a number of herpesviral infections, and are expected to play an important role in early escape from the CD8+ T cell response, allowing for the establishment of latent infection. These inhibitors act on various targets in the MHC class I pathway. Herpes simplex virus (HSV) expresses a targeted inhibitor of the MHC class I pathway, ICP47 (York 1994). ICP47 is a nonstructural, immediate-early HSV protein which has been shown to de-stabilize MHC class I molecules in the ER within 2 hours of infection (York 1994). The activity of ICP47 was found in vitro to be specific to cell type but not MHC class I species (York 1994, Früh 1995), suggesting that it did not act on MHC class I molecules directly but rather an up-stream or down-stream target within the pathway. ICP47 was subsequently found to inhibit TAP-dependent peptide transport in assays which provided non-proteasomally degraded peptides, thus establishing TAP as the target (Hill 1995; Früh 1995). ICP47 was also found to co-immunoprecipitate with TAP and MHC class I (Hill 1995; Früh 1995), and to co-localize with TAP in cellular immunostains (Früh 1995). Furthermore it was established that ICP47 inhibits TAP through competitive and non-competitive binding at the peptide-binding site (Hill 1995); thus, HSV provides a decoy molecule which binds to TAP and blocks peptides from reaching the MHC class I molecule. Interestingly, ICP47 was found to have little activity against murine TAP (York 1994, Früh 1995,

Hill 1995), a fact which complicates the use of mouse models in studying HSV infection, and also suggests that TAP genotypic diversity could contribute to resistance or susceptibility to some herpesvirus infections.

The importance of MHC class I-based immunity in HSV infection has been examined in mouse studies. In mice, which are not affected by ICP47, an MHC class I-based (CD8+ T cell) response was found to be important to both non-immune and immune challenge survival, although less so than an MHC class II (CD4+ T cell-stimulating) response (Ghiasi 1997). Interestingly, in a mouse model in which ICP47 was replaced by herpesviral proteins that can inhibit murine MHC class I (HCMV US11 or MCMV m152), immune-evasive HSV viruses were found to be more neuroinvasive and strongly induced paralysis relative to background virus. Similarly, CD8+ deficient mice also developed neuroinvasive disease when infected with background virus, indicating that MHC class I evasion contributes to HSV neurovirulence in the mouse model, although relevance to human infection is not proven (Orr 2005).

Another gene, UL49.5, has been found to be an inhibitor of TAP in a number of alphaherpesviruses, particularly the varicelloviruses (Koppers-Lalic 2005; Koppers-Lalic 2008). These varicelloviruses include BHV-1, BHV-1, BHV-5, BuHV-1, CvHV-1, PRV, EHV-1, EHV-4, and FHV-1 (Verweij 2011). Interestingly, homologous genes are present in beta and gamma-herpesviruses, as well as alpha-herpesviruses outside the varicellovirus lineage; however, UL49.5 from HSV-1 and 2, HCMV, and EBV were noted to not demonstrate TAP inhibitory activity, suggesting that this function of UL49.5 was novel within the varicelloviruses (Koppers-Lalic 2005). However, recently it has been noted that UL49.5 of MDV exhibits a small MHC down-regulating effect, contradicting a contemporary report, suggesting that UL49.5 might have at least some immune evasion function in non-varicellovirus alpha-herpesviruses (Jarosinski

2010, Verweij 2011). It should be noted that different cell types were used in the two contradicting studies, which could explain differences in MHC class I response.

Beta-herpesviruses also express modulators of MHC class I expression. HCMV has been found to express multiple MHC class I down-regulating gene products, which appear complementary in function. US11 and US2 are both able to cause degradation of MHC class I molecules by targeting them to the cytosol, where they are degraded by the proteasome rather than expressed on the cell surface (Wiertz 1996, Machold 1997). In a mouse model, these gene products were found to differentially degrade several mouse MHC class I alleles, suggesting different specificities which might complement each other by expanding specificity across hosts with varying HLA alleles in human infection. (Machold 1997) The HCMV US6 gene, on the other hand, inhibits TAP, reducing MHC class I by reducing peptide availability (Ahn 1997). Finally, the US3 gene product has been found to inhibit tapasin, a chaperone molecule important for peptide loading of class I molecules (Park 2004). A virus with multiple immune evasion functions all targeting the MHC class I pathway might be expected to be at risk for increased NK recognition; accordingly, HCMV also encodes evasins targeting NK activating ligands, including UL16, which reduces surface expression of MICB; and UL142, which similarly retains MICA intracellularly (Wu 2003, Ashiru 2009).

1.2.3.e. Conclusion

Herpesviruses have adapted to avoid immune recognition by multiple pathways, using a variety of viral gene products, and occasionally host proteins, to manipulate immune functions. This is expected to allow them a larger window of opportunity to establish permanent infection, and contributes to the difficulties encountered when attempting to prevent herpesviral diseases

through immunization. Future strategies developed to prevent and treat herpesviral diseases should take these mechanisms into account.

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CHAPTER 2

MDV012 down-regulates MHC class I in transfected chicken cells.

2.1. Abstract

Marek's Disease Virus (MDV) has been shown to significantly reduce the expression of major histocompatibility (MHC) Class I complexes on the surface of infected chicken cells, which is thought to be an immune evasion function. In order to identify and characterize the gene(s) responsible for the down-regulation of MHC class I, we selected nineteen viral genes with unknown function for cloning into plasmid vectors; 13 of these were eventually tested in vitro via cell transfection and flow cytometry. The gene MDV012, including its genomic and spliced (MDV012sp) variants, and to a lesser extent its first exon alone, were found to down-regulate MHC class I on the surface of both LMH and DF-1 cells in a target-specific manner. We also cloned a plasmid containing a signal-sequence-linked peptide, ssAR10V, in order to test candidate genes against the peptide translocation portion of the MHC class I pathway. Surface expression of MHC class I was rescued when MDV012sp was co-transfected with ssAR10V, suggesting that MDV012 targets the peptide translocation pathway and may be a novel TAP inhibitor.

2.2. Introduction

Marek's Disease is an economically important herpesviral disease in chickens which causes T cell lymphoid tumors, peripheral nerve enlargement, and may cause clinical disease such as paralysis, decreased productivity and carcass condemnation. Existing vaccines reduce the incidence of clinical disease but do not prevent latent infection or virus spread; emergence of

more virulent strains is a continuing challenge, necessitating the repeated development of new vaccines in response (Baaten 2004, Witter 1997, Witter 1998).

Herpesviruses modulate the host immune response using an exceptional array of methods across multiple arms of the immune system, as discussed in Chapter 1, Section 3. While these immunomodulatory functions are well-established, their relevance to immune evasion versus other benefits to virus survival (such as host cell targeting or enhancing viral replication) are not always clear; however, in several cases, it is clear that herpesviral immune evasion can enhance the virus' ability to escape immune control and cause greater disease (Kapadia 2002, Lubinski 1998, Lubinski 2011). One evasion method is down-regulating the expression of major histocompatibility complex (MHC) class I on the surface of infected cells, which is thought to prevent effective presentation of viral antigens to cytotoxic T lymphocytes and thus hinder cell-mediated immunity. Down-regulation of MHC class I has been shown to occur in multiple lineages, as noted below.

Responsible viral genes and their targets within the MHC class I pathway vary across and within the herpesviral subfamilies. For example, UL49.5 has been shown to block TAP, the transporter associated with antigen processing which transports antigenic peptides into the ER prior to MHC class I binding, in alpha-herpesviruses BHV-1, EHV-1, EHV-4, and PRV; in alpha-herpesvirus HSV, an unrelated gene product, ICP47, similarly inhibits TAP (Koppers-Lalic et al, 2008, York 1994) Other TAP inhibitors are found within the beta-herpesviruses (Ahn 1997). HCMV, a beta-herpesvirus, also encodes to genes that target MHC class I directly, as well as another gene that inhibits the chaperone protein tapasin (Wiertz 1996, Machold 1997, Park 2004). The proteasome, important for peptide processing, is inhibited by the gene EBNA-1 in EBV, a gamma-herpesvirus (Levitskaya 1997). The presence of multiple genes that inhibit

MHC class I in multiple herpesviral lineages suggests that this is an important immune modulatory function of herpesviruses.

Hunt, et al. (2001) showed that Marek's Disease Virus (MDV) causes MHC class I down-regulation on the surface of infected chicken cells; however the gene(s) and pathway capable of causing the down-regulation has not yet been identified. Viral immune evasion genes such as MHC class I down-regulation genes are expected to be present in both wild and vaccine strains of Marek's Disease Virus; if these genes are found, mutant viruses lacking immune evasion genes could be created which might have both enhanced immunogenicity and lowered pathogenicity and thus prove useful for vaccine production. The goal of this study is to identify and characterize the gene(s) responsible for MHC class I down-regulation in MDV and the point in the MHC class I pathway at which it acts.

2.3. Materials and Methods

2.3.1. Cell and virus lines

The MDV strain used for PCR amplification of candidate genes was tissue-culture-adapted Md11 contained in a bacterial artificial chromosome (Niikura et al, 2006; GenBank # AY510475) (kindly provided by Dr. Robert Silva, ADOL, East Lansing, MI). Cell lines used for transfection of MDV genes included LMH cells, a chicken hepatocellular carcinoma line (Kawaguchi et al, 1987); DF-1 cells, an immortalized chicken fibroblast line (Himley et al, 1998); and COS-K^b cells, an African green monkey kidney cell line stably transfected with mouse MHC class I H chain allele H-$2K^b$ (York et al, 2005). Cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen), with 10% Fetal Bovine Serum. DF-1 and LMH cells were passaged in ciprofloxacin HCl (10 ug/mL) approximately one week out of four to protect against

mycoplasma infection. DH5alpha sub-cloning efficiency E. coli cells (Invitrogen) were used for heat-shock-based transformation and growth of plasmid stocks.

2.3.2. Antibodies

Antibodies against chicken and mouse MHC class I were obtained. A mouse monoclonal antibody against chicken MHC class I, mAb C6B12, has been described (Shamansky et al, 1988) and was obtained from The Developmental Studies Hybridoma Bank, Iowa City, Iowa. Polyclonal antisera against the major and minor MHC class I loci of several MHC haplotypes have been developed using cloned sequences expressed in the RCASB-A retroviral system in RP9 cells and used to raise antisera in chickens (Fulton et al, 2001). As DF-1 cells are known to express B21 haplotype MHC class I, anti-BF2*21 specific (anti-major; H1222-RP9BFIV21m78) and anti-BF1*21 specific (anti-minor; E6338) antisera, were obtained from the USDA-ARS Avian Disease and Oncology Laboratory (USDA-ADOL), East Lansing, Michigan. The anti-major antibody is based on a mutated sequence that restricts the available epitopes to reduce cross-specificity between MHC class I haplotypes, and potentially between non-classical MHC loci (Fulton et al, 2001).

Different primary antibodies were used against the respective mouse and African green monkey MHC class I alleles expressed by COS-Kb cells. To analyze expression of the native green monkey MHC class I, the mouse anti-human MHC class I antibody PA2.6 (anti-HLA-A,B,C, which cross-reacts with African green monkey MHC class I alleles on COS cells) was used (Brodsky et al, 1979; York et al, 2005). In co-transfection experiments involving transfection of the H-2Kb-restricted ovalbumin epitope SIINFEKL, the monoclonal mouse anti-H-2Kb-SIINFEKL IgG antibody 25-D1-16 (Porgador 1997) was used. This antibody was also

used as an isotype control against C6B12 in DF-1 and LMH cell experiments. Normal chicken serum was obtained from USDA-ADOL for use as an isotype control against the anti-major and minor antibodies. Secondary antibodies used were anti-mouse and anti-chicken IgG conjugated with the Cy5 fluorophore, obtained from Jackson Immunoresearch (West Grove, PA).

2.3.3. PCR and plasmid cloning of MDV genes

Eighteen genes from Marek's Disease Virus were chosen as initial candidates (Table 2.1.). For the seventeen genes not previously amplified, pairs of amplification primer pairs were designed flanking these genes, and a unique restriction site was incorporated into the 5-prime end of each primer corresponding to a restriction site present in the multiple-cloning site of expression vector pTracer-CMV2 (Invitrogen), such that the gene could be inserted in the correct direction (Table 2.2.). These primers were obtained commercially from Integrated DNA Technologies (Corallville, Iowa). Initial amplification with Taq polymerase was performed to optimize the annealing temperature necessary to amplify each gene. PCR products were run on horizontal agarose gel electrophoresis and stained with ethidium bromide to verify product size (see Figure 2.1.). Subsequently, amplification with the higher-fidelity Pfu polymerase (Invitrogen) was performed to obtain a high-quality linear DNA sample of each gene.

Linear PCR products were purified using the Qiaquick PCR Purification kit (Qiagen) and ligated into the pTracer-CMV2 expression vector (Invitrogen) after restriction digestion of both the vector and the products with the appropriate enzyme pair matching the primers (Table 2.2.). Ligation was performed with T4 DNA Ligase (NEW ENGLAND BIOLABS; NEB) and the ligation products used to transform DH5alpha cells (Invitrogen) which were subsequently grown on LB/Ampicillin (100ug/mL) plates overnight at 37 degrees Celsius. Colonies were picked and

sub-cultured in 3 mL LB/Ampicillin (100ug/mL) broth and shaken overnight at 37 degrees Celsius and 225 RPM for DNA miniprep purification using the FastPlasmid Mini Kit (5Prime).

Miniprep DNA was analyzed through restriction digests using unique restriction sites external to the ligated PCR product and, where possible, an internal restriction site, to drop inserts in one or two fragments out of the plasmid DNA (Table 1.1.). Restriction-digested plasmid DNA was visualized via horizontal agarose gel electrophoresis and ethidium bromide staining to verify insert size (see Figure 2.2.).

Plasmids with inserts of approximately the expected size (see Table 2.2.) were amplified by subculturing 1 mL of the initial 3 mL culture into 250 mLs of LB/Ampicillin, grown overnight at 37 degrees Celsius and 225 RPM. Plasmid DNA was purified in quantity using the Qiagen Maxiprep Kit. Plasmid DNA purified via Maxiprep was commercially sequenced by MCLabs, Inc. (South San Francisco, California), using the CMV-Forward and BGH-Reverse primers (sequences available from Invitrogen), to verify that the MDV genes were inserted in the correct orientation and without mutation. Six genes were successfully cloned into pTracer-CMV by the author, while six more genes were later amplified by others (Table 2.1.).

Other plasmids used included in transfection testing included MDUL49.5 in several different vectors (obtained from USDA-ADOL; see Table 2.3.); pUG-S8L, a plasmid expressing SIINFEKL, the immunodominant peptide of ovalbumin for H2-Kb, as a ubiquitin-fusion protein; as well the parent vector, pUG-1 (both described in York et al. 2006). pUG-1 contains the ubiquitin sequence, as well as expressing GFP. Also included as another control was the plasmid pIG-I, the precursor plasmid to pUG-1 lacking the ubiquitin sequence (York 2006).

2.3.4. Transfection and flow cytometry

Plasmids containing candidate genes were used to transfect DF-1 and LMH cells in order to ascertain their affect on MHC class I surface expression in vitro. For both cell types, transfections were performed using approximately 1.5 x 10^5 cells per sample well in 3mL DMEM with 10% FBS. Cells were incubated at 39 deg C approximately 12 hours and refed in 2 mL fresh DMEM with 10% FBS (single gene experiments) or 2 mL RPMI without FBS (MDVUL49.5 co-transfection experiment). Transfection solution was made using 3 uL/ug DNA of Fugene 6 (Roche) (for DF-1 and Cos-Kb cells) or Transit-LT1 (Mirus) (for LMH cells) in 100 uL RPMI media per each sample, and 1 ug DNA per plasmid, per sample was added to the solution. The resulting DNA transfection mixture was added to each sample and the cells were incubated for an additional 24 hours at which time 1 mL (single gene experiments) or 3 mL (co-transfection experiment) of DMEM with 10% FBS was added to each sample well.

Cells were harvested after an additional 24 hours using 0.3 mL (co-transfection experiment) or 0.5 mL (single gene experiments) Trypsin-EDTA in the minimum amount of time needed for cell-cell and cell-surface de-adhesion (approximately 30s to 1 minute) and the Trypsin-EDTA was neutralized with a minimum of 1.4 mL DMEM with 10% FBS (later MDV gene expression tests used 4 mL DMEM per sample with 20% FCS to minimize trypsin exposure). Cells were pelleted via 30 s of microcentrifugation at 14,000 RPM, the pellets re-suspended and washed in 1 mL PBS, re-pelleted, and re-suspended in 50 uL of diluted primary antibody (1:50 dilution in PBS for concentrated C6B12, anti-major and anti-minor antibody stock; 50 uL supernatant for non-concentrated 25-D1-16 and normal chicken serum). The cells were allowed to stain on ice for 30 minutes, then re-pelleted and the supernatant removed, re-suspended and washed with 1 mL PBS, re-pelleted, and re-suspended in 25 uL of secondary

antibody dilution (1:150 in PBS using concentrated stock). The cells were again allowed to stain on ice for 15-30 minutes (consistent across samples within each experimental run), re-suspended and washed with 1 mL PBS, re-pelleted and the pellet re-suspended in 400 uL of PBS.

Sample data were collected with flow cytometry using either an LSR II or Vantage TurboSort SE flow cytometer. Data were analyzed using flowjo™ software. Visual gating on live cell populations was performed using irrelevant-antibody-stained, untransfected controls with FSC and SSC signals: gates were set to exclude very low populations on FSC and SSC, while including the largest population of cells. GFP (collected as FITC-A signal) and secondary staining (collected as APC-A or FL5-Cy5 signal) were compared for experimental and control-transfected cells, and visual gates for GFP-positive transfected cells were set on stained, untransfected controls to include 99.5% of live cells in the GFP-negative population in untransfected samples. This type of gating was used to allow histogram comparison of Cy5 staining within transfected populations only.

2.3.5. MHC surface rescue assay

Plasmids were built using similar methods and the same kits as above. The MHC class I B21-binding 10-mer peptide, REVDEQLLSV has been described in Koch et al, 2007. This peptide sequence was cloned into a plasmid containing an ER-targeting signal sequence as follows. Forward and reverse oligos were obtained of the N-terminally extended peptide sequence, AR10V. The peptide sequence was N-terminally extended with alanine to promote efficient cleavage from the signal sequence; the N-terminal A is expected to be efficiently cleaved off by ER-resident endopeptidases (Hearn A 2009). Restriction sites were incorporated into both ends of the oligos (obtained from Integrated DNA Technologies; see Table 2.4.). The

forward and reverse oligos were annealed together using a ramp-up annealing temperature cycle. The annealed oligos were restriction-digested (see Table 2.4.) and PCR-purified. A BlueScript (Stratagene) plasmid vector pBSss2, containing an ER-targeting signal sequence derived from the adenoviral E3/19K protein, (Hearn A 2009), was digested with the same restriction enzymes and purified with gel extraction. The cut oligos and plasmid were ligated, used to transform DH5alpha cells, and plasmid DNA was purified using the Maxi-prep technique. The resulting plasmid pBSssAR10V was then restriction-digested (see Table 2.4.) and plasmids pTracer-CMV2 (Invitrogen) and pTracer-RFP (a derivative of pTracer-GFP; Hearn A and Rock KL, unpublished) were digested with the same restriction enzymes. The ssAR10V oligo and the digested pTracer-CMV2 and pTracer-RFP vectors were gel-extracted and the ssAR10V oligo was ligated into each vector with subsequent transformation of DH5alpha cells. The resulting pTracer-CMV2-ssAR10V and pTracer-RFP-ssAR10V plasmids were Maxi-prepped and used in single and co-transfection experiments.

For single transfections, samples were transfected into DF-1 cells as in the MDV gene transfection experiments and refed with 3 mL per well of DMEM with 10% FBS at 24 hours post-transfection. The samples were harvested at 48 hours post-transfection with 0.3 mL of 0.25% Trypsin-EDTA, the trypsin was neutralized with 1.4 mL of DMEM with 10% FBS, and the cells were stained for flow cytometry as in the MDV gene transfection experiments. The insert sequence of pTracer-RFP-ssAR10V, which was found to increase surface MHC class I in DF-1 cell transfections, was verified by commercial sequencing (MCLabs Inc.).

For the MHC class I surface-rescue assay, the plasmids pEGFPN1 (Clontech) and pEGFPN1-MDV012 (obtained from Dr. Henry Hunt, USDA-ADOL), containing the mature spliced sequence of MDV012 as an EGFP fusion protein (at the C-terminus of MDV012), were

used alongside pTracer-RFP and pTracer-RFP-ssAR10V plasmids. In these experiments, DF-1 cells were plated at 1.5x10^5 cells/well, refed in 2 mL DMEM with 10% FCS at about 12 hours, and transfected similarly to above with the following modifications. Single transfections with 1 ug of each plasmid and co-transfections of combinations of 0.5 ug of an EGFPN1 construct and 1 ug of an RFP construct were performed, using 4.5 ug of Fugene 6 per sample. Samples were refed with 3 mL of DMEM with 10% FBS at 24 hours post-transfection, and harvested and stained at 48 hours post-transfection as in the single transfections performed with the same plasmids.

2.4. Results

2.4.1. Candidate genes

Candidate genes were chosen based on the assumption that genes with known roles were less likely to be involved in MHC class I down-regulation. Eighteen genes with unknown function which were conserved between MDV strains were selected as possible candidates to test for immune evasion (Table 2.1.). Of these, one (MDVUL49.5) was homologous to a known immune evasion gene in other herpesviruses. This gene has previously been cloned into several vectors which were obtained from Dr. Henry Hunt, USDA-ARS Avian Disease and Oncology Laboratory, East Lansing, MI (Table 2.3.). A nineteenth gene product of interest was predicted as the spliced product one of the candidates, MDV012, and the candidate MDV011 was noted to consist of the first exon of the complete MDV012 sequence; these three products, genomic MDV012, spliced MDV012, and the first exon of MDV012 (labeled as MDV011 in some annotations), have been tested separately. Of the seventeen initial genes of interest to clone, 12 genes were successfully amplified by PCR (see Table 2.1. and Figure 2.1.) and six were cloned

into the GFP-expressing plasmid, pTracer-CMV2 during this project (Table 2.1.). An additional 6 genes, including MDV011 and the spliced variant of MDV012 (abbreviated here as MDV012sp) were subsequently cloned by other members in the York lab (Table 2.1.). Table 2.1. lists the gene candidates and their current status with regard to cloning and testing. Plasmid sequences were verified with commercially available sequencing prior to or concurrent with use in transfections during this project.

2.4.2. Transfection tests

Transfection tests in cell culture were performed with 5 of the 6 successfully cloned genes, as well as the previously cloned MDVUL49.5; and the cells were stained for MHC class I and compared to untransfected and vector controls for effects on surface expression of MHC class I. MDVUL49.5 was tested in COS-Kb cells, while the other 5 genes were tested in chicken cells (DF-1 or LMH).

2.4.2.a. MDVUL49.5 may non-specifically inhibit MHC class I expression.

Initial transfections of COS-Kb cells with MDVUL49.5 cloned in six different vectors (Table 2.3.) suggested that transfection with this gene decreased expression of green monkey MHC class I on these cells as compared with vector (see Figure 2.3.), although the effect was variable depending on the vector used. Subsequently, a co-transfection experiment was performed in which COS-Kb cells were transfected with the MDVUL49.5 construct which showed the greatest effect initially, and also with a plasmid that expressed the immunodominant ovalbumin peptide (SIINFEKL) for the mouse H-2Kb MHC present on these cells, either in the cytosol in mature form via a ubiquitin-fusion construct (Bachmair et al, 1986), or as an N-terminally extended peptide (ASIINFEKL) with an attached endoplasmic-reticulum-targeting

signal sequence. The mild decrease in MHC class I expression was seen with staining for the MHC-associated peptide, and it was present regardless of whether the provided peptide was delivered to the endoplasmic reticulum via a signal sequence or required transport (Figure 2.4.). Further analysis of the same data indicated that co-transfection with MDVUL49.5 also decreased expression of GFP by the peptide-expression plasmids, suggesting that MDVUL49.5 may have an effect on overall protein expression within the cell (e.g. may be cytotoxic; Figure 2.5.). Further experiments in DF-1 and LMH cells by other members of the York Lab failed to show a consistent or significant effect with this gene product (see Figure 2.6.).

2.4.2.b. MDV012 down-regulates MHC class I in vitro.

The 5 newly cloned genes (see Table 2.1.) were transfected into DF-1 and LMH cells and the cells were stained at 2 days post-transfection for MHC class I. Surface staining for MHC class I, and transfection efficiency as indicated by GFP fluorescence, were analyzed by flow cytometry. Initial transfections exhibited low transfection efficiency, and data sufficient for analysis was collected only on MDV007 in DF-1 cells, and MDV087 in LMH cells. Figure 2.7. shows MHC class I expression in transfected cells for these two genes, as measured by geometric mean fluorescent intensity. In each case, MHC class I expression does not differ from cells transfected with the vector control. Further transfection experiments performed by other members of York Lab confirmed that these 2 genes as well as 7 others had no effect on MHC class I expression in DF-1 and LMH cells (see Figure 2.6.). MDVUL49.5 showed a mild decrease in MHC class I in LMH but not DF-1 cells. Of the thirteen total genes tested, only MDV012, its first exon (MDV011), and especially the spliced product MDV012sp showed consistent down-regulation in both cell lines (Figure 2.6.)

2.4.2.c. MDV012sp specifically reduces MHC class I surface expression.

Further experiments by other members of York Lab tested for specificity of MDV012sp against MHC class I versus general disruption of cellular processes. DF-1 cells were co-transfected with MDV012s and either mouse MHC class I SC-Kb, which is a single-chain Kb-b2m fusion construct and thus can be stably expressed in a non-mammalian system, or influenza hemagglutinin. Staining for hemagglutinin in the presence of MDV012sp showed no decrease in fluorescence (and actually a slight increase) relative to transfection with hemagglutinin alone, while expression of the MHC class I allele SC-Kb in the presence of MDV012sp was down-regulated relative to SC-Kb alone. MDV012sp continued to down-regulate both MHC class I major and minor isoforms (Figure 2.8.).

2.4.2.d. MDV012s inhibits peptide translocation to the ER.

Degradation of endogenous antigenic proteins into peptides and subsequent transport of these peptides into the endoplasmic reticulum via TAP for loading onto MHC class I molecules is necessary for these antigens to be presented on the surface bound to MHC class I (Townsend 1988, Powis 1991, Androlewicz 1993, Neefjes 1993**)**. Disruption of peptide loading generally causes a decrease in surface expression of MHC class I, as MHC class I molecules are not stable or transported to the surface if they do not bind peptide. Evaluation of MDV012s activity against this portion of the MHC class I pathway involved co-transfection with pTracer-RFP plasmid containing a peptide linked to a signal sequence which targets it directly to the endoplasmic reticulum at synthesis rather than requiring translocation by TAP. The peptide, REVDEQLLSV (R10V), has been shown to efficiently bind and stabilize chicken B21 haplotype MHC class I (Koch et al 2007).

Figure 2.9. shows the geometric mean fluorescence intensity of DF-1 cells stained with C6B12 anti-chicken MHC class I antibody when co-transfected with MDV012s and ssAR10V (peptide and signal sequence, noted as R10V in this figure), MDV012s and empty RFP vector, ssAR10V alone, or RFP vector alone. Relative to the vector control, transfection with ssAR10V increased MHC class I expression in DF-1 cells whereas transfection with MDV012s decreased MHC class I expression. However, co-transfection with both MDV012s and ssAR10V rescued MHC class I expression to about the same level as with ssAR10V alone, showing that the MHC class I pathway downstream of peptide translocation remains intact in the presence of MDV012s.

Further work by other members of the York Lab repeated and extended this experiment by comparing the results of co-transfection with the ER-targeted ssAR10V with co-transfection with R10V expressed as a ubiquitin-fusion protein, which targets the R10V peptide to the cytosol where it is cleaved to its mature form by ubiquitin C-terminal hydrolases (Bachmair 1986, Hearn A 2010). In this experiment, ssAR10V rescued the expression of MHC class I in the presence of MDV012sp, while ubiquitin-R10V failed to rescue MHC class I expression (Figure 2.10.). This strongly suggests that MDV012sp targets the MHC class I pathway by interfering with the transport of mature cytosolic antigenic peptides into the endoplasmic reticulum, a function localized to TAP.

2.5. Discussion

We have identified a gene in Marek's Disease Virus, MDV012sp, which (along with its first exon and un-spliced genomic form) are capable of down-regulating MHC class I expression in chicken cells, as demonstrated by transfection, staining for MHC class I surface expression, and flow cytometry. It is possible that more than one MHC class I down-regulation gene exists

in the MDV genome; to date, the only other likely candidate for MHC class I immune evasion is MDVUL49.5, which has been found to have a mild effect in some cell types (Jarosinski et al 2010).

Co-transfection of MDV012s with a peptide which bypasses peptide translocation showed rescue of MHC class I surface expression, whereas co-transfection with mature peptide provided in the cytosol fails to rescue MHC expression, strongly implicating TAP as the level at which MDV012s acts rather than an upstream target in the antigen processing pathway. TAP is a common target by several unrelated inhibitors across the spectrum of the alpha-herpesviruses (Koppers-Lallic et al, 2008). In chickens, the TAP gene is closely linked to the MHC class I loci and therefore can be expected to assort with MHC haplotypes which determine resistance or susceptibility to MDV. It has been suggested that tight coupling between the chicken MHC class I and TAP genes has allowed for co-evolution of TAP to create genetic resistance to infectious diseases in some MHC class I haplotypes which may be at least partially determined by TAP specificity (Kaufman, 2000). If this is the case, the interaction between a viral TAP inhibitor and genetically resistant versus non-resistant chicken strains could prove useful for further describing both viral pathogenicity and genetic strategies for disease resistance in the chicken.

We are in the process of designing knock-out viruses lacking functional MDV012sp for use in in vitro and in vivo testing. While MDV has been shown to reduce MHC class I expression in chicken cells, it is yet to be determined whether this is an immune evasion function in vivo. Assessing knock-out and revertant viruses for changes in pathogenesis will help to answer this question, as well as provide a starting point for research into the production of vaccine strains lacking immune evasion genes. Such vaccines could prove to be more effective

against the emerging MDV strains of increased virulence which continue to affect the poultry industry.

APPENDIX

TABLES

Table 2.1. Candidate genes in MDV.
(RefSeq NC_002229.3; GenBank accession # AY510475.1 for Md11BAC).
§ These genes were successfully cloned and/or used in transfection tests by other members of York Lab
¶ These genes were PCR-amplified by other members of York Lab but not successfully cloned.

Candidate Genes	Structure/Function/ Homologues	Cloned	Transfected DF-1 cells	Transfected LMH cells
MDVUL49.5§	Type 1 membrane protein; contains a signal peptide; complexed with envelope glycoprotein M (immune evasin in other viruses)	Previously completed	-	-
MDV002§	Originally annotated as Arg-rich protein	PCR	-	-
MDV006	Originally annotated as 14 kDa lytic phase protein	PCR	-	-
MDV007	Hypothetical protein	Yes	Yes	Attempted
MDV009§	Hypothetical protein	PCR	-	-
MDV011§	(First exon of MDV012)	No	-	-
MDV012§	Unknown function	Yes	No	No
MDV012s§	(Mature spliced form of MDV012)	No	-	-
MDV069	LORF4; uknown function	Yes	Attempted	Attempted
MDV081¶	Hypothetical protein	No	-	-
MDV082	Hypothetical protein	Yes	Attempted	Attempted
MDV083¶	Hypothetical protein	No	-	-
MDV085§	Hypothetical protein	PCR	-	-

Table 2.1. (cont'd)

MDV086[§]	Cytoplasmic protein	PCR	-	-
MDV087	SORF2; related to Gallus gallus hypothetical protein LOC422090	Yes	Attempted	Yes
MDV090	SORF3; unknown function	PCR	-	-
MDV091	Virion protein US2; possibly envelope-associated; interacts with cytokeratin 18	No	-	-
MDV092	serine/threonine protein kinase US3; tegument protein; involved in egress	No	-	-
MDV093	SORF4; unknown function	Yes	Attempted	Attempted

Table 2.2. Primers attempted for cloning MDV genes into pTracer-CMV2.

Gene	Primer	RE Site	OK*
MDV002	F: gcgaattcAGAAAGCCGAGACCACAAGA	EcoRI	-
	R: gctctagaGGGAGAAAGGACCGAAAGG	XbaI	-
MDV006	F: gcgaattcCAGGTTTGCAACTCCTCCAT	EcoRI	No
	R: gctctagaAATGTGCAAACAGCGACTCA	XbaI	No
MDV007	F: gcgaattCCACTCGAGGCCACAAGAAAT	EcoRI	Yes
	R: gctctagACCGCTTCATCATCAAATATCG	XbaI	Yes
MDV009	F: gcgaatTCGTGGCAAACCACGACTACCT	EcoRI	-
	R: gctctagaCATATCCGATTGGCTCACCT	XbaI	-
MDV012	F: gcgaattcCGGTGCTTTGTACTTCCTACG	Blunt-end (EcoRV for vector)	Yes
	R: gctctagaTGGATTTGCAATCACACAACA	XbaI	Yes
MDV069	F: gcgaattcGCCACCCTTAAGTCTTCGTG	EcoRI	Yes
	R: gctctagaTCTCAGATATGTCTTGTTAAAGTGTGG	XbaI	Yes
MDV082	F: gcgaattcCGGAAGCGATACTACTGCTG	EcoRI	Yes
	R: gctctagACGTAACATTCGAACAAGTACCAAG	XbaI	Yes
MDV085	F: gcgaattcGCTTAACCGGCAGTACAGGA	EcoRI	-
	R: gctctagaCGATGTGCTGAAAGTCGAAA	XbaI	-
MDV086	F: cggggtacCGGGCATGTCTGATCCTTCA	KpnI	-
	R: gctctagaTGGGCTACAATTCCCTTTTG	XbaI	-
MDV087	F: cggggtacCAGGCCATATCAGCTTTCACG	KpnI	Yes
	R: gctctagATCACGTGATATCCGGTCAAA	XbaI	Yes
MDV090	F: cggggtaccATCGAGCGCATTTGAAAGAC	KpnI	-
	R: gcggccgccGGCATTACAGTGTTGTCATGATT	NotI	-
MDV093	F: gcgaattcCGCGTTACCTGCAATAATGA	EcoRI	Yes
	R: gctctagaTCATATCCCGACGATGAAAA	XbaI	Yes

*Primers with a "yes" in this column were successfully used to clone MDV genes. Primers with a "no" were unsuccessful with repeated attempts. Primers with a dash were attempted, and were either sequenced later or cloning was re-attempted with other primers later by other members of York Lab.

Table 2.3. MDVUL49.5 clones. Obtained from Dr. Henry Hunt, USDA-ADOL.

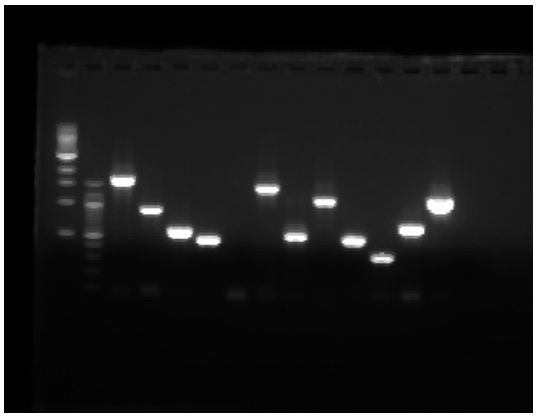
ID	Vector	Insert
16	pcDNA31+zeo	HA-UL49.5
17	pZeo-FLAG	FLAG-UL49.5
18	pCDNA	UL49.5 (F to I mutation present)
23	pZeoDNA3.1+zeo	Kozak HA-UL49.5
43	pEGFPN-1	UL49.5 C-terminal deletion (CT)
52	pEGFPN-1	UL49.5

Table 2.4. Primers for ssAR10V cloning.

ssAR10VF	aactgcagcgct GCC AGGGAGGTGG ACGAGCAGCT GCTGAGCGTG tag ttctagagc
ssAR10VR	gctctagaa cta CACGCTCAGC AGCTGCTCGT CCACCTCCCT GGC agcgctgcagtt
pUG-R10VF	AGGGAGGTGG ACGAGCAGCT GCTGAGCGTG TGA CTGCAG
pUG-R10VR	GATC CTGCAGTCAC ACGCTCAGCA GCTGCTCGTC CACCTCCCT

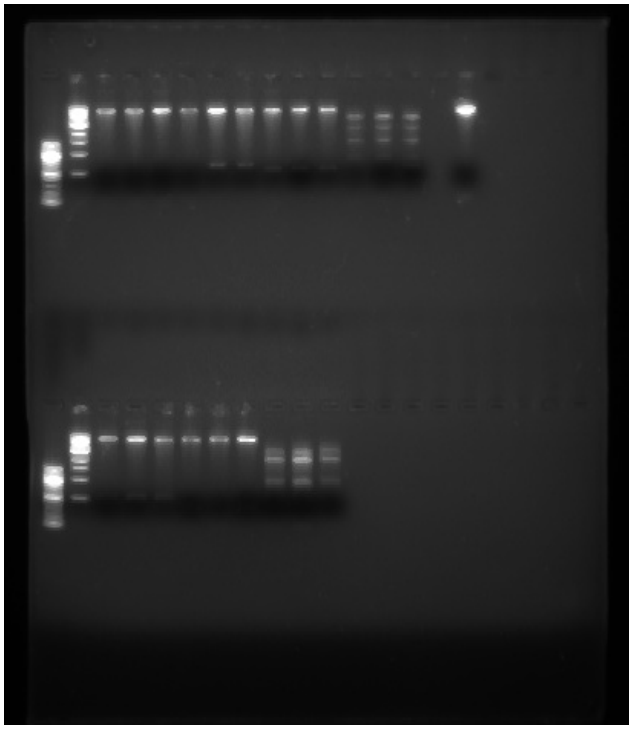
FIGURES

Figure 2.1. Gel electrophoresis of MDV gene PCR products. This representative gel shows successful PCR amplification products in lanes 3-6 and 8-14.



Lane	PCR product
1	Kb ladder
2	100 bp ladder
3	MDV012
4	MDV069
5	MDV093
6	MDV082
7	MDV002
8	MDV006
9	MDV007
10	MDV009
11	MDV085
12	MDV086
13	MDV087
14	MDV090
15	Negative control (no primers)

Figure 2.2. Example of analytic restriction digests of cloned pTracer-CMV2-MDV-genes. Gel electrophoresis. Lanes 7-11 above and 3-4 below show inserts of expected size and vector fragments.



Lane (above)	**Insert**
1	100 bp ladder
2	1 kb ladder
3-5	MDV012
6-8	MDV093
9-11	MDV082
12-14	MDV009
16	Vector (cut with PmeI)
Lane (below)	**Insert**
1	100 bp ladder
2	1 kb ladder
3-5	MDV085
6-8	MDV086
9-11	MDV090

Figure 2.3. MDVUL49.5 decreases MHC class I expression in COS-Kb cells. MDVUL49.5 constructs were used to transiently transfect COS-Kb cells, which were stained with PA2.6 and analyzed for MHC class I expression via flow cytometry. Geometric mean fluorescent intensity is shown as a percent of control (pTracer-CMV-2).

2.3.1. Experiment 1. One experiment is shown in which two UL49.5-expressing plasmids show 35-40% lower GMFI than the pTracer-CMV-2 control.

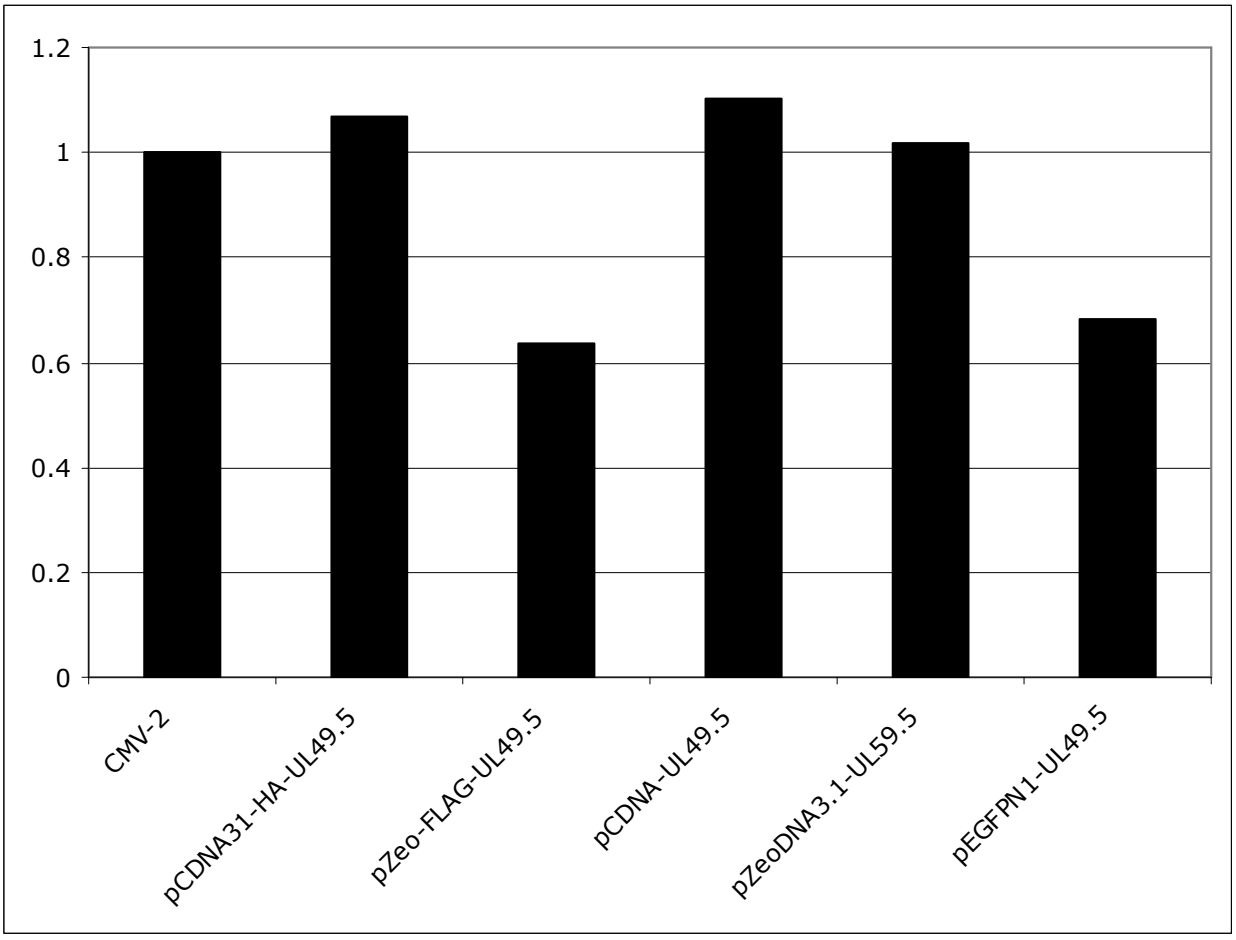


Figure 2.3 (cont'd)

2.3.2. Experiment 2. One experiment is shown in which two UL49.5-expressing plasmids show only 15-20% lower GMFI than the pTracer-CMV-2 control and approximately the same GMFI as the pIG-1 control.

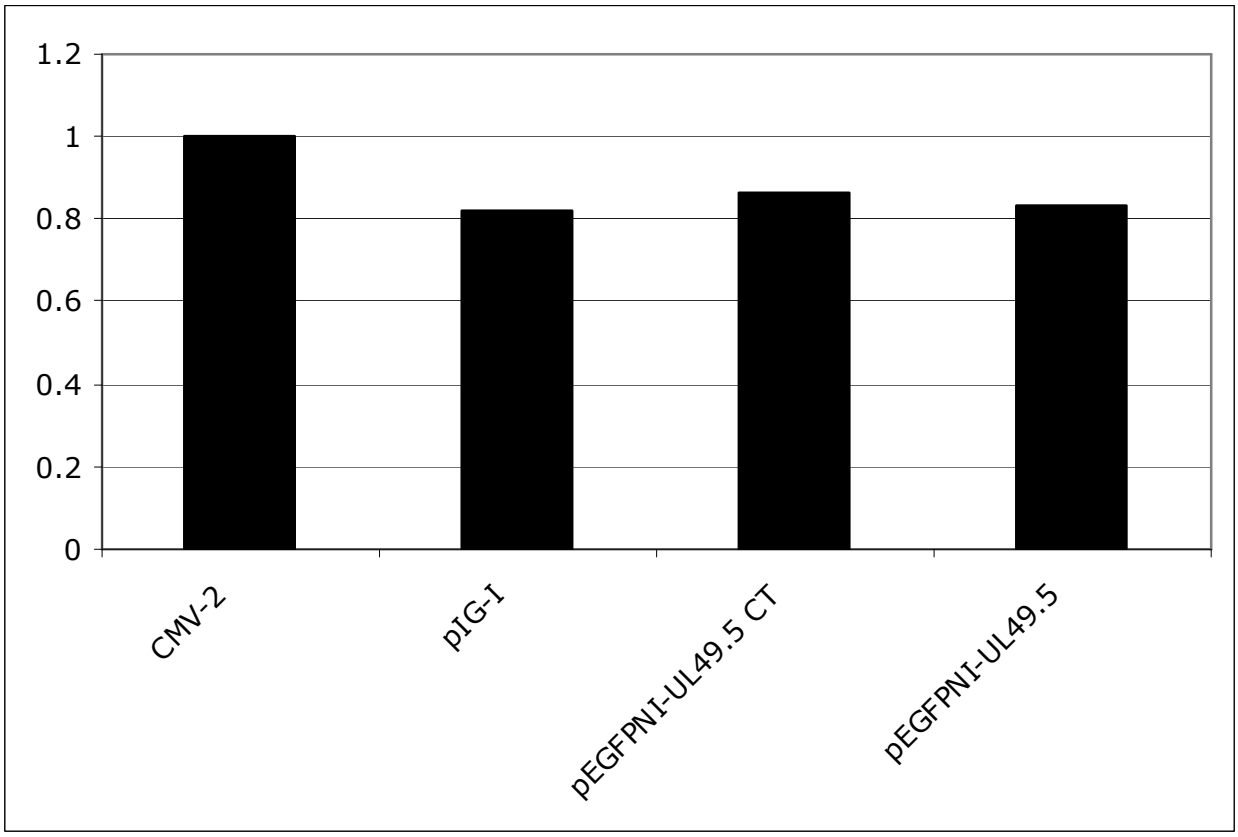


Figure 2.4. MDVUL49.5 reduces MHC class I on COS-kb cells regardless of whether mature peptide is provided in the cytosol or endoplasmic reticulum. COS-kb cells were transiently co-transfected with MDVUL49.5 and either pUG-1-S8L (SIINFEKL as a ubiquitin-fusion protein) or pTracer-CMV-2-ssAS8L (SIINFEKL with a signal sequence). Controls are SIINFEKL-expressing constructs alone. Samples were stained with 25:D1:16 and analyzed by flow cytometry. Data are shown as geometric mean fluorescent intensity (arbitrary scale). Results of 1 experiment are shown.

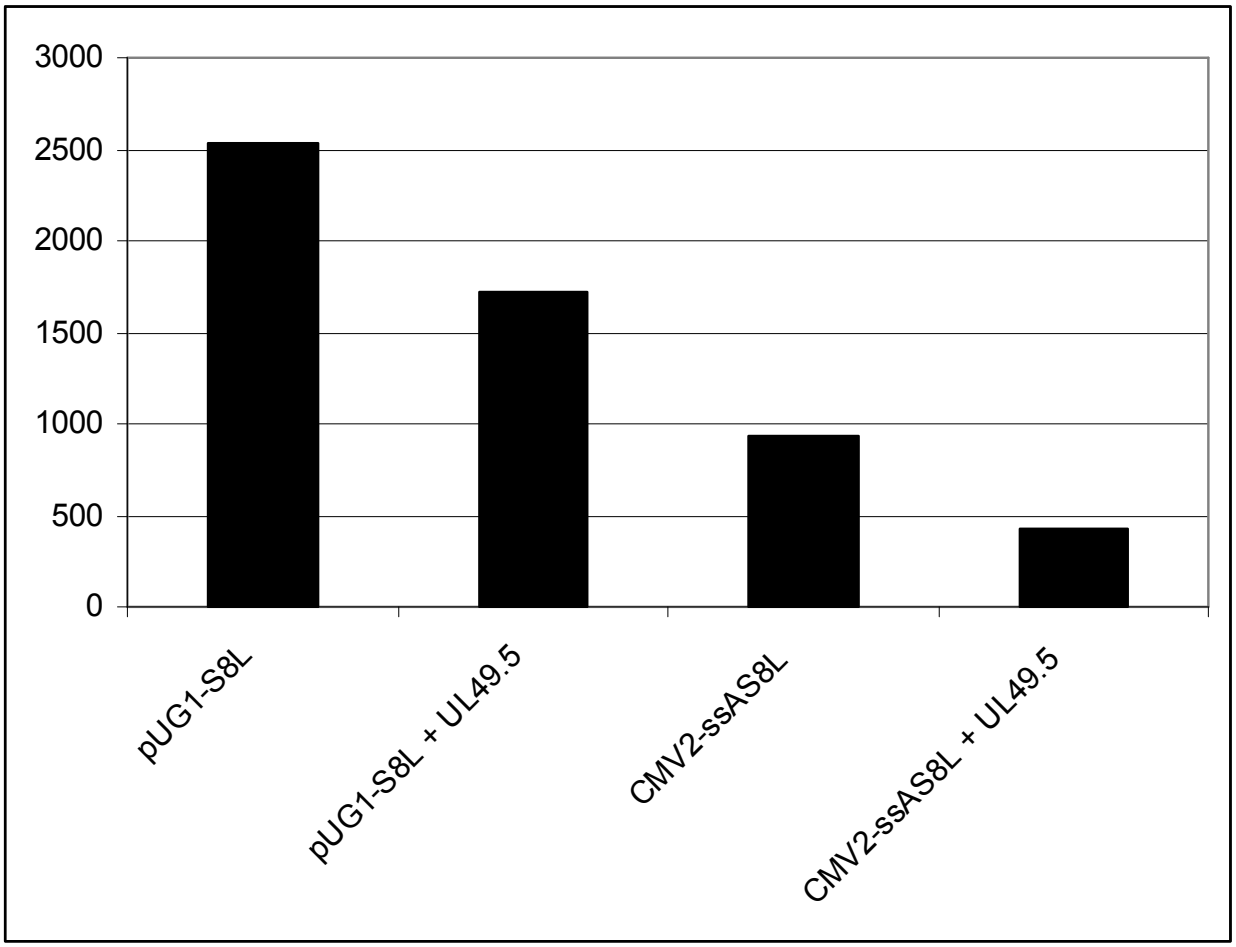


Figure 2.5. MDVUL49.5 reduces GFP expression in COS-Kb cells. COS-Kb cells were co-transfected with MDVUL49.5 and several GFP-expressing constructs (as shown in Figure 4). GFP expression was analyzed by flow cytometry. Sample data (dark bars) are shown as geometric mean fluorescent intensity of GFP normalized to controls for each sample (GFP-expressing plasmid alone; white bars). Results of 1 experiment are shown.

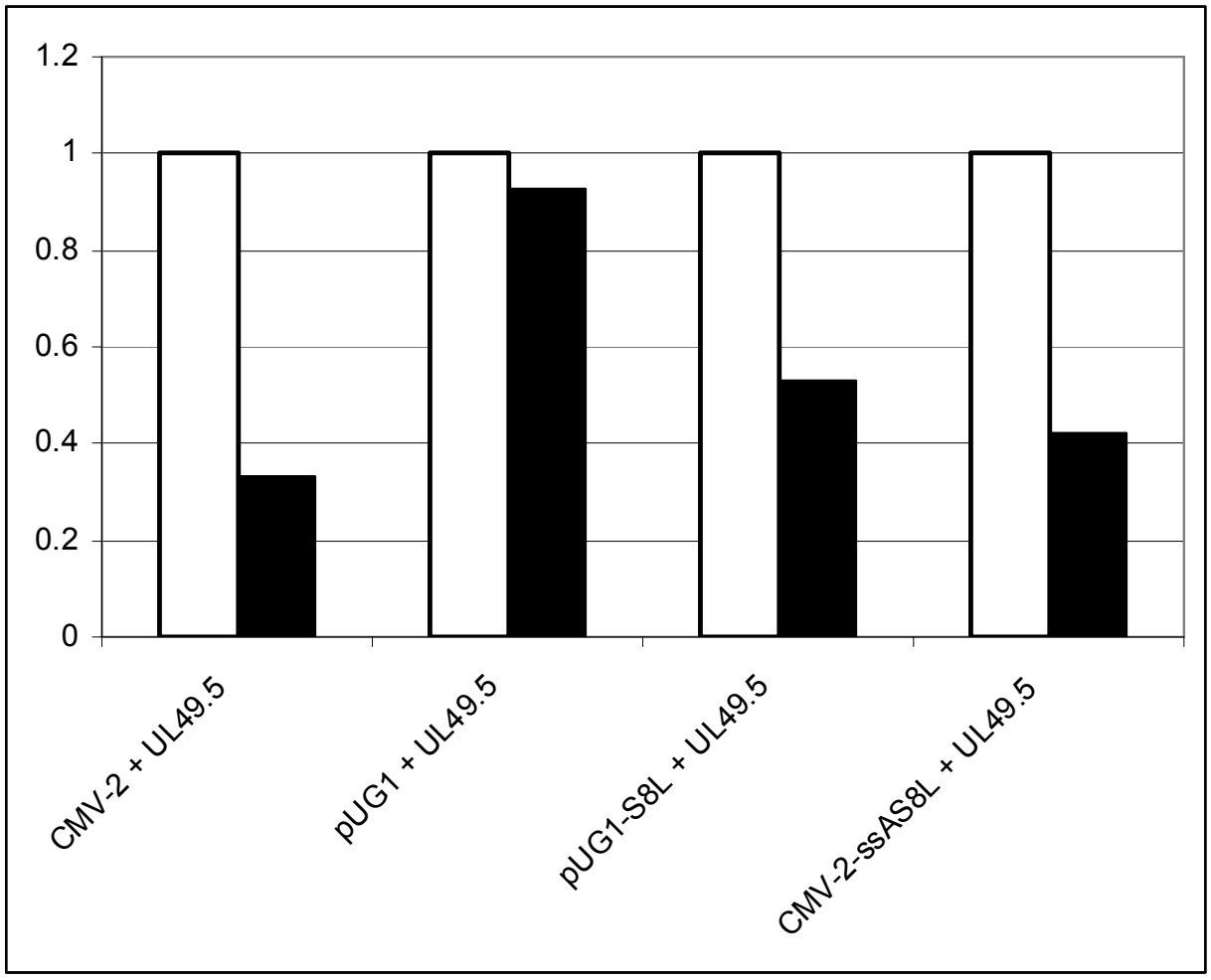


Figure 2.6. MDV012s decreases MHC class I expression in DF-1 cells more than other tested MDV genes. Twelve of the thirteen MDV genes transfected into DF-1 cells are shown in a representative experiment, with geometric mean fluorescence intensity (arbitrary units) measuring the intensity of staining for MHC class I. MDV011, MDV012, and especially MDV012s (noted here as MDV012b) consistently decreased MHC class I expression.

For interpretation of the references to color in this and all other figures in this document, the reader is referred to the electronic version of this thesis.

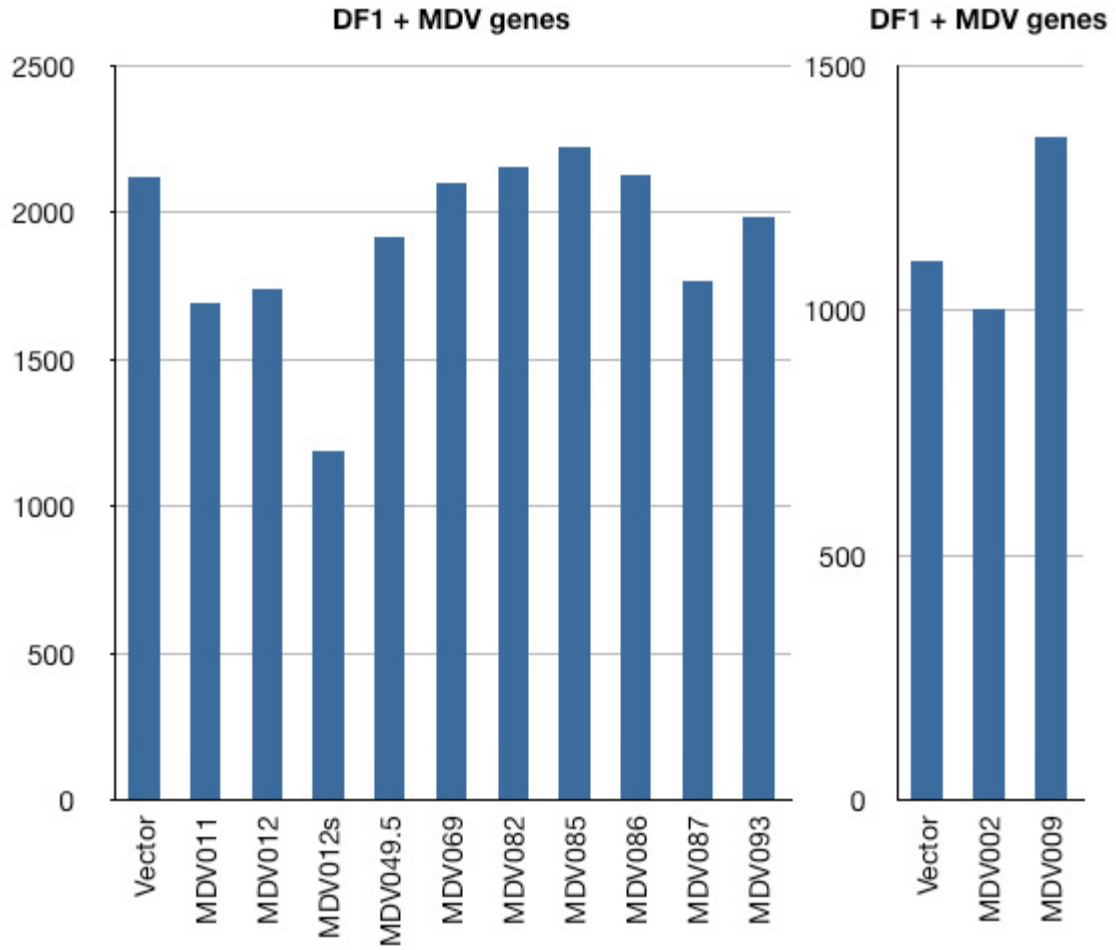


Figure 2.7. Neither MDV087 nor MDV007 have a significant effect MHC class I expression.

2.7.1. MDV087

LMH cells were transfected with pTracer-CMV-2 and pTracer-CMV2-MDV087. One experiment is shown.

2.7.1.a. Live gate. Live cells were gated on GFP ("FITC-A") at <0.5% in the untransfected control (back-gating is shown here to display the full live populations on GFP versus C6B12 ("APC-A"); gates were originally set on a GFP histogram as in Figure 7B).

2.7.1.a.i. Untransfected:

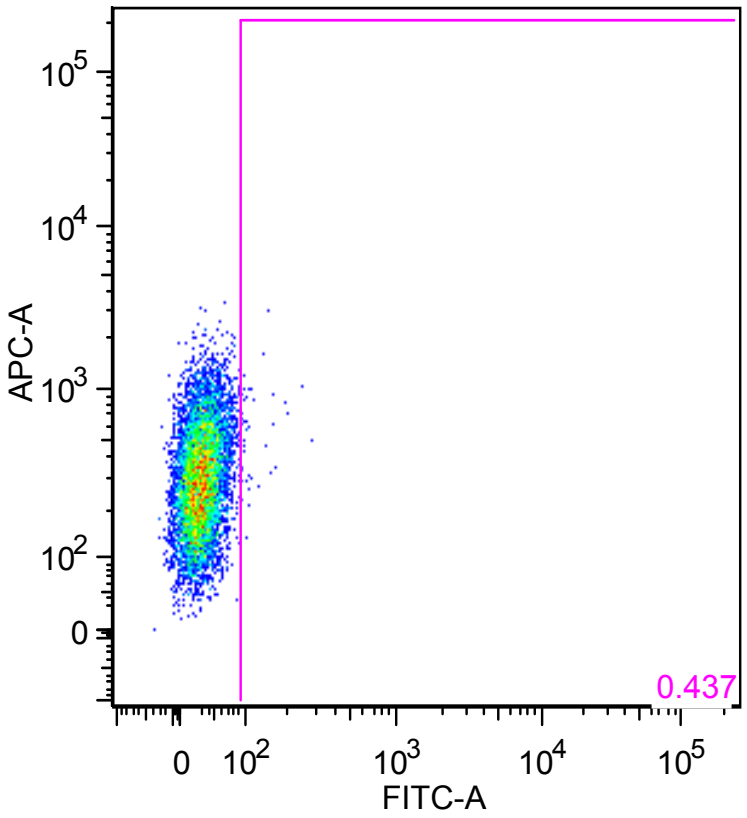
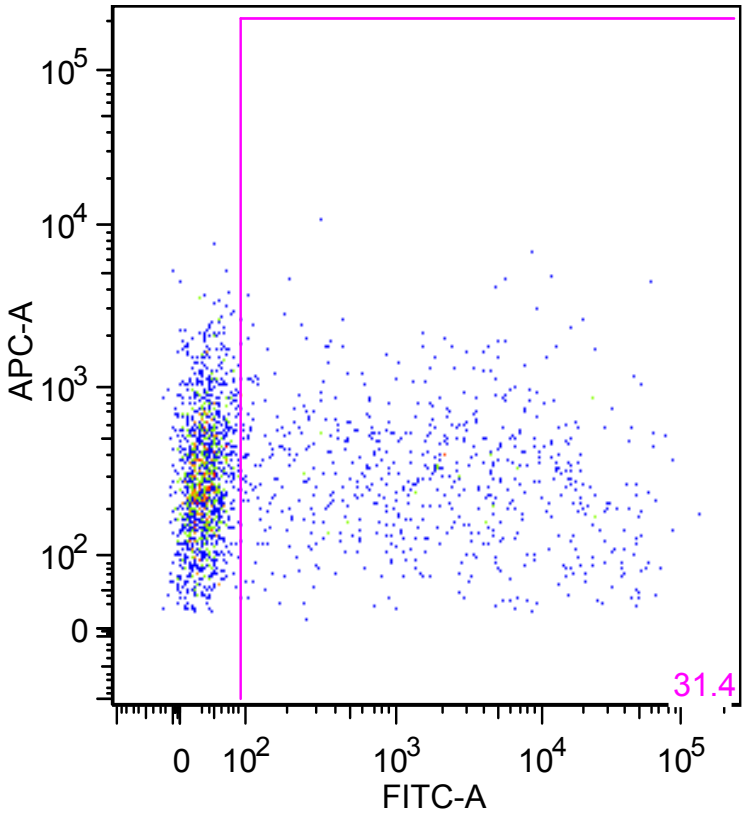


Figure 2.7. (cont'd)

2.7.1.a.ii. pTracer-CMV2:



2.7.1.a.iii. pTracer-CMV2-MDV087:

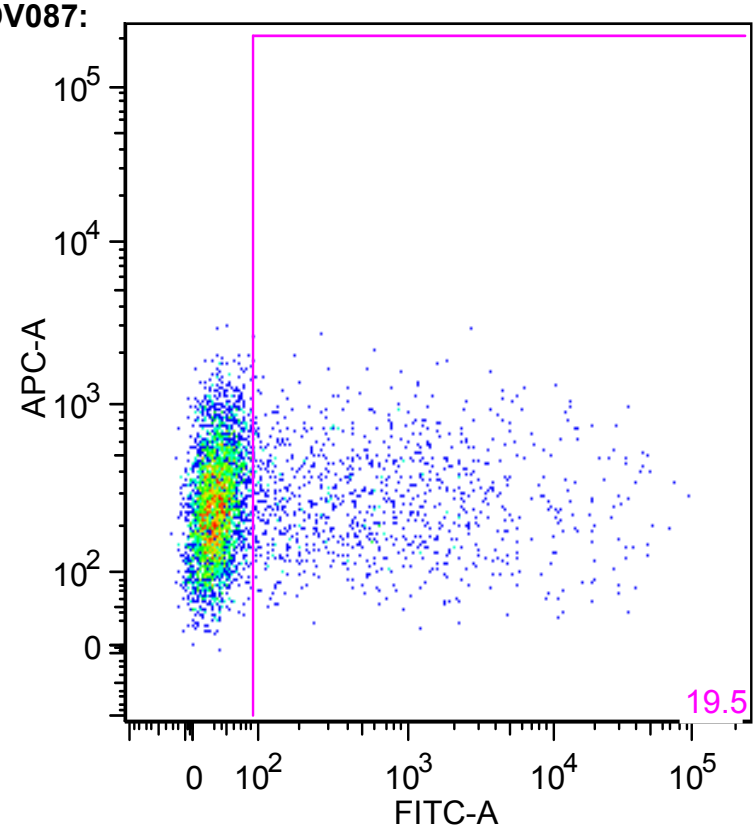


Figure 2.7. (cont'd)

2.7.1.b. MHC class I (histogram). Staining for MHC class I with C6B12 was compared between pTracer-CMV2 and pTracer-CMV2-MDV087 and is displayed as an overlay histogram of each GFP+ population as percent of maximum (right).

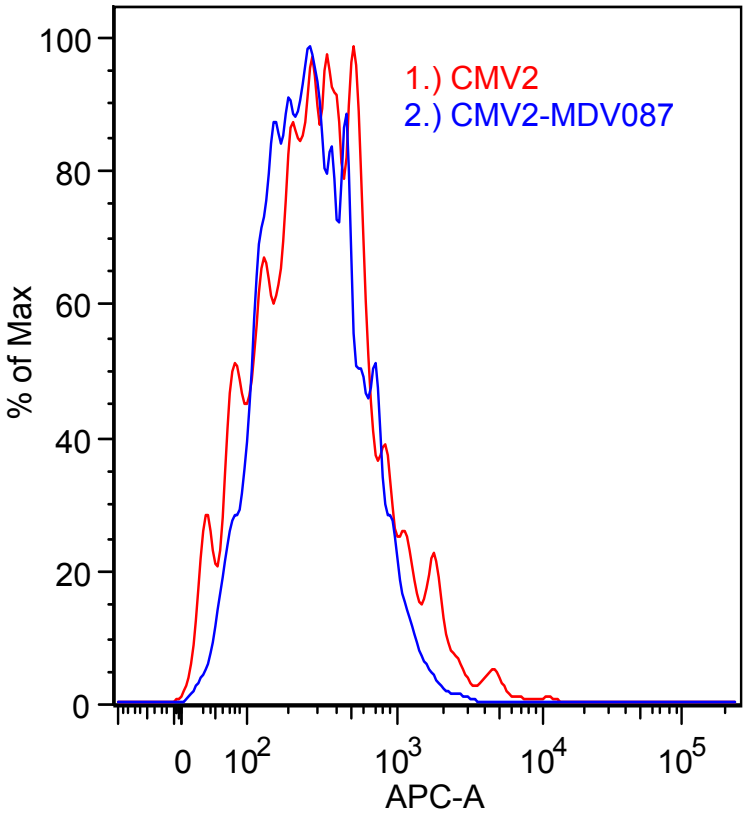


Figure 2.7. (cont'd)

2.7.1.c. MHC class I (bar graph). The geometric mean fluorescence intensity of C6B12 staining in the pTracer-CMV2-MDV087 sample was 91% that of pTracer-CMV2.

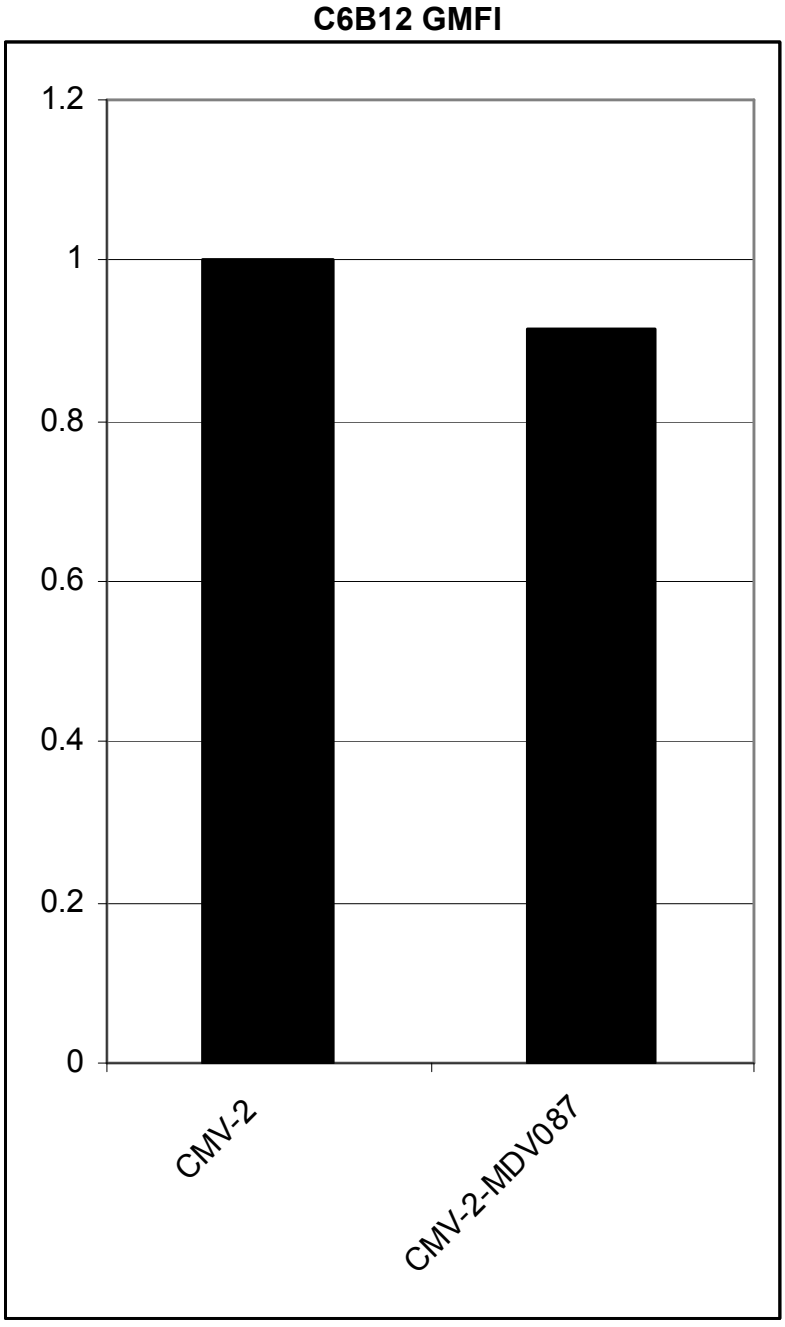


Figure 2.7. (cont'd)

2.7.2. MDV007

DF-1 cells were transfected with pTracer-CMV2 and pTracer-CMV2-MDV007, harvested after 2 days and stained with C6B12. One experiment is shown.

2.7.2.a. Live gate. Live cells were gated on GFP ("FL1") at <0.5% in the untransfected control.

2.7.2.a.i. Untransfected:

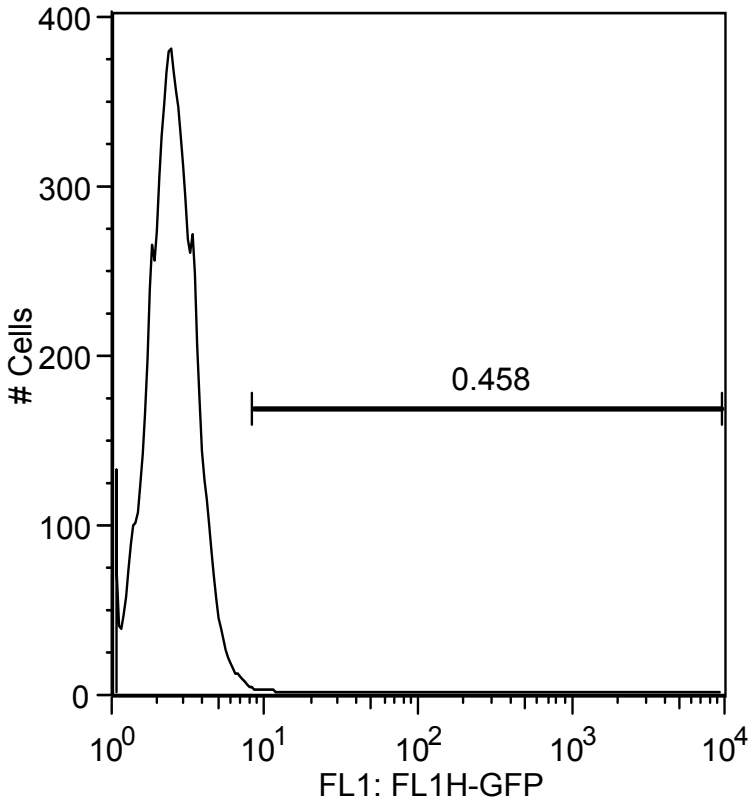
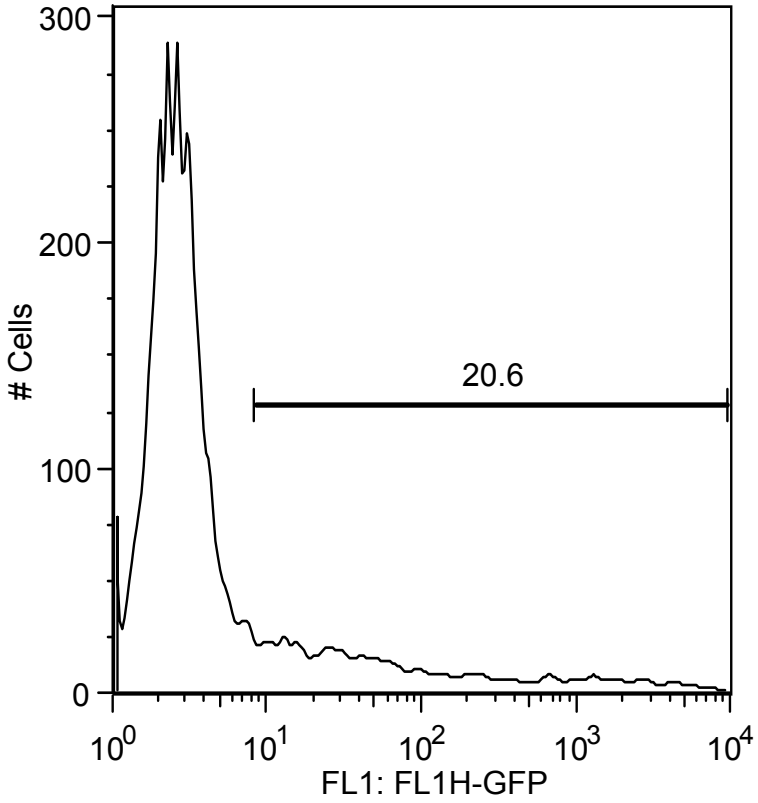


Figure 2.7. (cont'd)

2.7.2.a.ii. pTracer-CMV2:



2.7.2.a.iii. pTracer-CMV2-MDV007:

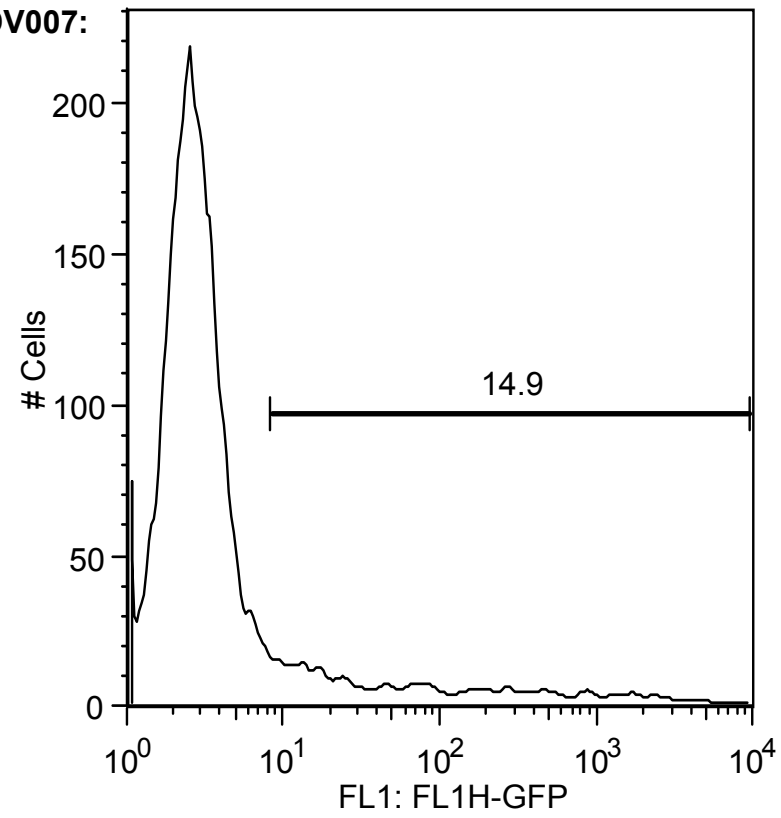


Figure 2.7. (cont'd)

2.7.2.b. MHC class I (histogram). Staining for MHC class I (C6B12 with secondary Cy5) was compared between pTracer-CMV2 and pTracer-CMV2-MDV087 and is displayed as an overlay histogram of each GFP+ gated population as percent of maximum.

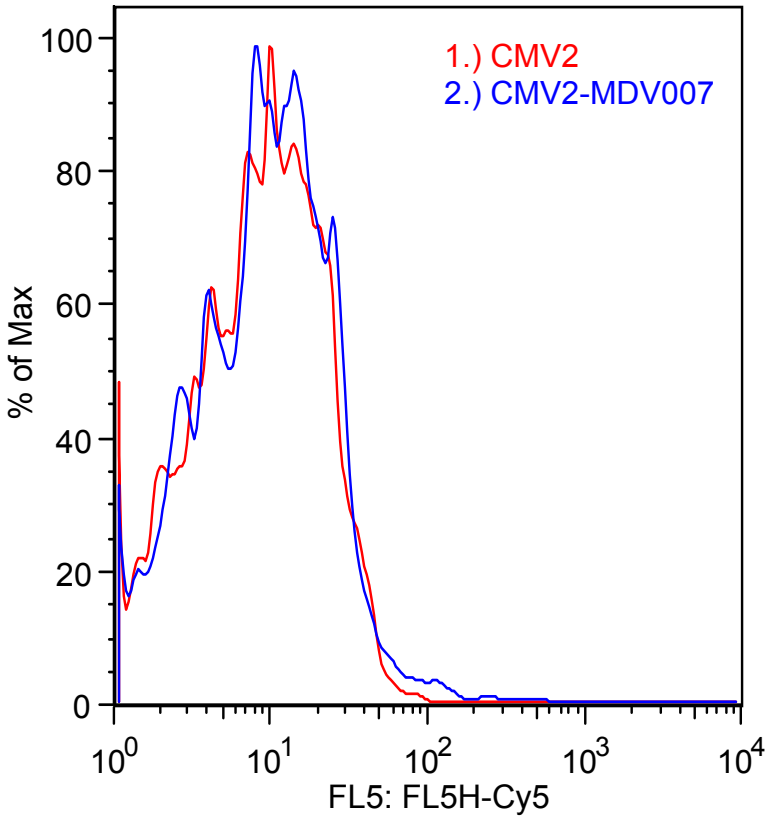


Figure 2.7. (cont'd)

2.7.2.c. MHC class I (bar graph). The geometric mean fluorescence intensity of C6B12 in the pTracer-CMV2-MDV007 sample was 110% that of CMV2 (left).

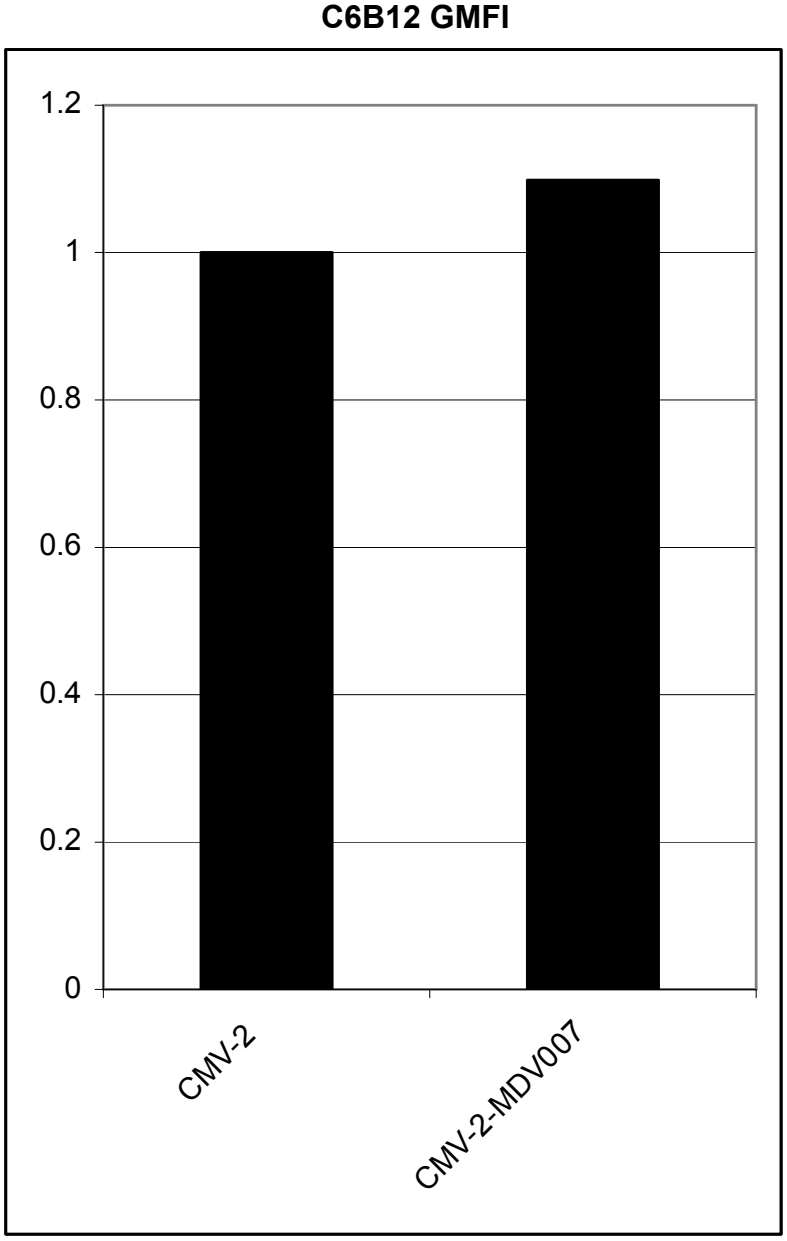


Figure 2.8. MDV012 reduces expression of H-2Kb, but not of influenza HA, in chicken cells. DF-1 cells were transfected with MDV012s (thick lines) and co-transfected with single-chain H-2Kb (SC-Kb) or influenza hemagglutinin (HA). After two days, cells were stained with A.) anti-major to confirm the effect of MDV012, B.) B8.24.3 to test H-2Kb expression, or C.) H36.5-4.2 to test HA expression. Thin lines represent controls not transfected with MDV012s.

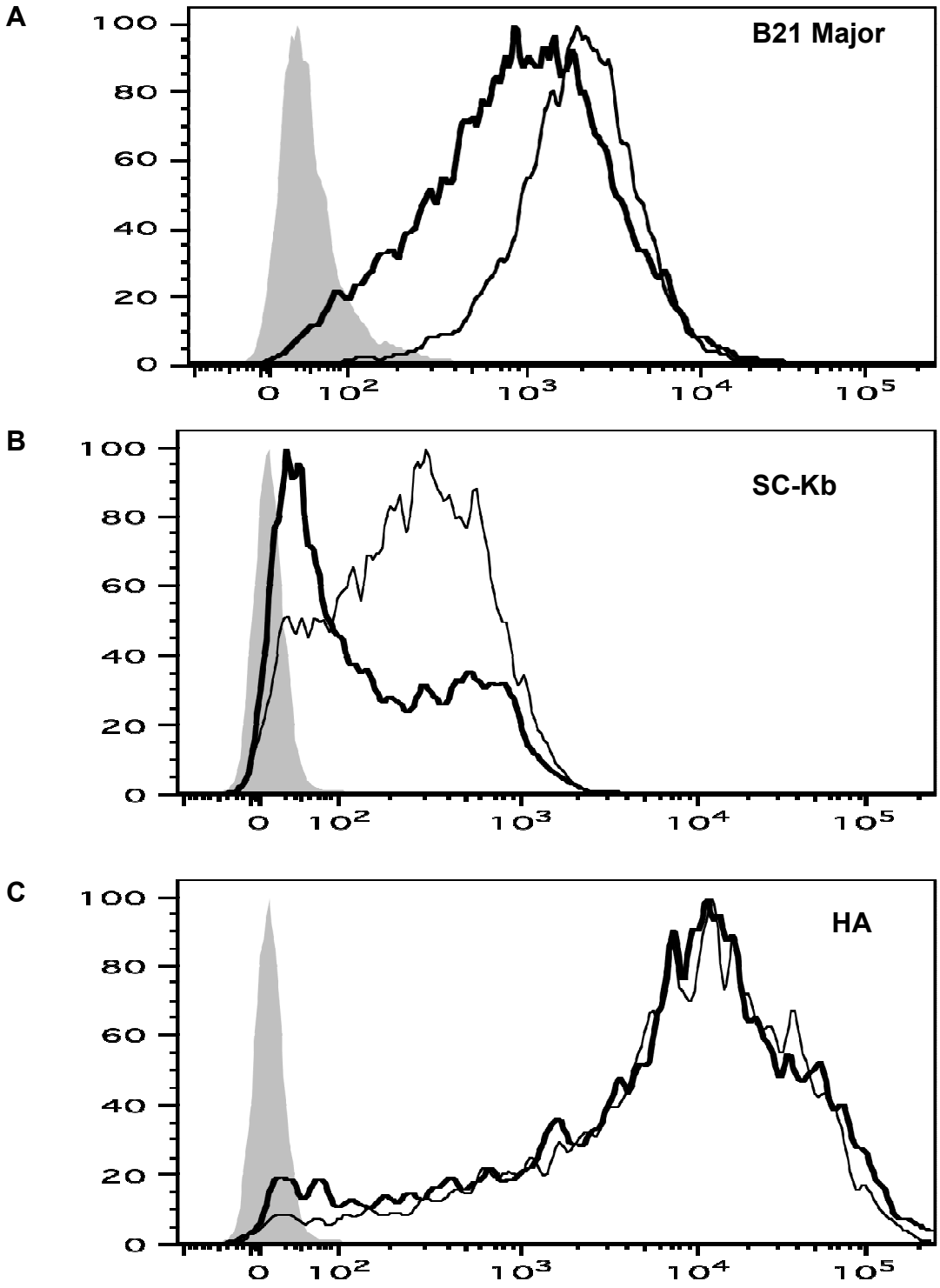


Figure 2.9. MHC class I down-regulation by MDV012s is rescued by ER-targeted MHC class I-binding peptide. Co-transfection of MDV012s with signal-sequence-linked R10V peptide in RFP vector elevated MHC class I expression to a level similar to transfection with the peptide alone in one experiment. MDV012s decreased MHC class I expression when co-transfected with the empty RFP vector. Transfections were performed in DF-1 cells, which were harvested and stained with C6B12 at 2 days. Data are shown as geometric mean fluorescence intensity of C6B12 on an arbitrary scale.

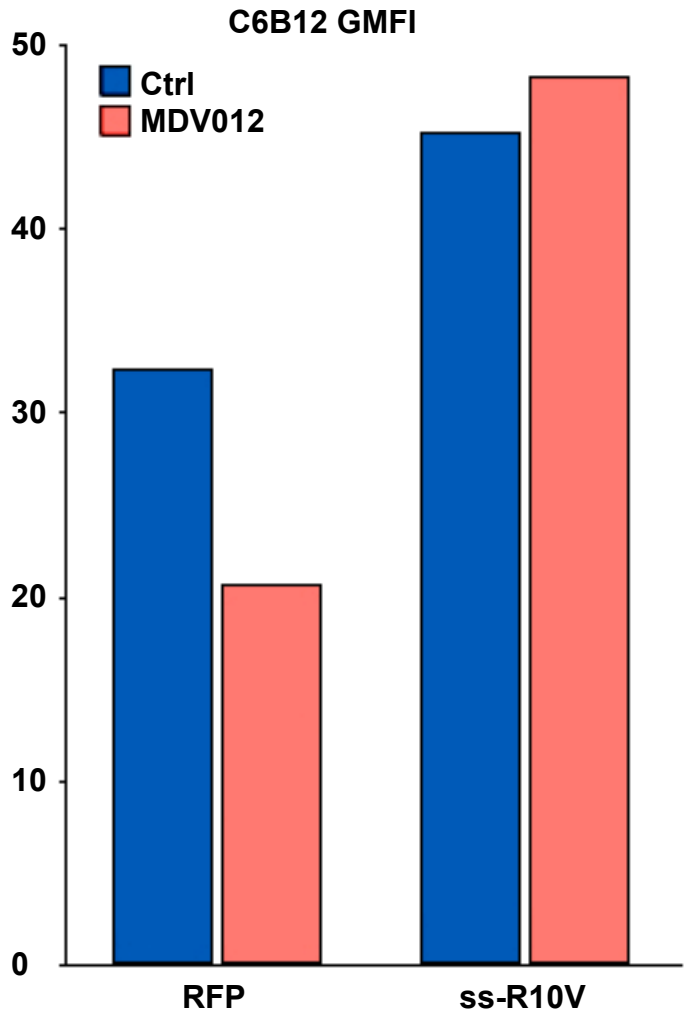
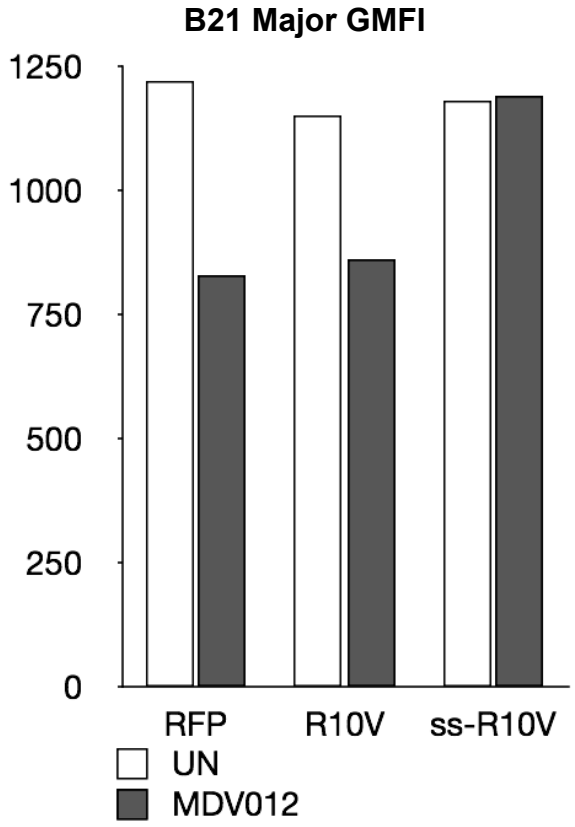


Figure 2.10. ER-targeted B21-binding peptide, but not cytosolic peptide, rescued MHC class I expression in the presence of MDV012. DF-1 cells were co-transfected with MDV012 (white bars) or control plasmid (dark bars) along with vector (RFP), R10V peptide as a ubiquitin-fusion protein (R10V), or R10V peptide with an ER-targeting signal sequence (ss-R10V). Cells were harvested at 2 days, stained with anti-major B21 chicken serum and analyzed by flow cytometry.



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CHAPTER 3

Creation of MDV012 start-codon mutants of a virulent MDV BAC and subsequent rescue of infectious virus.

3.1. Abstract:

Recent developments in molecular biological technology have facilitated the development of engineered herpesviral strains through selective mutation of large DNA constructs such as bacterial artificial chromosomes. The application of these advancements are expected to benefit rational vaccine development, as knock-out viruses are developed to assay the effects of specific genes on immunogenicity and pathogenesis. Marek's disease virus (MDV) presents an important challenge to vaccine development, as the use of highly effective but non-sterilizing vaccines is believed to drive the continued evolution of new, increasingly virulent strains. More information about MDV's interaction with and evasion of the chicken host immune system will be needed to continue producing vaccines effective against newly emerging strains. In this study, novel MDV viruses based on the virulent Md5 strain were developed which encode a mutated start codon for the MHC class I evasion gene MDV012, using two-step Red-mediated recombination of a bacterial artificial chromosome. Infectious viruses were subsequently rescued and passaged in tissue culture. These viruses may be useful for further characterization of the MDV012 gene, and viruses produced similarly could also prove useful as MHC class I evasin knock-out vaccine strains.

3.2. Introduction:

Historically, the development of novel attenuated virus strains for vaccination purposes has been accomplished through serial passage of virus in culture, a process which removes the selection pressures for virulence present in the original host and allows for relatively rapid loss of virulence factors and adaptation to a non-host environment. This has allowed the development of avirulent vaccine strains that cause none or only mild symptoms of disease, while eliciting a more complete immune response than inactivated or subunit viruses due to viral multiplication, presentation of intracellular antigen, and the presence of a variety of antigenic epitopes which stimulates a Th1 response, enhancing cellular immunity (Tizard 8th ed.). While this method has been very effective at developing vaccines to control many important human and animal diseases, attenuation by passage is an essentially un-targeted approach and therefore cannot easily be used to create vaccine strains that have specific functional changes to improve their safety or immunogenicity. While classically attenuated vaccine viruses are likely to have random mutations in nonessential genes, including virulence and immune evasion factors, they may also have changes in immunogenicity, such as loss of specific epitopes or replication defects that interfere with effective immune presentation. A more systematic approach would involve designing vaccine viruses that lacked the most important virulence factors, while maintaining high immunogenicity in vivo.

Herpesviruses cause a number of important diseases in humans and animals and present a unique challenge to vaccine development. These viruses have very large (125-245 kb) genomes which allow them to encode a variety of nonessential genes that enhance their infectivity and survival (Davison 2010). These genes can have functions in inducing latency, as specific virulence factors, or in evading the immune response (e.g. Fowler 2003, Ambagala 2010, Jones

1992, Lupiani 2004, Neidhart 1987, Dingwell 1995, Lubinski 2011, Parcells 2001). Live attenuated vaccine strains used to control herpesvirus infections have been unsuccessful at developing sterilizing immunity to natural infections. This allows continued circulation of wild-type viruses, and places strong selection pressures on the development of more virulent strains with higher replicative and transmission potential (Ganden 2001). This is complicated by the fact that vaccine viruses themselves may be transmitted between hosts or establish latent infection similarly to wild-type viruses. They can undergo recombination events which can interfere with testing attempts to distinguish vaccinated hosts from naturally infected hosts, as well as contribute to the diversity available within the circulating viral population (Muylkens 2006, Del Medico Zajac 2011).

Marek's Disease Virus (MDV), an alpha-herpesvirus of chickens with economic importance in the poultry industry, is a relevant example of the limitations of classically attenuated vaccines in controlling herpesviral infections. While this type of vaccine has been used very successfully in industry to prevent overt disease due to MDV, sterilizing immunity has proven unattainable and ubiquitously circulating viruses are still able to cause infection, including latent infection. Interestingly, the selection pressures posed by vaccine-induced non-sterilizing immunity has led to an overall increase in the virulence of circulating MDV strains, and new vaccines have been required about every two decades to meet this challenge. These vaccine strains have themselves needed to be less attenuated (more virulent) in order to provide immunity sufficient to prevent clinical disease (Witter 1997; Witter 1998; reviewed in Nair 2005).

Early methods of herpesviral mutagenesis were based on recombination techniques in viral culture, such as plasmid-based insertional mutagenesis, and required the use of selection

markers and plaque purification to isolate viral recombinants (Spaete 1987; Post 1981). Recently, methods of directly manipulating the large herpesviral genomes have been developed. Sets of overlapping cosmid clones have been designed for a number of herpesviruses, allowing identification and mutation of discrete genome fragments as well as rescue of intact virus (e.g. Arrand 1981; Van Zijl 1988; Craig 1990; Cohen 1993 Reddy 2002). A limitation of this approach is that reconstitution of the complete virus genome requires multiple recombination events between cosmids in cell culture and successful viral replication to occur.

More recently, bacterial artificial chromosomes (BACs) have also been constructed that contain an entire herpesviral genome in a single plasmid which can be manipulated with molecular techniques in E. coli and validated for genome integrity prior to transfection (Messerle 1997; Schumacher 2000; Zhou 2002; Rudolph 2002; Mahony 2002; Tanaka 2003; Costes 2006; Baigent 2006; Dewals 2006; Arii 2006). Furthermore, BACs have been designed with loxP sites flanking the vector sequences to allow excision of non-viral sequences (Wagner 1999; Adler 2000; Adler 2001). Herpesviral BACs have facilitated the design of novel viruses with targeted mutations, such as recombinant knock-outs of specific genes to test the functions of those genes. Novel recombinant strains of a number of herpesviruses, created either with BAC recombination or with older techniques, have been tested for vaccine efficacy and have shown promise in regard to improved safety or immunogenicity available through targeted mutagenesis (Kruger 1996; Tibbetts 2003; Fowler 2004; Cui 2005; Kayhan 2007; Crumpler 2009; Jia 2010; Lee 2010). BAC recombination techniques can help facilitate the development of these types of novel vaccine candidates.

We have identified an immune evasion gene in MDV which is a potential candidate for recombinant vaccine design. This gene, MDV012, appears at least partially responsible for the

down-regulation of MHC class I in MDV-infected cells, which is expected to interfere with effective antigen presentation and an effective T-cell response to infection (Hunt 2001). We anticipate that viruses lacking the ability to interfere with MHC class I antigen presentation may be attenuated while potentially also significantly more immunogenic. Evidence in favor of this approach exists for other herpesviral MHC class I evasins; deletion mutants of HSV-1 and HSV-2 viral host shutoff protein are were more immunogenic and protective than inactivated wild-type virus (Walker 1998) or background strains (Dudek 2008) in mouse challenge models, while a replication-defective, multiple immediate-early gene deletion mutant of HSV-1 incorporating an ICP47 deletion was a more immunogenic vaccine vector than a replication-defective single-gene (ICP8) deletion mutant (Watanabe 2007). Therefore, we suspect that vaccine viruses which incorporate deletion of MDV012 function might provide better immunogenicity without a corresponding increase in MDV vaccine strain virulence.

In this study, we have examined one method of creating a potential MDV012 knock-out, using two-step Red-mediated BAC recombination ("recombineering") (Tischer 2006) to incorporate a point-mutation in the start-codon of MDV012 into a virulent strain of MDV, the Md5 BAC strain, with subsequent viral rescue in tissue culture. The Red recombinase enzyme system mediates recombination at double-stranded breaks (or oligo ends); the two-step Red-mediated recombination technique uses two such events to insert and remove a selectable marker, leaving behind a scarless mutation site (Warming 2005; Tischer 2006). With this method, it should be possible to interrupt the function of a gene such as MDV012 with minimal or no impact on the expression and function of other MDV genes, thus providing the most information possible about the function of MDV012 and the effects of its deletion on MDV infection and vaccination.

3.3. Materials and methods:

3.3.1. Cells and viruses:

Secondary duck embryo fibroblasts (DEFs; cultured once post isolation) were obtained from USDA-ARS ADOL, and cultured in 50% Leibowitz/50% McCoy's medium supplemented with penicillin/streptomycin and 1% GlutaMAX (Invitrogen, Carlsbad, CA) ("LM medium"), with 5% heat-inactivated FBS for expanding cultures ("4% hiFBS medium") and 1% for confluent cultures (1% hiFBS medium"). The MDV strain used for recombineering was the Md5B40BAC strain (GenBank # HQ149525.1; Niikura 2011). SW105 cells containing Md5B40BAC (hereafter abbreviated as "Md5"), were obtained from Dr. Robert Silva, USDA-ARS ADOL, East Lansing, MI. The SW105 cells are a DH10B-based strain, with a defective lamda prophage expressing Red recombinase enzymes exo, bet and gam under the control of a temperature-dependent promoter; SW105 cells also express the gal operon except for galK, and express flp*e* under an arabinose-induced promoter (Lee 2001; Warming 2005). Neither galK selection or flp-frt recombination were used in this study. The SW105 cells and Md5 BAC were used for BAC recombineering. The Md11BAC strain of MDV (GenBank # AY510475; Niikura et al, 2006), also obtained from Dr. Robert Silva, was used as a negative screening PCR control. FHV-1BACΔUL49.5kan in SW105 cells, an intermediate 2-step recombineering product developed in the York Lab from an FHV-1BAC strain obtained from Dr. Roger Maes, MSU, East Lansing MI, was used as a positive screening PCR control.

3.3.2. Recombineering:

MDV012 start-codon mutants were created using two-step Red-mediated BAC recombination ("BAC recombineering") using methods similar to those described in Tischer, et.

al, 2006. A PCR product for point A>C mutagenesis at the MDV012 start codon was prepared using Phusion High-Fidelity DNA Polymerase (Finnzyme/Thermo Scientific). A pair of primers were designed containing overlapping regions around the MDV012 start site, as well as regions flanking the kanamycin resistance gene aphAI of the pEPkan-S plasmid (Tischer 2006) and overlapping its upstream I-SceI site (see Figure 3.1. and Table 3.1.). Phusion PCR using pEPkan-S as template was performed with these primers at 69 degrees C, and the product was digested with DpnI to cleave the Dam-methylated pEPkan-S template, then purified with the Qiagen Gel-extraction Kit (Qiagen, Valencia, CA).

SW105 cells containing the Md5 BAC were thawed, made recombinase- and electrocompetent and electroporated with the PCR product as follows. Unless otherwise specified, all incubations in media or on plates were performed between 30 and 32 degrees C to prevent induction of recombinase activity. A 3 ml Luria-Bertani medium (LB)/chloramphenicol (30 ug/ml) overnight culture was grown, then subcultured 1:12 and grown to an A600 optical density of 0.55. The culture was heat-shocked by shaking in a 42 degree water bath at 200 RPM for 18 minutes to induce the Red recombinase system, shaken in an ice-bath for 30 minutes, then spun down at ~1800 rcf at 4 degrees C for 5 minutes. The pellet was washed 2 times with 10 ml's ice-cold water and spun again each time, then washed with 1 ml ice-cold water and microcentrifuged at ~16100 rcf for 1 minute. The resulting 50 ul pellet of cells was electroporated at 1.4 kV, 25 uF, and 200Ω in a 0.1 cm cuvette (BioRad) for about 6 ms with 100 ng of PCR product. The cells were then incubated in 1 mL LB/chloramphenicol media for 90 minutes, and plated on LB/chloramphenicol/kanamycin (30 ug/ml each antibiotic) at 100 uL on 1 plate and 900 uL on a second plate, incubating for 2 days.

Colonies of putative SW105-Md5-PCR were screened onto fresh LB/chloramphenicol/kanamycin plates. Positive colonies were screened with Taq PCR using a forward primer internal to the MDV012 sequence and a reverse primer internal to the aphAI kanamycin resistance cassette (MDV012mutF1 and MDV012mutR1; see Table 3.1.). A colony of SW105-Md5-PCR, which was positive for the kanamycin gene by both plate screening and PCR screening, was subcultured in 3 mL of LB/chloramphenicol/ampicillin overnight for an electroporation protocol the following day with the pBad-SceI plasmid (expressing arabinose-inducible SceI restriction enzyme; Tischer 2006), performed similarly to the first electroporation, which proved unsuccessful. The remaining overnight culture of SW105-Md5-PCR, which was stored for less than 1 week at 4 degrees C, was subcultured again at about 0.5 mL per 12 mL sample to an A600 optical density of 0.65-0.75, and made recombinase- and electrocompetent via heat shocking, incubation on ice, and washing similarly to the first electroporation with the PCR product (induction of recombinase at this step was unnecessary but did not prevent successful transformation in this case). Following the two 10-mL washes with ice-cold water, the samples were resuspended in 1 mL of 10% glycerol in water and frozen at -80 degrees C. 50 uL of thawed, recombinase- and electrocompetent SW105-Md5-PCR cells was electroporated with 100 ug of pBad-SceI plasmid (obtained from Dr. Robert Silva at USDA-ARS ADOL), similarly to the first electroporation. The electroporated cells were shaken by hand at room temperature in 1 mL of LB media for 20 minutes, then at 200 RPM at 31 degrees C for 45 minutes and plated on LB/chloramphenicol/kanamycin/ampicillin (30, 30, and 100 ug/ml) plates at 100 uL on 1 plate and 900 uL on a second plate. Plates were incubated for 2 days at 31 degrees C.

A positive colony on the SW105-Md5-PCR/pBad-SceI plates was cultured overnight in LB/chloramphenicol/ampicillin and subcultured 100 ul into 2 mls of the same media. At 3 hours, 2 mls of the same media with 1% L-arabinose was added and the culture was further incubated for 1 hour, in order to induce SceI expression and restriction of the BAC. It was then heat shocked by shaking in a 42 degree C water bath for 30 minutes to induce recombination. Finally the culture was incubated at 31 degrees C for 2 hours and plated at 1:1, 1:10, and 1:100 dilutions on LB/chloramphenicol/ampicillin/1% arabinose and incubated for 26 hours. In order to obtain separate colonies, subcultures from the 1:1 and 1:100 plates were re-streaked on LB/chloramphenicol/ampicillin and grown for 2 days. Colonies were screened onto matched LB/chloramphenicol/ampicillin and LB/chloramphenicol/kanamycin/ampicillin plates, and colonies positive only on kanamycin-negative plates were selected, subcultured and frozen at –80 degrees C as putative MDV012ctg recombinants.

3.3.3. Screening and sequencing:

Taq PCR screening was performed on frozen putative SW105-Md5-MDV012ctg/pBad-SceI cultures using the MDV012mutF1 and R1 primers for aphAI kanamycin resistance cassette amplification. Recombinants that screened negative for aphAI were subsequently screened by Taq PCR using primers flanking the MDV012 gene, and the MDV012 gene from 3 recombinants were sequenced using flanking and internal sequencing primers (Table 3.1.).

3.3.4. BAC DNA preparation and restriction digests:

BAC DNA was prepared from SW105-Md5-MDV012ctg/pBad-SceI clones and SW105-Md5/pBad-SceI using the PSIΨClone BAC DNA kit (Princeton Separations, Freehold, New

Jersey). For transfection and viral rescue, "dirty" preps were considered sufficient, eliminating the column binding steps as any remaining pBad-SceI plasmid should not be sustained through viral passages in non-immortalized cells. 5 mL overnight cultures in LB/chloramphenicol were centrifuged for 10 minutes at ~2000 rcf**.** The pellets were resuspended, lysed and neutralized with 300 uL of each of respective buffers 1-3, with gentle mixing and incubation for 10 minutes on ice for lysis and neutralization reactions. The samples were microcentrifuged at 16100 rcf for 10 minutes, and the pellets were precipitated in 1 mL isopropanol overnight at 4 degrees C. The samples were re-pelleted for 30 minutes at 16100 rcf, washed with 200 uL of 70% ethanol with microcentrifugation at 16100 rcf for 5 minutes, air dried and resuspended in 40-50 uL of TE buffer.

In order to obtain pure BAC DNA for restriction analysis, Md5-MDV012ctg clones were first electroporated into DH10B cells using BAC DNA from "dirty" preps. The BAC DNA was diluted 1:100 in TE buffer, then 25 uL of competent DH10B cells were electroporated with 1 uL of the BAC dilution at approximately 2 kV. The cells were incubated in 1 mL of LB medium for 1 hour at 37 degrees C and 250 RPM, then plated overnight on LB/chloramphenicol at 37 degrees C. Colonies were screened onto LB/carbenicillin to identify clones that lacked the pBad-SceI plasmid. These colonies were subcultured and frozen at -80 degrees C.

"Clean" BAC preps were obtained using the full PSIΨClone kit protocol, with the following specifics. Overnight 3 mL cultures of DH10B-Md5 or DH10B-MDV012ctg were grown in LB/chloramphenicol from plated colonies. Centrifuged cultures were resuspended, lysed and neutralized using 500 uL of each of buffers 1-3. Following the 10 minute microcentrifugation after neutralization, the samples were moved to 15 mL tubes and 1 mL of sterile water was added for dilution. The samples were then bound to prepared columns and

washed twice with 1 mL of wash buffer. Columns were dried by centrifugation at ~120 rcf for 2 minutes and eluted with 50 or 100 uL of elution buffer via centrifugation at ~120 rcf for 2 minutes. An equal 50 or 100 uL volume of isopropanol was added to precipitate the pellets overnight at 4 degrees C, and subsequent steps were the same as for "dirty" preps.

Restriction digests were performed to verify that BAC DNA was intact with no visible rearrangements. 0.5 uL EcoRI-HF **(**New England Biolabs, Ipswich, MD) was used in 25 uL reaction volumes with 1X Buffer 4 and 16 uL of "clean" BAC DNA. Reactions were performed at 37 degrees C overnight, and subsequently 10 uL of each sample was electrophoresed at low voltage (0.8-1.3V/cm) at 4 degrees C on a 1% agarose gel in TBE buffer for 18 hours. The resulting restriction fingerprints were analyzed by ethidium bromide staining and U/V visualization

3.3.5. Virus rescue and passaging:

Transfection: Secondary DEF cells were cultured in 60 mm plates at about 1.5x10^6 cells per plate for 24 hours in 5 mL's of medium (as described above). The plates were re-fed with 4 mL's each of MEM with 5% hiFBS and incubated for 30 minutes. They were then transfected using a calcium-phosphate precipitation transfection method (Graham and Van der Eb 1973). Approximately 1 ug of BAC DNA was added to 250 uL of sterile, deionized water, followed by 250 uL of filtered 0.5M CaCl and mixed. Then, 500 uL of filtered 2X Hepes-Buffered-Saline was added slowly while mixing gently to encourage DNA-calcium-phosphate complex formation. The mixture was incubated for 15-20 minutes and 0.5 mL was added to each of 2 separate plates of DEFs. The transfected DEFs were incubated at 41 degrees for 3-4 hours. Then media was removed, and the cells were shocked with 2 mL of 15% glycerol in

deionized water per plate for 2 minutes. The plates were then washed twice with sterile PBS and re-fed with 5 mL of LM medium with 4% hiFBS without antibiotics. Transfected plates were incubated at 41 degrees overnight. After 24 hours the transfected plates were re-fed with 5 mL of LM with 1% hiFBS, supplemented with Pen-Strep.

Passage 1: After another 48 hours, cells were trypsinized, resuspended in 4 mL of LM medium with 4% hiFBS, pelleted at ~200 rcf for 4 minutes **a**nd the pellet resuspended in 3 mL of complete medium. The cells from each plate were seeded onto a 1-day-old 100 mm plate of secondary DEFs, about 4.5x10^6 cells per plate in 10 mL of LM medium with 4% hiFBS and Pen-Strep. The following day the 100 mm plate was re-fed with 10 mL of LM media with 1% hiFBS and Pen-Strep.

Passage 2: 48 hours later, the cells from each of 2 100 mm plates were harvested and centrifuged similarly to the 60 mm plates, and the pellet resuspended in 5 mL of complete medium. The cells from both plates were seeded onto a single 1-day-old 150 mm plate of secondary DEFs, about 1.5x10^7 cells per plate, freshly re-fed in 20 mL of LM medium with 4% hiFCS and Pen-Strep. The cells were again re-fed with 20 mL of LM medium with 1% hiFCS and Pen-Strep the following day.

Passage 3: After 2 more days, the cells were again harvested, centrifuged in 10 mL of LM with 4% hiFBS, and resuspended in 12 mL of LM with 4% hiFBS and Pen-Strep. $1/6^{th}$ of the cells (for heavily-infected wild-type Md5) or $1/3^{rd}$ of the cells (for recombinants) were plated onto each of 3 1-day-old 150 mm plates of freshly re-fed secondary DEFs. The cells were again re-fed with 20 mL of LM with 1% hiFCS and Pen-Strep the following day. After 2 more days (wild-type Md5) or 3 more days (recombinants), cells were harvested and spun, and resuspended in 12 mL (wild-type Md5) or 9 mL (recombinants) of freezing medium (LM with 10% DMSO

and 17% hiFBS). Cells were frozen in 1 mL vials at -70 degrees C for 2 days and stored in liquid nitrogen; thus, 1/4th of a plate was frozen in each 1 mL vial of wild-type Md5, and 1/3rd of a plate in each 1 mL vial of DEF-Md5ctgMDV012 (abbreviated "DEF-Md5Δ12").

Passage 4 for Reverse-Transcriptase PCR: In order to obtain sufficient RNA for extraction, one clone (#32) was re-passaged as follows. 300 uL of thawed passage 3 stock was plated onto each of 3 1-day-old 150 mm plates of secondary DEFs in freshly re-fed DMEM (Gibco) with 10% hiFBS and 10 ug/mL of ciprofloxacin, 30 mL per plate. The following day the plates were re-fed with 30 mL of the same media but with 1% FBS. Two days later each plate was harvested with 3 mL of 0.25% Trypsin-EDTA, resuspended in 10 mL DMEM with 10% hiFBS, and centrifuged for 4 minutes at ~150 rcf. The cells were resuspended in 15 mL (1/5 plate per mL) of freezing medium (DMEM with 18% FBS and 10% DMSO), aliquoted into 1.5 and 1 mL vials, and frozen at -80 degrees C prior to use in RT-PCR.

Further passages: Virus stocks were grown up in 150 mm plates of secondary DEFs and frozen down for each additional passage after passage 3, out to 11 total passages for mutants and wild-type MDV, as described in Table 3.2. In general, 1-day-old plates of just-confluent secondary DEFs were refed in 20-30 mL's of 4% hiFBS. The previous passage was harvested with 3 mL 0.25% Trypsin-EDTA per 150 mm plate, resuspended in 10-12 mL of medium with 4% hiFBS, and passaged onto the new plates at the split ratios recorded in Table 3.2. The remainder was centrifuged at ~200 rcf, resuspended in 10 mL of freezing medium, and frozen in 1 mL vials (for most passages, approximately 1/10 of a plate per vial, except Passages 8-11, which were frozen at ¼ plate per vial). The new passage was generally re-fed the following day in 1% hiFBS, although several final passages were re-fed at lower serum concentrations to slow cell growth and enhance plaque production (see Table 3.2.). Two passages were repeated

(Passages 8 and 9) due to low preliminary titers measured in the frozen stock of the initial Passage 9a. Passage 4, Passage 9a for Md5 and Md5-MDV012ctg #20, Passage 8a for Md5-MDV012ctg #32, and Passage 8 were initiated from frozen stock of the previous numbered passage.

3.3.6. Titrations:

Titers in plaque-forming-units/mL of passage 11 of wild-type Md5 and recombinants in DEF cells were measured as follows. 1-day-old, confluent 60 mm plates of DEF cells were infected with 1 uL, 0.1 uL, 0.01 uL, or 0.001 uL of thawed viral stock per plate in replicates of 3 plates per titration. Plates were re-fed in 0.5% hiFBS medium the following day and again 2 days after that. Plates were fixed at 7 days post-infection, by washing with excess PBS and then fixing with 2 mL of 3 parts acetone/2 parts ethanol for 5 minutes. The fixative was pipetted off and the plates were air-dried. Plates were stored at 4 degrees C until staining.

Titration plates were stained with the H19 antibody, which binds to the pp38, pp24 and pp41 viral antigens, as follows (Silva 1984). The plates were first re-hydrated with 2 mL of PBS per plate for 5 minutes. Then, the plates were stained with 1.5 mL of PBS with H19 at 1 uL/mL for 30 minutes. The plates were then rinsed with excess PBS and de-stained with 2 mL of PBS for 5 minutes. The plates were then counter-stained with 1.5 mL PBS containing 1 uL/500 uL of Alexa-Fluor 488 goat anti-mouse IgG antibody for 30 minutes. Finally, the plates were rinsed again with an excess of PBS and plaques were counted under FITC fluorescent microscopy for plates with discreet plaques (see Table 3.2.). Titers were estimated as the average number of plaques per 1 mL of viral stock.

3.3.7. Reverse-Transcriptase PCR and sequencing:

RNA was extracted from DEF cells infected with virus rescued from 2 recombinant BAC clones (#20 passage 3 and #32 passage 4 "high titer" stock), using the Qiagen RNEasy Mini Kit (Qiagen, Valencia, CA) as follows. DEF cells were lysed via 4 freeze-thaw cycles using hand-warming and -80 degree C freezing. 250 uL from thawed viral stock was pelleted at 300 rcf for 5 minutes, washed in 1 mL PBS, repelleted and lysed with 175 uL of RLN lysis buffer at 4 degrees C for 5 minutes. The samples were then pelleted at 300 rcf for 2 minutes. The supernatants were collected and mixed with 600 uL of RLT (binding) buffer. 430 uL of 90% ethanol was added to each sample and the samples were bound to a spin column by microcentrifugation at 16,100 rcf for 15 seconds with excess sample bound in a second spin to the same column. The columns were washed in 700 uL of RWI wash buffer and re-spun, then washed with 500 uL RPE (de-salting) buffer for 15 seconds and again for 2 minutes. Columns were spin-dried for 1 minute and eluted with 40 uL of RNAse-free deionized water.

Reverse-transcriptase PCR for cDNA synthesis was performed on the resulting RNA using RNA SuperScript III (Invitrogen, Carlsbad, CA). For each reaction, 1 uL of oligo dT, 5 uL of RNA sample, 1 uL of 10 mmM dNTP's and 13 uL deionized water were heated on a thermal cycler at 65 degrees for 5 minutes. Then, 4 uL of 5x First-Strand Buffer, 1 uL of 0.1 M DTT, 1 uL of RNAse inhibitor, and 1 uL of Superscript III RT enzyme was added. The mixture was heated to 50 degrees C for 59 minutes and then at 70 degrees C for 15 minutes on a thermal cycler to allow cDNA amplification.

Taq PCR was performed on the cDNA using MDV012 cloning primers (see Table 3.1.). Gel electrophoresis was performed to verify successful amplification from cDNA (see Figure 3.1.). The products were purified using the Qiagen Gel-Extraction Kit and sequenced using

commercially available sequencing with the cloning primers to verify the presence of a mutated start codon in MDV012.

3.3.8. Allele-Specific PCR:

A PCR-based assay to differentiate the wild-type and mutated start codon alleles of Md5 and Md5-MDV012ctg BACs was attempted based on the PASA technique (reviewed in Bottema 1993). Forward primers with a single base-pair difference at the 3' end, corresponding to the A or C at the beginning of the MDV012 start codon, were designed and tested with a single reverse primer in standard Taq PCR assays, optimizing for template concentration, magnesium concentration and annealing temperature. Optimal values for amplification with these primers was with 2.5 ng of template and 37.5 nmoles of MgCl2 per 25 uL reaction performed at an annealing temperature of 49 degrees C.

3.4. Results:

Figure 3.1. shows a diagram of the two-step Red-mediated recombineering process. Primers beginning with 60 overlapping base pairs from MDV012 surrounding and including the desired A-to-C start codon mutation were designed to amplify a PCR product from pEPkan-S similar to that shown in Figure 3.1. (primers MDV012_CTG-1 and -2; see Table 3.1.). Figure 3.2. shows successful amplification of this product, including the aphA1 kanamycin resistance gene and SceI site from pEPkan-S, using these primers at three different annealing temperatures and using the high-fidelity Phusion enzyme. Subsequent PCR reactions with these primers and template were performed at 69 degrees C in order to maximize specific product (visible as the

approximately 1500 bp top band) and minimize non-specific product (smaller bands below the specific product, particularly visible at lower annealing temperatures.)

Several screening primers, for which the forward primer overlapped with the MDV012 start codon mutation, and the reverse primer primed an internal site in the aphA1 kanamycin gene (see Table 3.1.), were optimized for annealing temperature and tested for specificity using Md11BAC, SW105-Md5, SW105-FHVdeltaUL49.5-kan as negative controls and the MDV012ctg recombineering PCR product as a positive control (Figure 3.3.). This PCR was performed using Taq polymerase. All three primer pairs were found to be specific for the PCR product sequence, as they did not amplify templates containing only MDV012 (the Md11BAC prep and SW105-Md5 culture) or containing only the kanamycin sequence (SW105-FHVdeltaUL49.5-kan culture). Screening reactions were subsequently performed using only the first set of primers at the highest successful annealing temperature (65 degrees C) to maximize specificity.

Six colonies from the initial electroporation step which grew on kanamycin-positive plates were screened using the optimized MDV012mut screening primers (Figure 3.4.). All screened positive for the approximately 250 bp screening product, indicating that successful recombination between the PCR product and the region surrounding the MDV012 start codon of the Md5 BAC had occurred, transferring the SceI-kanamycin resistance cassette flanked by overlapping MDV012ctg sequences into the BAC. Colony #1 as shown in Figure 3.4. was used for subsequent electroporation with pBad-SceI, restriction at the SceI site upstream of the aphAI cassette, and second recombination step between the matching MDV012 sequences surrounding the aphAI cassette. This recombination event removes the aphAI cassette and leaves the

MDV012 sequence intact except for the introduction of the A-to-C point mutation in the MDV012 start codon (see Figure 3.1.).

Colonies from the second recombination step were screened onto kanamycin selection media. Ten screened colonies showed no growth under kanamycin selection, and thus lacked the aphAI cassette, indicating either appropriate step-2 recombination or inappropriate rearrangement of the Md5 BAC resulting in loss of the aphAI cassette. These clones were first screened with the MDV012mut screening primer pair; negative results verified loss of the aphAI cassette but did not provide further information about the integrity of the MDV012 gene (Figure 3.5.). The clones were screened again using primers external to the MDV012 gene (MDV012 Forward and Reverse; see Table 3.1.). PCR products were amplified from all except 1 of these clones and showed sizes consistent with the MDV012 gene; the negative sample likely had a BAC rearrangement that led to a deletion of the MDV012 gene. Three clones were chosen for sequencing of the MDV012 PCR products, using the external MDV012 primers and several more sequencing primers internal to the MDV012 gene (see Table 3.1.). These genes clones were found to have intact MDV012 genes except for the presence of the A-to-C point mutation at the start codon.

BAC DNA was isolated from the SW105 cells (also containing the pBad-SceI plasmid) using the PsiΨClone kit and analyzed via restriction analysis using EcoRI-HF. Figure 3.6. shows the EcoRI restriction pattern of 5 Md5-MDV012ctg clones as compared to wild-type Md5. The 3 clones which were chosen for sequencing (#20, 32 and 26) are visualized to have no losses or gains of major restriction fragments, while one clone (#19) is poorly visualized but appears normal, and another is grossly rearranged (#21, the same clone noted to have lost the MDV012 gene on PCR screening). The intact clones #20, 32, and 26 were used for attempted viral rescue.

Two out of the three clones of Md5-MDV012ctg (#20 and 32), as well as wild-type Md5, were successfully transfected into secondary duck embryo fibroblasts, resulting in the production of infective virus. An estimated 1 ug of each BAC was used in the transfection, based on expected kit yields; however, the wild-type Md5 generally produced visible plaques 1-2 days earlier than either of the Md5-MDV012ctg clones and produced subjectively larger and more numerous plaques. Formal growth curves have not yet been performed. Eleven total passages of each virus were carried out, with the wild-type virus frequently requiring passaging at about half the split ratio of the other two viruses to maintain growth at a similar number of days per passage. Multiple attempts to transfect secondary DEF's with the third clone of Md5-MDV012ctg (#26) were unsuccessful.

Reverse-transcriptase PCR was performed on viral passage 3 of clone #20 and viral passage 4 of clone #32 to amplify the MDV012 gene from cDNA (see Figure 3.7.). This allowed verification that the gene was still transcribed, and the PCR products were sequenced to confirm that the A-to-C mutation was present in the start codon. The MDV012 transcript was successfully amplified from cDNA for each clone, and the A-to-C mutation was present in sequences from each PCR product. A forward sequence chromatograph from Md5-MDV012ctg #20 spanning over 800 bp showed an un-spliced product spanning at least MDV nucleotides 17,431-18,231 (RefSeq NC_002229.3).

Titers were performed on viral passages 11 of Md5-MDV012ctg clones #20 and #32, as well as wild-type Md5. Despite the wild-type Md5 undergoing 3 fewer days in culture to reach passage 11, and, for 5 of those 11 passages, having been split at a ratio of 50% or less than the split ratios of the two mutant viruses, the Md5 virus still reached a titer approximately 15 times greater than either mutant virus (see Table 3.3.). While the original quantities of DNA used for

transfection were estimated rather than measured, the consistently higher apparent titers of Md5 despite repeated relative reductions in the amount of wild-type virus passaged suggests that a significant growth difference may be apparent when growth curves are performed.

A PASA (PCR amplification of specific alleles) assay to differentiate BAC DNA from wild-type and MDV012 A-to-C start codon mutation clones was attempted. Taq PCR using allele-specific forward primers with the A or C at the 3' end successfully amplified the MDV012 start codon alleles in a differential pattern, although in the same conditions the wild-type primer proved more specific than the mutant-type primer which amplified either allele (see Figure 3.8.). It was thus possible to differentiate between the two alleles in an Md5-based BAC with this assay based on the success of the wild-type primer (stringent amplification) or the mutant-type primer (permissive amplification of either allele) as long as separate reactions with each forward primer were run.

3.5. Discussion:

We have successfully developed a novel Md5-based BAC for MDV with a mutated MDV012 start codon using the scar-less BAC recombination techniques described in Tischer, 2006. Based on the results of whole BAC restriction analysis and sequencing of the region of interest from both BACs and cDNA we are reasonably confident that no major genome rearrangements have occurred and no mutations have been introduced into MDV012 other than the start codon mutation.

While 2 clones of Md5-MDV012ctg were successfully rescued in tissue cultk4ure, a third clone was resistant to multiple transfection attempts. Given that the other two clones were rescued without difficulty, one hypothesis that could explain this is that the third clone had

another novel mutation in a gene necessary for viral replication which prevented the formation of plaques in tissue culture. Another hypothesis is that the initial 2 clones which were successfully transfected were contaminated with wild-type Md5 BAC, which could have rescued replication of the mutant clones if an intact MDV012 gene is required for viral replication. However, since cDNA sequencing at passage 3 and 4 of the successfully transfected Md5-MDV012ctg clones indicated unambiguously that the MDV012 gene contained the A-to-C mutation, and Md5-MDV012ctg viruses continued to grow to lower titers than wild-type Md5 over multiple passages, this hypothesis seems less likely.

More interesting is the apparent defect in growth present in two independent Md5-MDV012ctg viruses, each of which produced subjectively smaller and fewer plaques at each passage than wild-type virus. Formal growth curves are needed to determine definitively if these viruses are partially growth defective in tissue culture. Given that MDV is highly cell-associated in tissue culture (Churchill 1967), a defect in growth of this nature could indicate either a replication defect or a spread defect in viruses with reduced or deleted MDV012 function given that plaque number is restricted by the number of infected cells plated at each passage.

In these assays we have not yet shown whether MDV012 function is completely abrogated by mutation of the initial ATG start codon to CTG. We designed these viruses with the smallest possible mutation likely to cause a full functional deletion, based on the lack of any nearby in-frame start codons that could be expected to cause translation of a fully or partially functional MDV012 product. Using reverse-transcriptase PCR we have verified that a large MDV012 transcript is produced, which was not suprising given the minimal nature of the introduced mutation, however functional assays or protein expression assays will be necessary to determine whether translation of an MDV012 gene product is still occurring in these viruses.

The MDV012 gene was initially annotated as two separate open reading frames, "MDV011" and "MDV012"; however, more recently it has been described as a single gene with two exons of unknown function (RefSeq: NC_002229.3; first annotated as a single gene on Feb. 20, 2007), and the spliced transcript has been cloned and sequenced from cDNA (Henry Hunt, unpublished). Interestingly, our cDNA sequencing showed apparent unspliced transcripts produced; while it is possible that genomic viral DNA was unintentionally amplified, this appears less likely as we were unable to directly amplify genomic MDV012 from cell lysates, which could be expected to contain more viral DNA than column-purified RNA samples. This may suggest that alternate splicing of MDV012 occurs. If the longer second exon is also able to be alternatively transcribed and/or translated independently, this may in effect make our Md5-MDV012ctg viruses MDV012 exon 1 knock-out viruses only, and will limit the interpretation of results obtained using these viruses accordingly.

As yet, no function has been attributed to MDV012, other than our recent findings that this gene is able to decrease the expression of MHC class I in vitro on transfected cells. Homologous sequences are present in non-oncogenic (type-2) MDV strains, but have not been found in other alpha-herpesvirus genomes (Tulman 2000); thus it is a unique potential immune evasion gene of avian herpesviruses. We anticipate using recombinant viruses such as the MDV012 start codon mutants developed in this study to investigate the function of MDV012 in the immunocompetent host, to determine the in vivo relevance of this gene in the pathogenesis of MDV, and to determine the potential of MDV012 knock-out viruses in the development of new MDV vaccines.

APPENDIX

TABLES

Table 3.1. Primers for recombineering.

a. Referenced within the Md5 genome (Refseq: NC_002229.3).
b. See Tischer et. al. 2006 (Supplementary data) for further examples of recombineering primers that amplify aphAI from pEPkan-S.
c. Referenced against the aphAI gene out of pACYC177 (Genbank: X06402.1).
d. Referenced within the Md5 genome (Refseq: NC_002229.3).
e. See Tischer et. al. 2006 (Supplementary data) for further examples of recombineering primers that amplify aphAI from pEPkan-S.
f. Referenced against the aphAI gene out of pACYC177 (Genbank: X06402.1).

Name	PCR Target	Sequence	*Use, Features*
MDV012R	MDV012 bp 19015-18995 (minus strand)[a]	GC*TCTAGA*TGGATTT GCAATCACACAACA	Cloning/Sequencing; XbaI site
MDV012F	MDV012 bp 17361-17381 (plus strand)[a]	GC*GAATTC*CGGTGCT TTGTACTTCCTACG	Cloning/Sequencing; EcoR1 site
MDV012CTG_1	pEPkan-S (plus strand)[b]	*TATAAAAACGAGAGG TTGGTAACAAACAGC TTTTGAAAA**C**TGACT AGCGAGAGAGCTCTT A*GGATGACGACGATA AGTAGGG	Recombineering; MDV012 bp 17392-17451 (plus strand); A-to-C mutation at 17431[a]
MDV012CTG_2	pEPkan-S (minus strand)[b]	*ACTTTACCAGGCGCG AGAGTAAGAGCTCTC TCGCTAGTCA**G**TTTT CAAAAGCTGTTTGTT* CAACCAATTAACCAA TTCTGATTAG	Recombineering; MDV012 bp 17471-17412 (minus strand); complementary T-to-G mutation at 17431[a]
MDV012mutF1	MDV012ctg bp 17409-17431 (plus strand)[a]	GGTAACAAACAGCTT TTGAAAAC	Screening
MDV012mutR1	aphAI bp 30-11 (minus strand)[c]	CAAGACGTTTCCCGT TGAAT	Screening
MDV012mutR2	aphAI bp 253-234 (minus strand)[c]	GATGGTCGGAAGAGG CATAA	Screening

Table 3.1. (cont'd)

Name	PCR Target	Sequence	*Use, Features*
MDV012mutR3	aphAI bp 126-107 (minus strand)[c]	CGATAGATTGTCGCA CCTGA	Screening
MDV012SeqF1	MDV012 bp 17368-17389 (plus strand)[a]	TTGTACTTCCTACGT CGGATCA	Sequencing
MDV012SeqR1	MDV012 bp 17529-17510 (minus strand)[a]	TTGGCGTACAAATTC ACGAC	Sequencing
MDV012SeqR2	MDV012 bp 17689-17671 (minus strand)[a]	CTTAGGCGGACGCTG ACAT	Sequencing
VYG 2197-MDV012SeqR3	MDV012 bp 17734-17715 (minus strand)[a]	GGCTTCATGGTGGGC TATTA	Sequencing
StartFn	MDV012 bp 17407-17431 (plus strand)[a]	TTGGTAACAAACAGC TTTTGAAAAA	Allele-specific PCR
StartF	MDV012ctg bp 17407-17431 (plus strand)[a]	TTGGTAACAAACAGC TTTTGAAAAC	Allele-specific PCR
StartR	MDV012 bp 17798-17779 (minus strand)[a]	CCCATAACCCATCAA CGATT	Allele-specific PCR

Table 3.2. Viral passages performed.

3.2.1. Viral passages of Md5:

Passage Number	Date Frozen	Split ratio	Days in culture	Days refed post-infection and % serum
Transfected	Not frozen	2x60 mm plates	4 days in culture	Refed 1 day post-transfection in 1% hiFBS
Pass 1	Not frozen	All onto 100 mm plate	3 d.	1 d. 1% hiFBS
Pass 2	Not frozen	All onto 150 mm plate	3 d.	1 d. 1% hiFBS
Pass 3	4/16/2010	1:6 passage	3 d.	1 d. 1% hiFBS
Pass 4	6/7/2010	1:4	5 d.	1 d. 1% hiFBS
Pass 5	6/11/2010	1:20	4 d.	1 d. 1% hiFBS
Pass 6	6/15/2010	1:20	4 d.	1 d. 1% hiFBS
Pass 7	6/19/2010	1:20	4 d.	1 d. 1% hiFBS
Pass 8a	6/23/2010	1:20	4 d.	1 d. 1% hiFBS
Pass 9a	7/2/2010	1:10	4 d.	1 d. 1% hiFBS
Pass 8	12/20/2010	1:30	5 d.	1 d. 5% hiFBS for 2 hours; then 1% hiFBS
Pass 9	12/25/2010	1:4; 1 newly plated 150 mm plate in 4% FBS, 2 one-day-old 150 mm plates in 4% hiFBS	5 d.	1 day old plate – 1 d. 4%hiFBS, 2 d. 1% hiFBS, 4 d. 0.5% hiFBS. 2-day-old plates – 1 d. 1% hiFBS, 4 d. 0.5% hiFBS.
Pass 10	12/30/2010	1:4 plated on newly plated 150 mm plates	5 d.	2 d. 0.25% hiFBS
Pass 11	1/4/2011	1:4	4 d.	1 d. 0.25% hiFBS

Table 3.2. (cont'd)

3.2.2. Viral passages of Md5-MDV012ctg #20:

Passage Number	Date Frozen	Split ratio	Days in culture	Days refed post-infection and % serum
Transfected	Not frozen	2x60 mm plates	4 days in culture	Refed 1 day post-transfection in 1% hiFBS
Pass 1	Not frozen	All onto 100 mm plate	3 d.	1 d. 1% hiFBS
Pass 2	Not frozen	All onto 150 mm plate	3 d.	1 d. 1% hiFBS
Pass 3	4/18/2101	1:3 passage	5 d.	1 d. 1% hiFBS
Pass 4	6/7/2010	1:3	5 d.	1 d. 1% hiFBS
Pass 5	6/11/2010	1:10	4 d.	1 d. 1% hiFBS
Pass 6	6/15/2010	1:10	4 d.	1 d. 1% hiFBS
Pass 7	6/19/2010	1:10	4 d.	1 d. 1% hiFBS
Pass 8a	6/23/2010	1:10	4 d.	1 d. 1% hiFBS
Pass 9a	7/5/2010	1:5	7 d.	1 d. 1% hiFBS
Pass 8	12/20/2010	1:10	5 d.	1 d. 5% hiFBS for 2 hours; then 1% hiFBS
Pass 9	12/25/2010	1:4; 1 newly plated 150 mm plate in 4% FBS, 2 one-day-old 150 mm plates in 4% hiFBS	5 d.	1 day old plate – 1 d. 4%hiFBS, 2 d. 1% hiFBS, 4 d. 0.5% hiFBS. 2-day-old plates – 1 d. 1% hiFBS, 4 d. 0.5% hiFBS.
Pass 10	12/30/2010	1:4 plated on newly plated 150 mm plates	5 d.	2 d. 0.25% hiFBS
Pass 11	1/5/2011	1:4	5 d.	1 d. 0.25% hiFBS

Table 3.2 continued:

3.2.3. Viral passages of Md5-MDV012ctg #32:

Passage Number	Date Frozen	Split ratio	Days in culture	Days refed post-infection and % serum
Transfected	Not frozen	2x60 mm plates	4 days in culture	Refed 1 day post-transfection in 1% hiFBS
Pass 1	Not frozen	All onto 100 mm plate	3 d.	1 d. 1% hiFBS
Pass 2	Not frozen	All onto 150 mm plate	3 d.	1 d. 1% hiFBS
Pass 3	4/18/2010	1:3 passage	5 d.	1 d. 1% hiFBS
Pass 4a	5/17/2010 (-80°C only)	1:9	3 d.	1 d. 1% FBS
Pass 4	6/7/2010	1:3	5 d.	1 d. 1% hiFBS
Pass 4a		1:9	3 d.	1 d. 1% FBS
Pass 5	6/11/2010	1:10	4 d.	1 d. 1% hiFBS
Pass 6	6/15/2010	1:10	4 d.	1 d. 1% hiFBS
Pass 7	6/19/2010	1:10	4 d.	1 d. 1% hiFBS
Pass 8a	Not frozen	1:10	4 d.	1 d. 1% hiFBS
Pass 9a	7/10/2010	1:5	7 d.	1 d. 1% hiFBS
Pass 8	12/20/2010	1:10	5 d.	1 d. 5% hiFBS for 2 hours; then 1% hiFBS
Pass 9	12/25/2010	1:4; 1 newly plated 150 mm plate in 4% FBS, 2 one-day-old 150 mm plates in 4% hiFBS	5 d.	1 day old plate – 1 d. 4%hiFBS, 2 d. 1% hiFBS, 4 d. 0.5% hiFBS. 2-day-old plates – 1 d. 1% hiFBS, 4 d. 0.5% hiFBS.
Pass 10	12/30/2010	1:4 plated on newly plated 150 mm plates	5 d.	2 d. 0.25% hiFBS
Pass 11	1/5/2011	1:4	5 d.	1 d. 0.25% hiFBS

Table 3.3. Titrations. 60-mm plates of DEF cells were infected in triplicate with several dilutions of passage 11 rescued Md5 virus and Md5-MDV012ctg virus clones #20 and #32. Plates were fixed at 7 days after infection, stained for the pp38 MDV antigen at and plaques were counted under FITC-fluorescence microscopy. The dilution which produced within 10-100 plaques per plate was averaged to determine plaque-forming units per 1 mL vial.

Md5:

uL virus/plate	plaques counted/dish	ave/plate	pfu/vial/mL
1	604/NC/NC		
0.1	56/46/39	47	4.7x10^5 pfu/vial/mL
0.01	NC/NC/NC		

Md5-MDV012ctg #20:

uL virus/plate	plaques/dish	ave/plate	pfu/vial/mL
1	36/26/35	32.3	3.23x10^4 pfu/vial/mL
0.1	2/5/2		
0.01	NC/NC/NC		

Md5-MDV012ctg #32:

uL virus/plate	plaques/dish	ave/plate	pfu/vial/mL
1	44/31/18	31	3.1x10^4 pfu/vial/mL
0.1	7/11/3		
0.01	NC/NC/NC		

FIGURES

Figure 3.1. Schematic of the 2-step red-mediated recombineering process. Adapted from Tischer, 2006. A linear PCR product containing overlapping regions of interest with the desired mutation (represented by a red star), surrounding a positive-selection marker ('Kan-r'; kanamycin-resistance cassette) and with an adjacent unique restriction site (SceI; represented by the green bracket) is homologously recombined with the original BAC (recombination is represented in the figure by thin arrows). This produces a modified BAC containing the original PCR product in place of the region of interest. Restriction at the SceI site is induced, and a second recombination event removes the positive-selection marker. The result is a mutated BAC containing a single copy of the desired mutation with no other modifications after 2 recombination events.

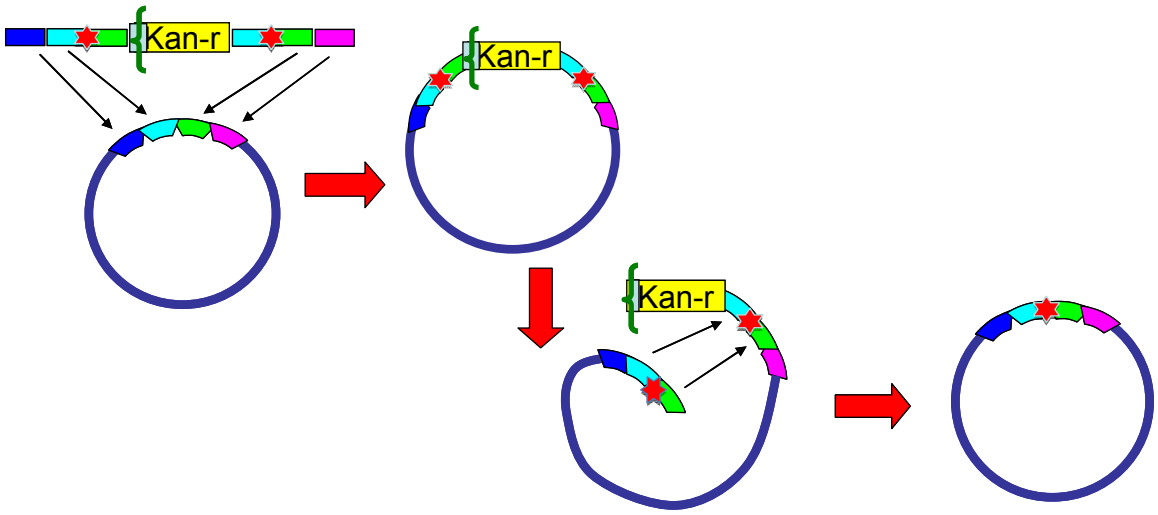


Figure 3.2. Gel electrophoresis of PCR amplification of recombineering product using MDV012gtc primers and pEPkan-S. Lanes 1 and 8 contain 100 bp ladder and 1 kb ladder, respectively. Lanes 3-5 contain products of PCR performed at three different annealing temperatures using recombineering primers described in Figure 1 and Table 1. Annealing temperatures were 65, 69 and 72 degrees C respectively for Lanes 3-5. Thirty-five PCR cycles were performed according to the Phusion manual's instructions. Lane 8 contains 1 kb ladder (New England Biolabs).

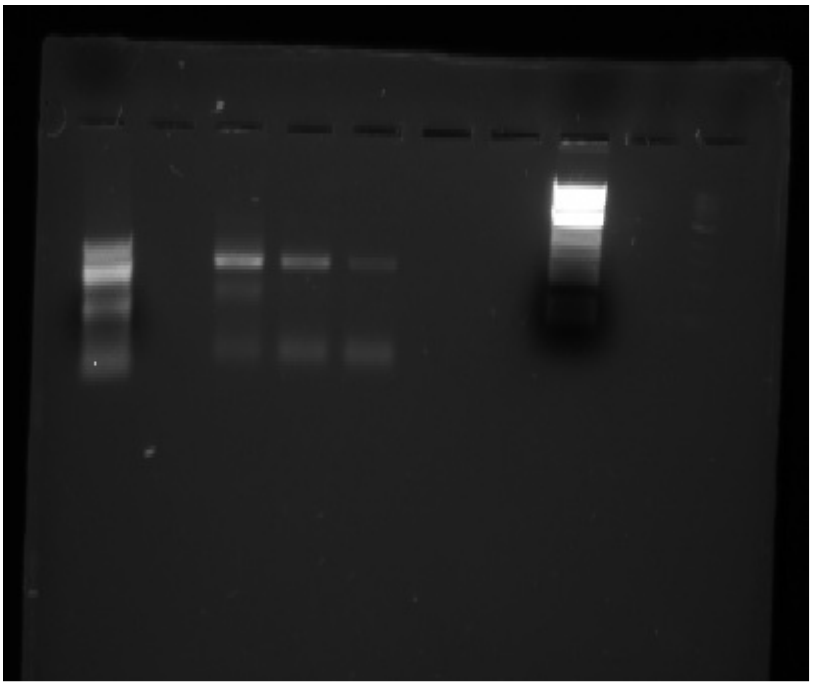


Figure 3.3. Gel electrophoresis showing optimization of screening primers for amplification of step 1 recombineering product from the forward recombineering primer sequence to the middle of the aphAI kanamycin resistance cassette. Primer pairs MDV012mut F1:R1, F1:R2, and F1:R3, which had predicted melting temperatures between 51 and 55 degrees C, were optimized for annealing temperature and tested for template specificity in a standard 25 uLTaq PCR reaction. Lanes 1-7 and 9-15 above, and lanes 1-7 below, represent primer pairs F1:R1, F1:R2, and F1:R3 respectively. Within each primer pair optimization, the first 3 lanes represent annealing temperatures 61, 63.1, and 65.1 degrees C respectively, using the initial recombineering PCR product as a positive sample. The following 4 lanes in each set represent negative template controls Md11BAC plasmid prep, SW105-Md5 culture, SW105-FHVdeltaUL49.5-kan colony, and sterile water. The last 2 lanes in each row represent 100 bp and 1 kb ladders (New England Biolabs), respectively.

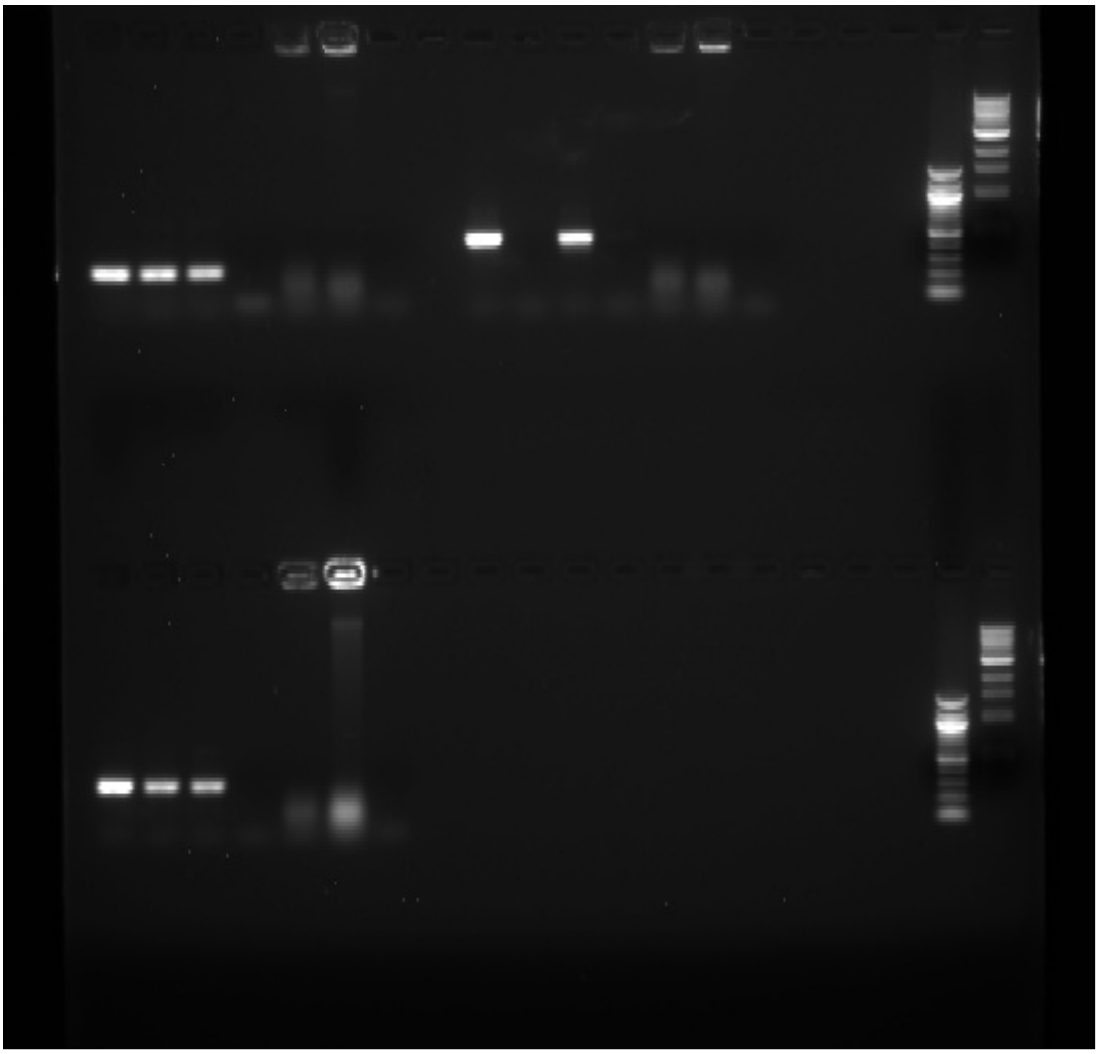


Figure 3.4. Gel electrophoresis showing screening PCR of step 1 recombineering product SW105-Md5-PCR. Primer pair MDV012mutF1:R1 (see Table 1 and Figure 3) was used in a standard 25 uL Taq PCR reaction to screen kanamycin-resistant colonies from the initial recombination reaction between Md5 BAC and MDV012ctg Forward:Reverse PCR product containing the SceI restriction site and aphAI kanamycin resistance cassette, in SW105 cells. Colonies were transferred to the PCR reaction on micropipette tips, and the reaction was carried out at 65 degrees C. Lanes 1-6 represent six colonies tested, lane 7 represents SW105-Md5 as a negative control, and lane 8 ;2represents the recombineering PCR product as a positive control. Lanes 9 and 10 represent 100 bp and 1 kb ladders (New England Biolabs), respectively.

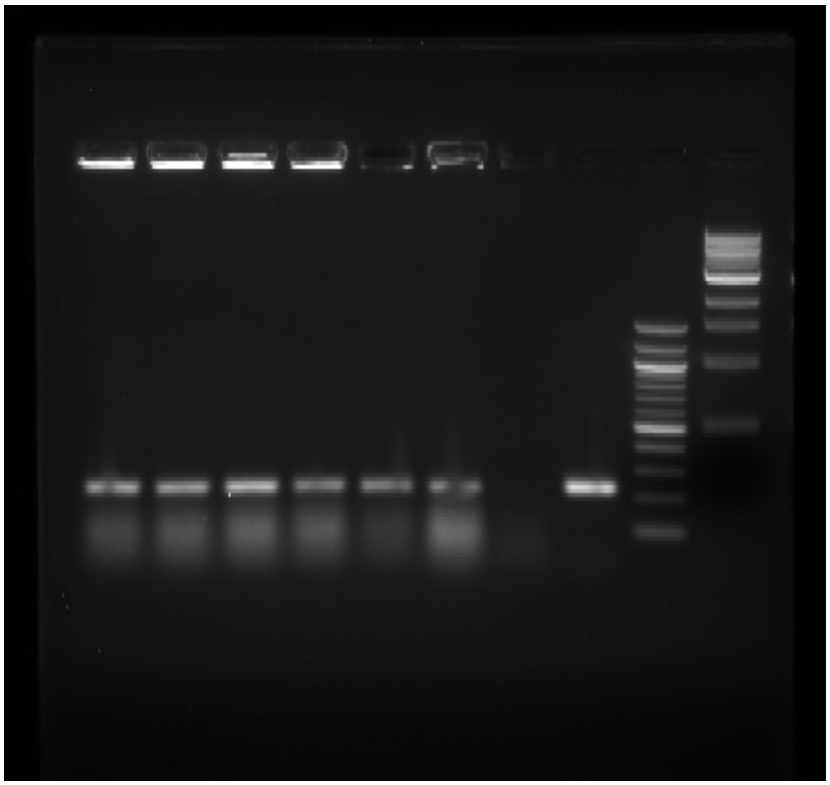


Figure 3.5. Gel electrophoresis showing screening PCR of step 2 recombineering product SW105-Md5-MDV012ctg/pBad-SceI. Primer pair MDV012mutF1:R1 was used in a standard 25 uL Taq PCR reaction to screen kanamycin-susceptible colonies from the second recombination reaction between matching MDV012ctg-region sequences in the BAC flanking the aphAI cassette, in SW105 cells. A. Lanes 1-8 above and lanes 1 and 2 below represent ten colonies of SW105-Md5-MDV012ctg/pBad-SceI (0.5 uL of frozen subcultures), lane 3 below represents SW105-Md5/pBad-SceI (0.5 uL of frozen culture) as an aphAI-negative control, and lane 4 below represents the recombineering PCR product as an aphAI-positive control. The last 2 lanes in each row represent 100 bp and 1 kb ladders (New England Biolabs), respectively. B. On a separate gel, an identical PCR reaction was performed with non-recombination-induced SW105-Md5-PCR/pBad-SceI as an additional aphAI-positive control.

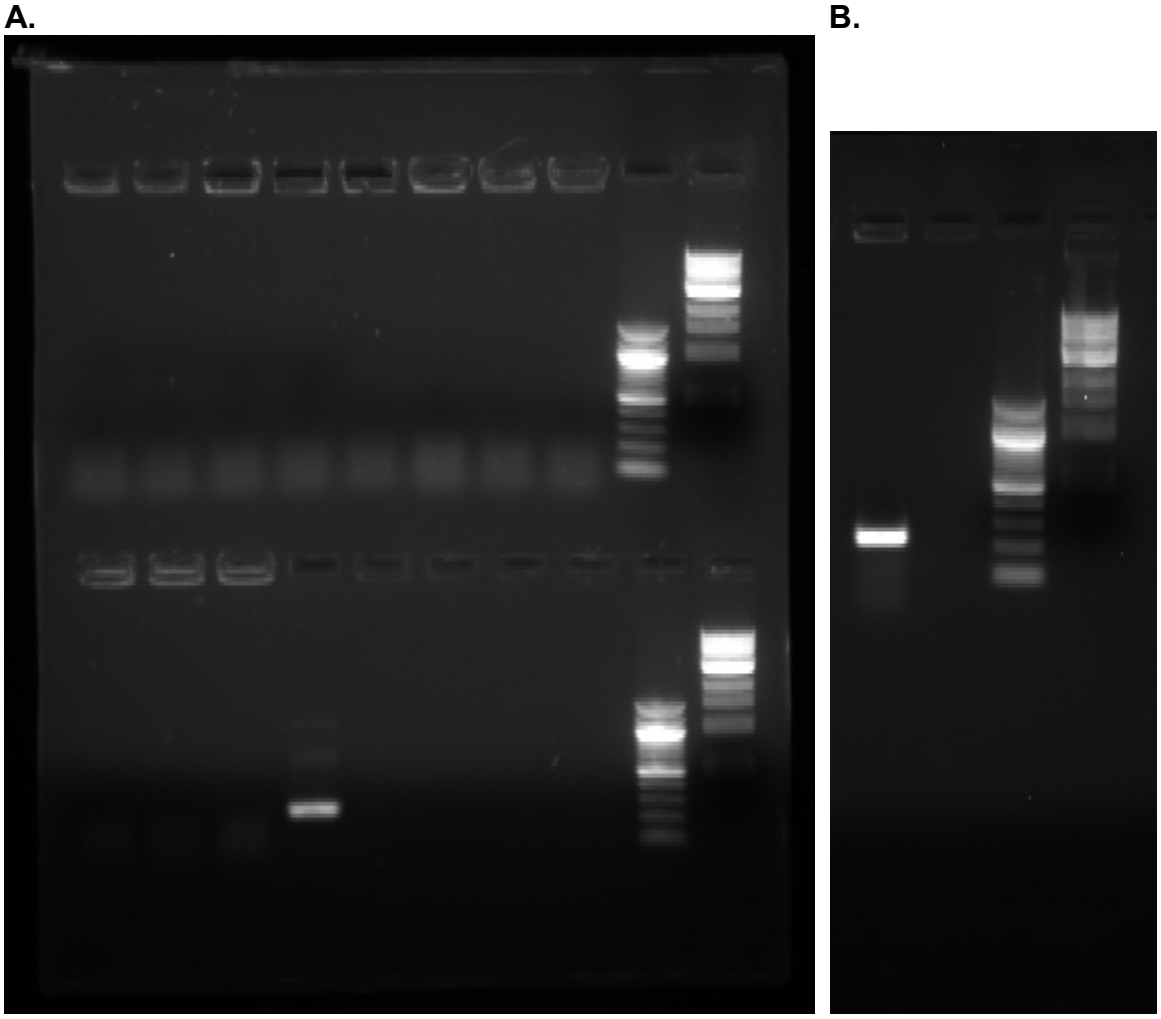


Figure 3.6. Gel electrophoresis of EcoRI-HF fingerprints. BACs were digested with EcoRI-HF (NEB) overnight and run on a 1% gel for 18 hours at 0.8-1.3 V/cm**.** Lane 1 contains Md5, lanes 2-6 are Md5-MDV012ctg clones #20, 32, 19, 21, and 26 respectively, and lanes 8 and 9 are 1 kb ladder (NEB) and 100 bp ladder (Invitrogen).

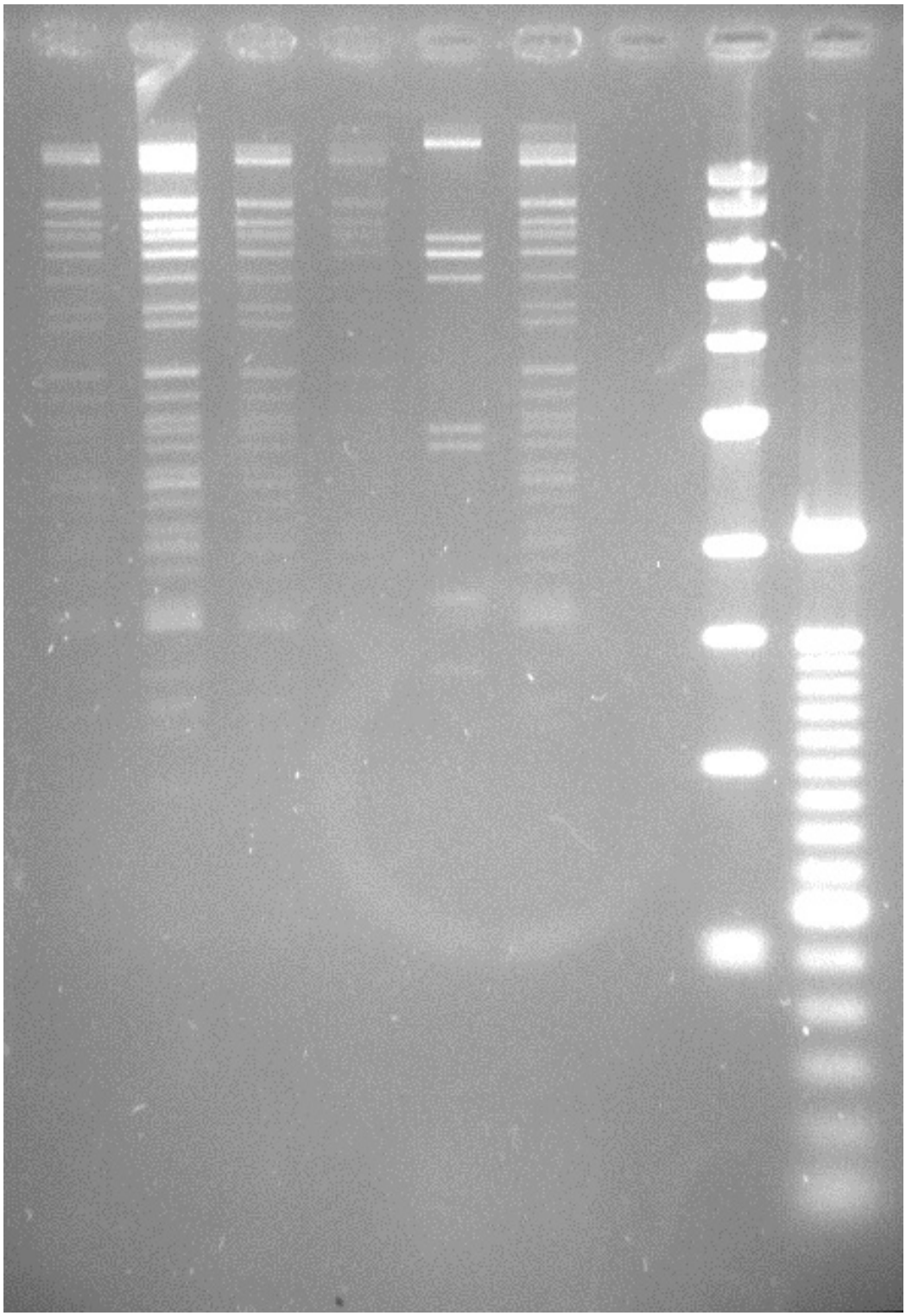


Figure 3.7. Gel Electrophoresis of Reverse-Transcriptase PCR of MDV012 from Md5-MDV012ctg virus clones. cDNA was amplified from infected DEF cells at passage 3 (#20) and passage 4 (#32), and standard Taq PCR was subsequently performed using MDV012 Forward and Reverse primers. Lane 1 and lanes 5-8 are not loaded; lane 2 represents Md5-MDV012ctg #20 and lane 3 represents Md5-MDV012ctg #32. Lane 4 represents negative (water) control, and lanes 9 and 10 represent 100 bp and 1 kb ladders respectively (NEB).

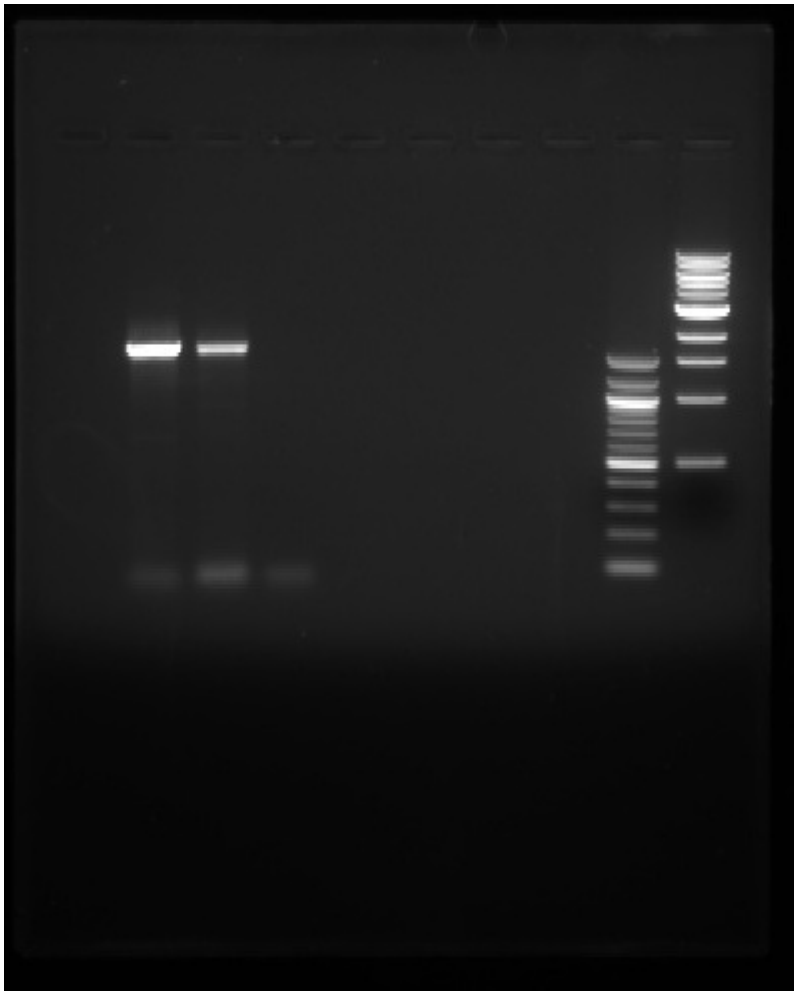
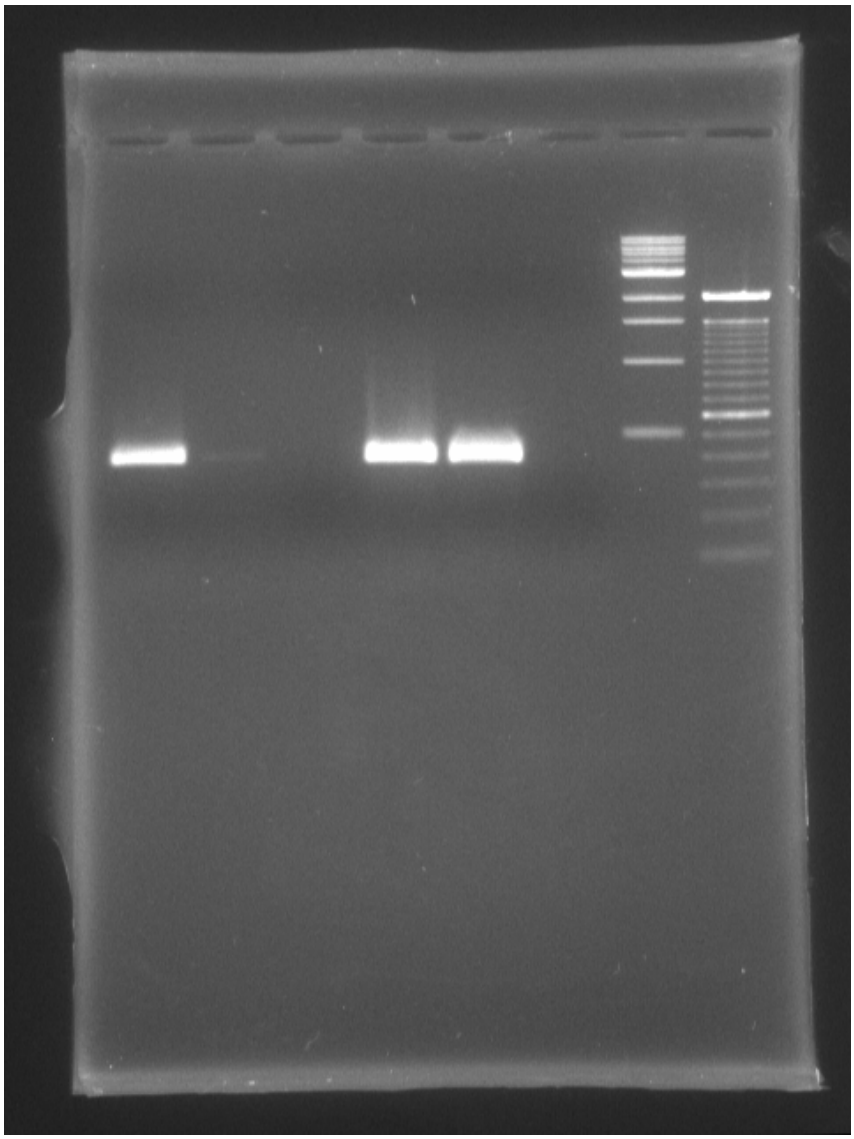


Figure 3.8. Gel electrophoresis of PASA (PCR-based amplification of specific alleles) of Md5 and Md5-MDV012ctg #20. Primers StartFn, StartF and StartR were used to amplify a 391 bp product, with the forward primers overlapping the first nucleotide of the MDV012 start codon of either wild-type Md5 BAC or A-to-C start codon mutant Md5-MDV012ctg BAC clone #20. Taq PCR was performed in 25 uL reactions at 94 degrees for 5 minutes; 30 cycles of 94 degrees C for 30 seconds, 49 degrees C for 30 seconds, and 65 degrees C for 30 seconds; and 65 degrees for 7 minutes. Lanes 1-3 are amplified with primers StartFn and StartR. Lanes 4-6 are amplified with primers StartF and StartR. Lanes 1 and 4 are amplified from Md5 BAC, and lanes 2 and 5 are amplified from Md5-MDV012ctg BAC #20. Lanes 3 and 6 are water (negative) controls. Lane 7 contains 1 kb ladder (NEB) and lane 8 contains 100 bp ladder (Invitrogen).



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CHAPTER 4

A start codon mutant of MDV012 still encodes a functional immune evasion gene.

4.1. Abstract:

MDV012 has been shown to down-regulate MHC class I in transfected chicken cells, which is expected to have an immune-evasive effect on cellular immunity to MDV (Chapter 2). Viruses with the start codon of MDV012 mutated to potentially delete MDV012 expression were created (Chapter 3), and the start-codon-mutated MDV012ctg gene was cloned from these Md5-MDV012ctg viruses into a plasmid vector. Transfection assays were performed to determine whether MDV012ctg is a functional knock-out relative to wild-type MDV012. Overall expression of MHC class I (as measured by C6B12 monoclonal antibody staining) indicated that MDV012ctg retained MHC class I down-regulation function. Effects of MDV012 and MDV012ctg on the major MHC class I were inconsistent with overall MHC class I expression and raise the possibility that non-classical MHC class I might also be affected. The minor MHC class I was not affected by transfection with either MDV012 or MDV012ctg, indicating that differential susceptibility to down-regulation effects exists between the more highly expressed major MHC class I gene and the poorly expressed minor gene. Due to the lack of differences between MDV012ctg and MDV012 in transfection tests, we conclude that Md5-MDV012ctg is not a functional knock-out of MDV012, and that a gene product capable of down-regulating MHC class I can still be expressed from MDV012ctg, perhaps as a functional product from the second exon.

4.2. Introduction:

We demonstrated in Chapter 2 that the Marek's disease virus (MDV) gene, MDV012, is capable of down-regulating the surface expression of MHC class I complexes on transfected chicken cell lines. This effect, noted previously to occur with infection by virulent MDV virus, is suspected to contribute to evasion of CD8+ T-cell immunity, and potentially to the failure of MDV vaccination to prevent viral latency, shedding and continued circulation of MDV strains (Hunt 2001). Viral down-regulation of MHC class I is a well-established phenomenon found among multiple herpesviruses, including cytomegalovirus (Beersma 1993, Wiertz 1996, Besold 2009), gamma-herpesviruses (Adang 2007), and other alpha-herpesviruses (York 1994, Ambagala 2000, Koppers-Lalic 2005). However, the importance of this function to in vivo infection and immunogenesis is still being established.

Another MDV gene, MDVUL49.5, has also been found to have a mild MHC class I down-regulatory effect in chickens; knock-out viruses were found to be less pathogenic to chickens with a genetically resistant MHC class I haplotype, but was unimportant for infection in more susceptible chickens (Jarosinski 2010). Interestingly, infection of calves with a bovine herpesvirus 1 (BHV-1) lacking a functional UL49.5 gene of bovine showed a more rapid CD8+ and virus neutralizing antibody response than in calves infected with wild-type viruses, although pathogenicity was similar to wild type infection (Wei 2012). This suggests that deletion of MDV012 in MDV might similarly enhance immune response in vaccine viruses, although attenuation of pathogenicity may require deletion of multiple immune evasion genes as well as virulence genes.

Further analysis of MHC class I evasion by MDV012 in MDV will require the development of knock-out viruses that lack a functional copy of the MDV012 gene, in order to

determine the importance of MDV012 in mediating this effect against the background of the rest of MDV genome; to determine the in vitro and in vivo effects of MDV012 deletion on MDV pathogenicity; and to compare the immunogenicity and protectiveness of MDV strains lacking immune evasion genes such as MDV012 and MDVUL49.5 in vaccination and challenge studies. In Chapter 3, the development of potential knock-out MDV viruses for the MDV012 gene was described, using the two-step Red-mediated bacterial artificial chromosome (BAC) recombination ("recombineering") method (Tischer 2006) to design minimally mutated viruses based on the virulent Md5B40BAC strain (Niikura 2011). These viruses, which contain an A-to-C point mutation at the MDV012 start codon, were found to express mRNA transcripts from the MDV012 gene, with the expected start codon mutation present in cDNA sequences. The MDV012 start codon mutants, referred to as Md5-MDV012ctg, may be useful in in vitro and in vivo assays to determine the significance of MHC class I down-regulation by MDV012 in infection and immunization with MDV.

Further characterization of these viruses requires validation that MDV012 gene expression is deleted prior to use in infection assays. We chose to use a similar system to that originally used to identify MDV012 function, with cloning of the mutant MDV012ctg gene into the same plasmid vector and comparison of MHC class I expression in transfected chicken cells. If MDV012ctg is not expressed due to the introduced start codon mutation, then we would anticipate rescue of MHC class I expression in MDV012ctg-transfected cells relative to down-regulated expression of MHC class I in cells transfected with wild-type MDV012.

4.3. Methods:

4.3.1. Antibodies:

Antibodies against the chicken MHC class I were used as described in Chapter 2. A mouse monoclonal antibody against chicken MHC class I, mAb C6B12, has been described (Shamansky et al, 1988) and was obtained from The Developmental Studies Hybridoma Bank, Iowa City, Iowa. Polyclonal antisera against the major and minor MHC class I loci of several MHC haplotypes have been developed using cloned sequences expressed in the RCASB-A retroviral system in RP9 cells and used to raise antisera in chickens (Fulton et al, 2001). As DF-1 cells are known to express B21 haplotype MHC class I, anti-BF2*21 specific (anti-major; H1222-RP9BFIV21m78) and anti-BF1*21 specific (anti-minor; E6338) antisera, were obtained from the USDA-ARS Avian Disease and Oncology Laboratory (USDA-ADOL), East Lansing, Michigan. The anti-major antibody is based on a mutated sequence that restricts the available epitopes to reduce cross-specificity between MHC class I haplotypes, and potentially between non-classical MHC loci (Fulton et al, 2001). The monoclonal mouse anti-H-2kb-SIINFEKL IgG antibody 25-D1-16 (Porgador 1997) was used as an isotype control against C6B12. Normal chicken serum was obtained from USDA-ADOL for use as an isotype control against the anti-major and anti-minor antibodies. Secondary antibodies used were anti-mouse and anti-chicken IgG conjugated with either the Cy5 flourophore (Jackson Immunoresearch, West Grove, PA) or the AlexaFluor 633 fluorophore (Invitrogen).

4.3.2. Cells, viruses and plasmids:

DF-1 cells are an immortalized chicken fibroblast cell line, developed from USDA-ADOL Line 0 primary fibroblasts, described in Himley 1998; and known to homozygously

express the B21 MHC haplotype. These cells were routinely cultured in T25 and T75 tissue culture flasks at 39 degrees C and 5% CO2, in Dulbecco's Minimal Essential Medium (DMEM; Gibco) with 10% fetal bovine serum (FBS), and were split routinely at 90% confluence at a ratio of 1:10, but ranging as high as 1:2 when larger working stocks were needed or when cells were split at sub-confluence immediately prior to use in transfection assays. Transfection assays were performed using DF-1 cells obtained from the American Tissue Culture Collection (ATCC) at passage 150, which were cultured in regular media for 3 passages (8 days), then in DMEM/10% FBS/ciprofloxacin HCL 10 ug/mL for 3 additional passages (14 days), and then recovered in regular medium for 3 passages (10 days) prior to use. Prior experiments with this cell line indicated that transfection efficiencies were significantly reduced in cells not treated on a regular schedule with ciprofloxacin, and expression of cell surface markers also appeared to be affected. Mycoplasma contamination was suspected to be the cause of this problem, therefore the newly obtained cells were treated prophylactically. Extended culture in ciprofloxacin-containing medium had the anticipated effects of slowing cell growth and increasing indicators of cellular toxicity such as vacuolization during passage in this medium (Fleckenstien 1996; Freshney 4th ed.); cell growth and morphology recovered with subsequent passaging in antibiotic-free medium. Transfection efficiencies were high (30-50%) in treated cells.

The Md11BAC clone of MDV was the source of MDV012 cloned into the pTracer-CMV2 vector (Invitrogen) to create pTracer-CMV2-MDV012 (see Chapter 2). Md11BAC is a tissue-culture adapted strain cloned into a bacterial artificial chromosome (GenBank # AY510475; Niikura 2006). The Md5B40BAC clone was used to create the recombinant Md5-MDV012ctg BACs described in Chapter 3. Md5B40BAC is a bacterial artificial chromosome containing the very virulent oncogenic Md5 strain of MDV (GenBank # HQ149525.1; Niikura

2011), and according to published sequence data contains the same DNA sequence for the MDV012 gene as the Md11BAC. The Md5-MDV012ctg BAC #32 is a recombinant Md5B40BAC which contains a single nucleotide A-to-C start codon mutation in the MDV012 gene, as described in Chapter 3. Md5-MDV012ctg BAC #32 was used as a cloning template for MDV012ctg, and was maintained in SW105 E. coli cells (a DH10B strain with a temperature-dependent lamda Red recombinase system, and expressing arabinose-inducible flp*e*; more fully described in Chapter 3; Lee 2001; Warming 2005). These cells also contained the plasmid pBAD-SceI (Tischer 2006), and were cultured in LB/chloramphenicol/ampicillin (30 ug and 100 ug/mL respectively) prior to storage at -80 degrees C in 15% glycerol. Plasmids were amplified in subcloning-efficiency DH5α E. coli cells (Invitrogen).

4.3.3. Cloning of MDV012ctg:

The recombinant MDV012ctg gene was PCR-amplified from Md5-MDV012ctg BAC #32 using Phusion PCR (Finnzymes/Thermo Scientific) and MDV012 cloning primers (see Table 3.2.) as follows. A 52.3 uL PCR reaction was performed, containing 4 uL of thawed stock SW105-Md5-MDV012ctg/pBAD-SceI #32 stock culture as template, 10 uL of HF Buffer, 10 nmoles each mixed DNTPs, 100 pmoles each of MDV012F and MDV012R primers, and 0.5 uL of Phusion enzyme. PCR was performed using the following protocol: 98 degrees C for 30 seconds; 35 cycles of 98 degrees C for 10 seconds, then 53 degrees C for 30 seconds, then 72 degrees C for 40 seconds; and finally 72 degrees for 10 minutes. PCR product was run on agarose gel electrophoresis and gel extracted using the Qiagen Gel Extraction kit (Qiagen).

pTracer-CMV2 and the MDV012ctg PCR product were each restriction-digested in a double-digest with EcoRV and XbaI enzymes (digesting with EcoRV was unnecessary for the

PCR product, which does not contain an EcoRV site). The vector backbone of pTracer-CMV2 was gel extracted, while the large PCR fragment was purified using the Qiagen PCR Purification kit. The restriction-digested PCR fragment was treated with T4 PolyNucleotide Kinase enzyme (NEB) to phosphorylate the 5' end prior to blunt-end ligation into the EcoRV site of the vector. Finally, the vector and PCR fragments were ligated for 2 hours at room temperature using T4 DNA Ligase (NEB) and subcloning-efficiency DH5α cells (Invitrogen) were transformed with the ligation product by heat shock and plated on LB/ampicillin (100 ug/mL) overnight at 37 degrees. Colonies were picked and sub-cultured in 3 mL LB/ampicillin broth and shaken overnight at 37 degrees C and 250 RPM for plasmid miniprep purification using the FastPlasmid Mini kit (5Prime).

Miniprep DNA was restriction-digested using the PmeI and KpnI enzymes to screen for correct plasmid and insert sizes (see Figure 4.1.). A 250-mL overnight LB/ampicillin culture at 37 degrees C and 250 RPM was subcultured from one of the original mini-prep cultures and plasmid DNA was prepared in quantity using the Qiagen MaxiPrep kit. Maxiprep DNA of pTracer-CMV2-MDV012ctg was commercially sequenced using the CMVForward and BGHReverse sequencing primers, to ensure that the insert was intact and in the correct orientation.

4.3.4. Plasmids for transfection assays:

pTracer-CMV2 and pTracer-MDV012 are described in Chapter 2. pTracer-CMV2 is a commercially available expression vector (Invitrogen) which expresses green fluorescent protein (GFP) under control of EM-7 promoter and contains a multiple cloning site for expression of an insert under control of the highly active CMV promoter. pTracer-MDV012 contains the wild-

type sequence of the MDV012 gene (bp 17361-19015; reference genome Md5, RefSeq: NC_002229.3) inserted in the multiple cloning site. Fresh maxi-prep DNA of pTracer-MDV012 and pTracer-MDV012ctg was prepared for re-sequencing just prior to use in transfection assays. Subcloning-efficiency DH5α cells (Invitrogen) were heat-shock transformed with about 1 ug of each plasmid and plated overnight on LB/ampicillin (100 ug/mL). Overnight 3 mL cultures in LB/ampicillin were grown at 35 degrees C and 220 RPM from well-isolated colonies and minipreps were performed using the Qiagen Mini Prep kit (Qiagen). Minipreps were restriction-digested with PmeI and KpnI to screen for correct insert sizes. New 3 mL starter cultures were grown overnight from the same plates, and 220 mL overnight cultures were subcultured from 1 mL each of the starter cultures at 35 degrees C and 220 RPM. Plasmid DNA was purified in quantity using the Denville Isopure Maxi II kit, eluted in TE buffer, and restriction digests were repeated. Fresh plasmid preps of the pTracer-CMV2 plasmid were performed, with expected restriction digest fingerprints, but had too low of yield (<200 ng/uL) to be used in volume-standardized transfection assays so the original plasmid stock, which had a DNA concentration of >700 ng/uL, was used.

Ethanol precipitations were performed on the new plasmid maxipreps prior to insert re-sequencing to remove TE buffer salts, using the following procedure. 35-40 ug of DNA (50-100 uL) was mixed with an equal volume of 7.5 M ammonium acetate, followed by 5 times the original DNA volume of 70% ethanol, and incubated at 4 degrees C for a minimum of 5 minutes. For pTracer-CMV2-MDV012, which had a low initial concentration of ~400 ng/uL, 1 uL of 20 mg/mL glycogen was added to encourage precipitation during the incubation period, which was performed overnight. Subsequently, samples were microcentrifuged at 14000 RPM for 15 minutes. 1 mL of 70% ethanol (90% for pTracer-CMV2-MDV012) was then added and the

sample was microcentrifuged for 5 minutes. The ethanol wash was repeated and the sample was microcentrifuged for 10 minutes, and the pellet air-dried completely. DNA pellets were re-suspended in 20 uL of deionized water and plasmid samples were submitted for sequencing of the insert regions using the CMV-Forward and BGH-Reverse primers.

4.3.5. Transfections:

DF-1 cells were plated for transfection in 35 mm sterile plates or equivalent-sized 6-well culture dishes in 2 mL (plates) or 3 mL (6-well dishes) DMEM/10% FBS at $1.5x10^5$ cells/well. 22-24 hours later, the plates were transfected with pTracer-CMV2, pTracer-CMV2-MDV012, and pTracer-CMV2-MDV012ctg, using Fugene 6 according to the following protocol. DF-1 cells were refed in 2 mL fresh DMEM/10% FBS immediately prior to transfection. Transfection solution was made using 3 uL of Fugene 6 in 100 uL of RPMI media per sample, and 1 ug plasmid DNA (diluted in sterile water to 200 ng/uL) per sample was added to the solution after 5-7 minutes of incubation at room temperature. The resulting mixture was incubated for an additional 15-20 minutes to allow the formation of DNA-transfectant complexes, and then the full 108 uL of mixture per sample was added to the appropriate sample dish/well. Samples were incubated at normal culture conditions for 24 hours at which time 3 mL of fresh DMEM/10% FBS was added to each well.

Cells were harvested after an additional 12 hours using 0.5 mL of 0.25% Trypsin-EDTA; trypsinization was stopped as soon as cells began to detach, using 1 mL per sample of DMEM/20% FBS. Cells were pelleted via 30 s of microcentrifugation at 14,000 RPM, the pellets re-suspended and washed in 1 mL PBS, re-pelleted, and re-suspended in 50 uL of diluted primary antibody (1:200 dilution in PBS for concentrated C6B12; 1:150 anti-major and anti-

minor antibody stock; and 50 uL undiluted supernatant for non-concentrated 25-D1-16 and normal chicken serum). The cells were allowed to stain on ice for 30 minutes, then re-pelleted and the supernatant removed, re-suspended and washed with 1 mL PBS, re-pelleted, and re-suspended in 25 uL of secondary antibody dilution (1:100 in PBS, excepting one assay where a 1:150 dilution was used). The cells were again allowed to stain on ice for 30 minutes, re-suspended and washed with 1 mL PBS, re-pelleted and the pellet re-suspended in 300-400 uL of PBS (depending on apparent pellet sizes and consistent across samples within each experiment).

Sample data were collected with flow cytometry using either an LSR II or Vantage TurboSort SE flow cytometer. Data were analyzed using flowjo™ software. Visual gating on live cell populations was performed using irrelevant-antibody-stained, untransfected controls with FSC and SSC signals: gates were set to exclude very low populations on FSC and SSC, while including the largest discrete population of cells. GFP (collected as FITC-A signal or FL-1) and secondary staining (collected as APC-A signal or FL-5) were compared for experimental and control-transfected cells, and visual gates for GFP-positive transfected cells within each experiment were set on stained, untransfected controls to include 99.5% of live cells in the GFP-negative population in untransfected samples. This type of gating was used to allow histogram comparison of Cy5 or AlexaFluor 633 staining within transfected populations only.

4.4. Results:

4.4.1. Cloning:

The recombinant MDV012ctg gene was successfully amplified from SW105-Md5-MDV012ctg BAC #32/pBAD-SceI using Phusion PCR and cloned into pTracer-CMV2, as demonstrated by the two insert fragments of 1044 bp and 680 bp respectively which is produced

when pTracer-CMV2-MDV012ctg is cut with the PmeI and KpnI restriction enzymes (Figure 4.1.; a large 6.1 kb vector backbone fragment is also visible as expected). Sequencing data confirmed the presence of the A-to-C mutation at the MDV012 start codon in this plasmid. DF-1 cell transient transfection tests were subsequently performed using this plasmid, the vector pTracer-CMV2, and the wild-type plasmid pTracer-CMV2-MDV012.

4.4.2. Transfections:

4.4.2.a. C6B12:

Figure 4.2. shows the results of four transient transfection experiments of DF-1 cells with these plasmids, stained with C6B12 for overall surface MHC class I and secondarily stained with Alexa-Fluor 633 to allow flow cytometric analysis. Geometric mean fluorescence intensity (GMFI; arbitrary units) was calculated for MHC class I for each sample (Sub-Figures 4.2.1.d., 4.2.2.d., 4.2.3.d., 4.2.4.d.). In some samples (transfectants and controls) a small population of cells with high C6B12 staining was present, in both untransfected and transfected cell populations; therefore GMFI was chosen as a robust measure of central tendency to compare MHC class I expression in the overall transfected cell population. DF-1 cells transiently transfected with either wild-type pTracer-CMV2-MDV012 or the mutant pTracer-CMV2-MDV012ctg showed a small but consistent decrease in C6B12 staining as compared to cells transfected with vector (Sub-Figure 4.2.5.). This decrease averaged about 20% of vector GMFI for both wild-type and mutant alleles, varying from 7% to 30% depending on the experiment.

Analysis by Friedman's test for non-parametric blocked data (in this case, logarithmic data in repeated measures) just barely reached statistical significance for difference between the three plasmids ($p=0.0498$), however, sample sizes ($n=4$ experiments) were not large enough to

determine significant differences between individual plasmids by post-hoc Wilcoxon signed rank testing (which requires a minimum of 5 samples per block). Nonetheless, the difference in C6B12-staining between pTracer-CMV2-transfected DF-1 cells and pTracer-CMV2-MDV012 cells was similar to that seen in earlier experiments (see Chapter 1) and can therefore be expected to be significant, whereas no difference can be observed between pTracer-CMV2-MDV012 and pTracer-CMV2-MDV012ctg (Sub-Figure 4.2.5). Therefore, these data appear to confirm the previously noted decrease in MHC class I expression in MDV012-transfected cells, while indicating that MDV012ctg inhibits MHC class I equally to the wild-type allele.

4.4.2.b. MHC class I major:

DF-1 cells transiently transfected with the same three plasmids were compared for major allele expression of the MHC class I B21 haplotype, which is known to be present in the chicken line these cells were derived from. Figure 4.3 shows the results of four experiments stained with anti-BF2*B1 antibody H1222-RP9BFIV21m78. Suprisingly, results from only the initial 3 out of 4 experiments mirrored the results of C6B12 experiments, with the fourth experiment showing the opposite effect of transfection with either MDV012 or MDV012ctg (Sub-Figure 4.3.4.). This experiment was performed at the same time as the fourth C6B12 experiment (Sub-Figure 4.2.4.), which showed the expected down-regulation of MHC class I, indicating that the major B21 allele and overall MHC class I expression were differentially affected. MHC class I major expression, averaged for all four experiments, did not appear significantly different between MDV012 and MDV012ctg plasmids and vector control (Sub-Figure 4.3.5.).

4.4.2.c. MHC class I minor:

DF-1 cells transiently transfected with the three plasmids were compared for expression of the MHC class I B21 minor allele. Figure 4.4. shows the results of three transfection

experiments stained with anti-BF1*21 antibody E6338. On average, no differences in MHC class I minor expression were seen between MDV012 and MDV012ctg plasmids and vector control (Sub-Figure 4.4.4.).

4.5. Discussion:

Transfection experiments with plasmids expressing wild-type MDV012 and a start-codon mutant, MDV012ctg, showed that both alleles similarly mildly decreased overall MHC class I expression on DF-1 cells. This result for MDV012 is similar to results obtained previously (see Chapter 1). Expression of the major MHC class I molecule followed a similar pattern in 3 out of 4 experiments but was increased in MDV012 and MDV012ctg-transfected DF-1 cells in the final experiment, although overall MHC class I expression in cells plated and transfected from the same stock at that time continued to show the previous pattern of down-regulation in these samples. Transfection with MDV012 and MDV012ctg had no consistent effect on B21 minor MHC class I. This may not be surprising given that MHC class I minor alleles are expressed at a significantly lower level in general, which might reduce the sensitivity of the assay to small effects. On the other hand, the minor chicken MHC class I alleles behave somewhat differently than the major alleles in that they are apparently undergoing separate selection (Shaw 2007), differ in peptide specificity from the major allele and peptide-translocation machinery (Kaufman 2008), and might have different roles in immune function (Livant 2004). It is possible that the BF1*21 MHC class I allele might have a different susceptibility than the BF2*21 MHC class I allele to down-regulation by MDV012 due to differences in reliance on TAP complex function, consistent with our observation that MDV012 down-regulates MHC class I molecules through inhibition of TAP (see Chapter 2).

The unexpected result that B21 major MHC class I allele did not follow the same pattern as the results for overall MHC class I expression suggests that the antibodies used may not be seeing the all same targets on this cell line. DF-1 cells are an immortalized B21-haplotype chicken embryonic fibroblast cell line which is readily transformed by oncogene expression (Himly 1998) and can spontaneously transform with continuous passage (Silva lab, unpublished observations). Given that these cells were prophylactically treated for 2 weeks with an anti-mycoplasma antibiotic which has been found to have genotoxic effects in some eukaryotic cells (Itoh 2006), it is possible that the changes seen in MHC class I major allele expression in later transfections were due in part to a transformation event with subsequent accumulation of cells with an altered MHC class I expression phenotype (and in fact, an apparent increase in cell growth rate which was noted prior to the last transfection experiment which might support this hypothesis). However, this would not fully explain differences between overall MHC class I (decreased in MDV012 transfectants) and major and minor MHC class I (increased in MDV012 transfectants) in late experiments, unless another MHC class I-like epitope is also being seen by one of the antibodies used. The chicken MHC locus contains non-classical MHC class I (Rfp-Y locus) genes with significant homology to classical MHC class I (BF) genes (Affanasief 2001); it is possible that one of these genes is being differentially regulated by transfection with MDV012 as well, and might cross-react with one of these antibodies.

Previous experiments showed a substantially greater down-regulation effect on MHC class I expression when the spliced cDNA form of MDV012 was transfected than when the genomic form was used. The small effects seen in this study with C6B12 correlate well with effects seen previously with genomic MDV012; it is likely that larger effects would have been seen if plasmids containing spliced MDV012 were used in this study, and the system used in this

assay was thus more stringent. A second potential limitation on effect size in this study was the use of B21 haplotype chicken cells, as the B21 MHC class I haplotype is known to confer resistance to MDV in chickens (Briles 1977). Given that the major MHC class I molecule is closely linked to its co-evolving TAP complex (discussed in Kaufman 2000), resistance or susceptibility of MHC haplotypes to MDV could be due not only to peptide specificity of the expressed MHC class I alleles (mediated by both MHC binding and peptide transport by TAP), but also to differences in susceptibility to viral evasins such as TAP inhibitors. Comparing MDV012's effect in cells differing in MHC haplotype, such as CEFs from MHC-congenic chicken lines, could help determine whether MHC class I down-regulation by MDV012, an effect we believe to be mediated through TAP inhibition, plays a differentiating role in MHC haplotype-based resistance to MDV.

Using this assay, we determined that mutation of the MDV012 start codon did not prevent down-regulation of MHC class I in transfected chicken cells, suggesting that an alternative translation occurred. As discussed in Chapter 3, MDV012 is a spliced gene with a short exon 1 and longer exon 2; the most likely case is that in MDV012ctg the long second exon is translated independently, preserving a significant portion of the protein's function. Less likely would be use of an alternative start codon; as there are no in-frame ATG codons within the putative first exon, use of an alternative start site would require an alternative splice donor start site as well, or a truncated protein would likely result; even so, such a translation product would have a missense N-terminal. We previously determined that exon 1 provides part, but not all, of the spliced MDV012 protein's function, in transfection assays (see Chapter 2, Figure 6: "MDV011"); therefore it appears likely that MDV encodes an immune evasion gene with two exons which each contribute to MHC class I down-regulation function, and which could

potentially be alternatively transcribed or translated to produce 2 or more separate gene products with immune evasion activity.

Given the above results, we predict that the MDV012 start codon mutant MDV viruses we designed (described in Chapter 3) will not completely lack MHC class I down-regulation function, due to expression of at least the second exon of MDV012 which provides significant MHC class I evasion ability. In order to fully attenuate MHC class I evasion in these viruses, it will likely be necessary to further mutate the MDV012 coding sequence, which could be accomplished through several means using the same methods described in Chapter 3. Possible approaches to consider include point mutation of the initiation codon in the second exon to prevent alternative translation; incorporation of early stop codons in both exons; or full deletion of MDV012 from the MDV genome. Furthermore, development of antibodies which recognize MDV012, or the peptides encoded by the individual exons, will help determine whether MDV012 translation has been deleted successfully. Viruses lacking MDV012 function will be necessary to determine in vitro and in vivo effects of this gene on immune function.

APPENDIX

FIGURES

Figure 4.1. Restriction fragment analysis of pTracer-CMV2-MDV012ctg clones. DNA from pTracer-CMV2-MDV012ctg plasmid minipreps were double-digested with PmeI and KpnI and electrophoresed on a 1% agarose minigel. Lanes 1-5 represent digests from 5 separate plasmid minipreps, all of which showed the expected restriction fragment pattern. Lane 9 contains 100 bp ladder (NEB) and lane 10 contains 1 kb ladder (NEB).

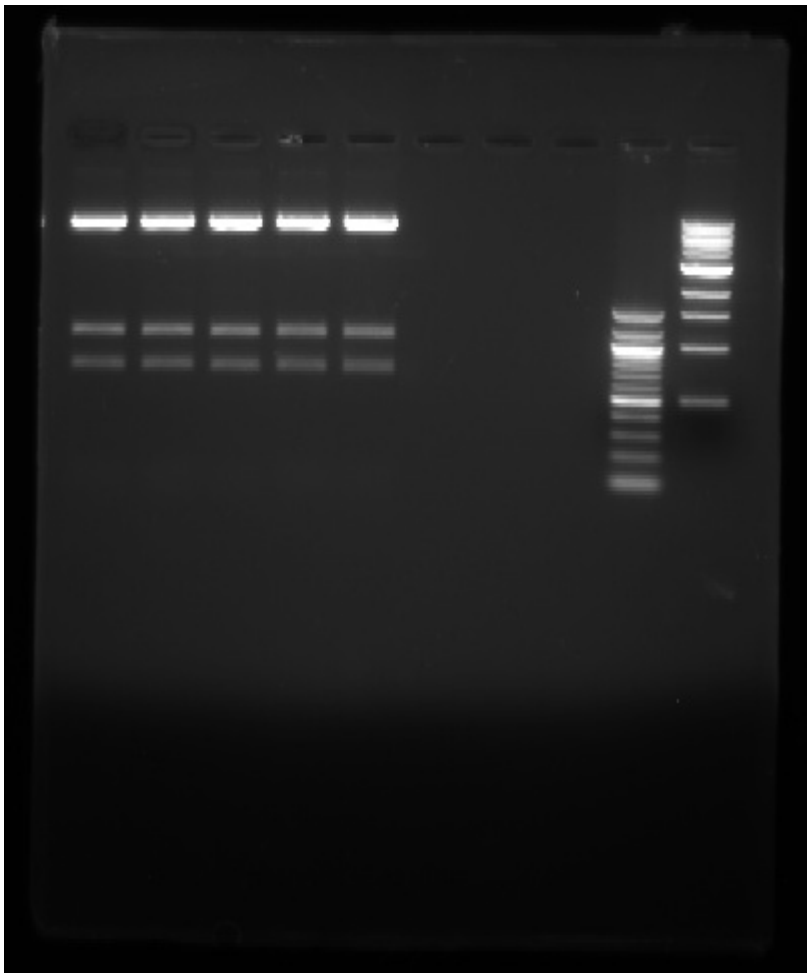


Figure 4.2. DF-1 cells transiently transfected with MDV012 and MDV012ctg plasmids express less MHC class I (as measured by C6B12 staining) than vector controls. DF-1 cells were transfected with pTracer-CMV2, pTracer-CMV2-MDV012, or pTracer-CMV2-MDV012ctg plasmids and stained with C6B12 (mouse anti-chicken MHC class I IgG), then with Alexa-Fluor 633-conjugated anti-mouse IgG. Cells were analyzed by flow cytometry, gated on live and transfected cell (GFP+) populations, and GFP+ cells were compared for C6B12 staining as measured by Alexa-Fluor 633 intensity. Four experiments are shown.

4.2.1: Experiment 1.

4.2.1.a. Live gate. Live cell gate shown on isotype control and C6B12-stained non-transfected cells.

4.2.1.a.i Isotype control:

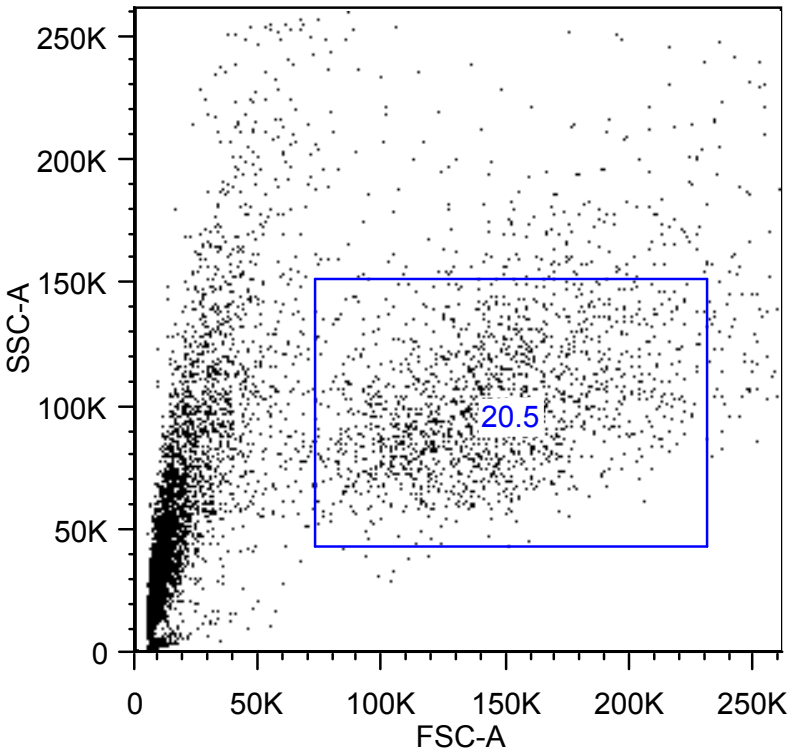


Figure 4.2. (cont'd)

4.2.1.a.ii C6B12:

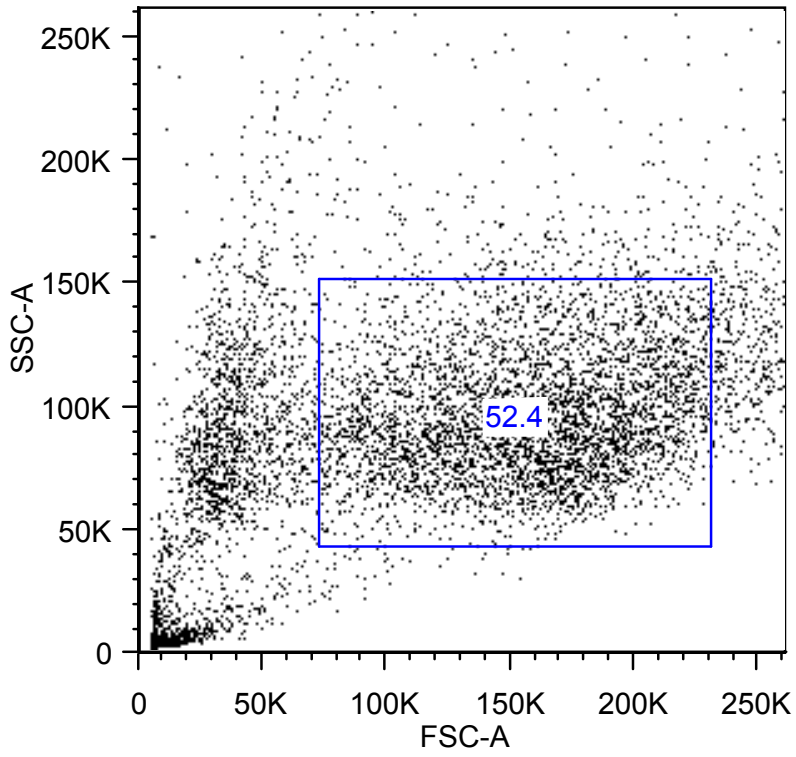


Figure 4.2. (cont'd)

4.2.1.b. Transfected gate. Transfected gate set on non-transfected C6B12-stained cells (<5% positive).

4.2.1.b.i. C6B12:

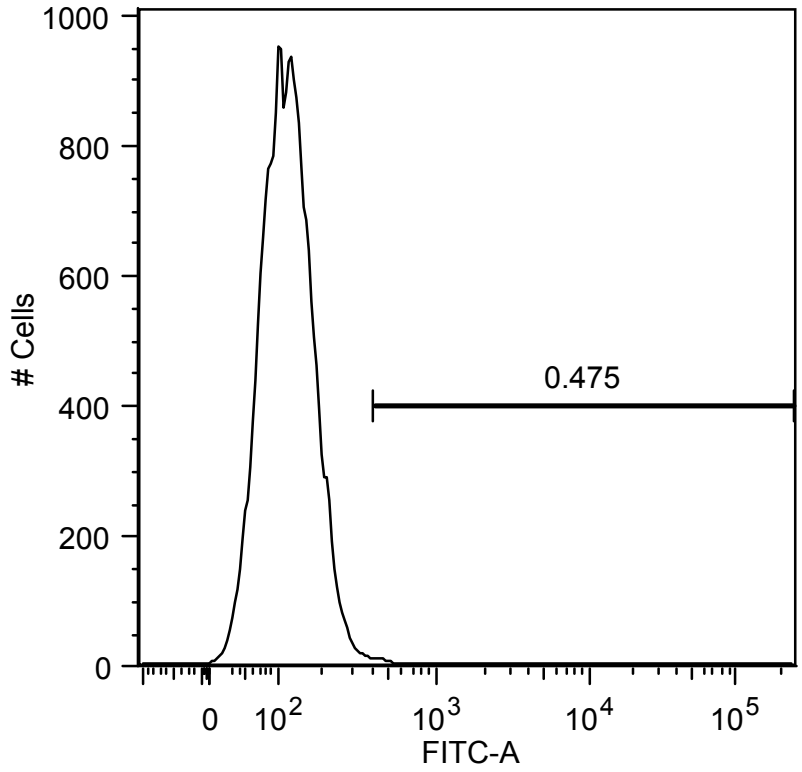
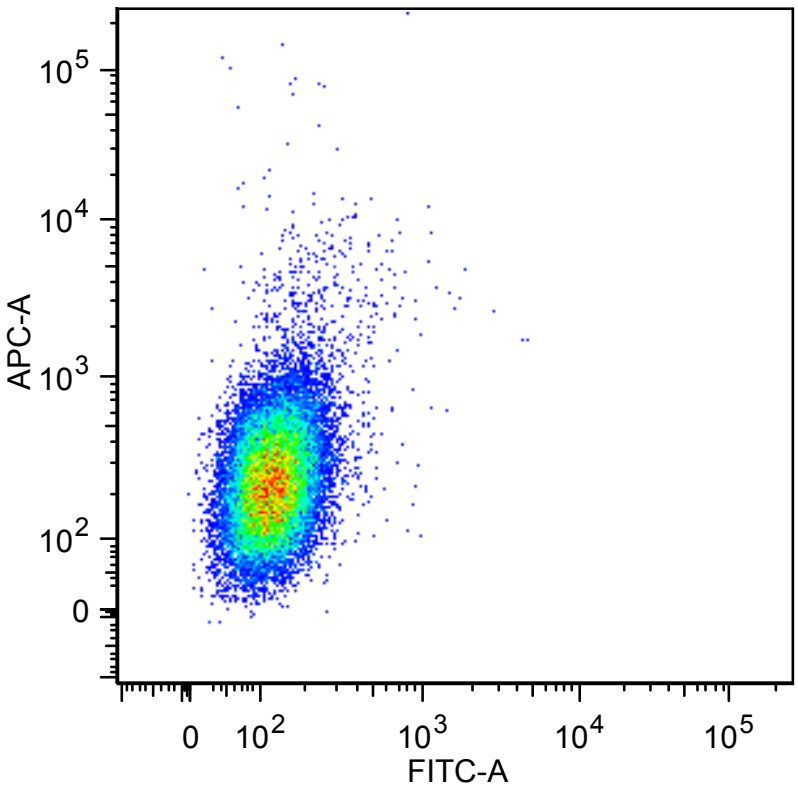


Figure 4.2. (cont'd)

4.2.1.c. GFP (FITC-A) vs. Alexa-Fluor 633 (APC-A). Live cells (pseudocolor dot plots).

4.2.1.c.i. C6B12:



4.2.1.c.ii. pTracer-CMV2:

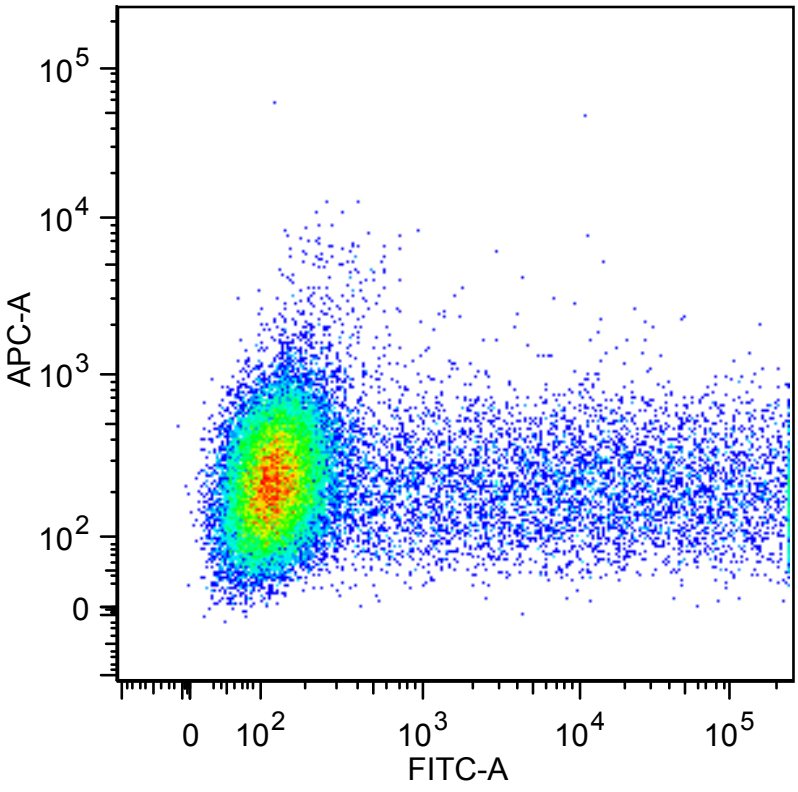
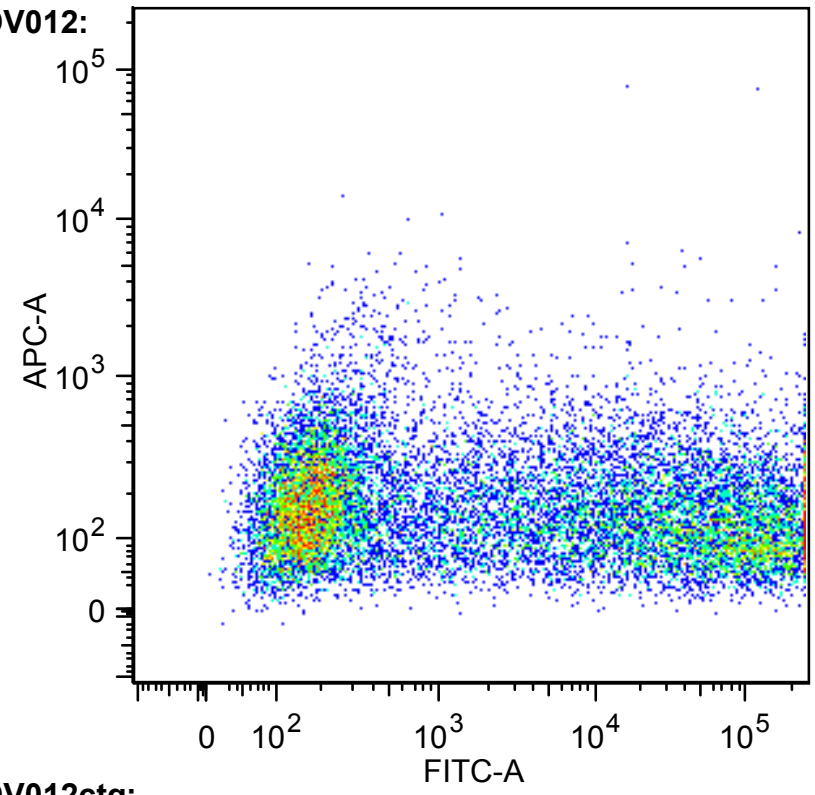


Figure 4.2. (cont'd)

4.2.1.c.iii. pTracer-CMV2-MDV012:



4.2.1.c.iv. pTracer-CMV2-MDV012ctg:

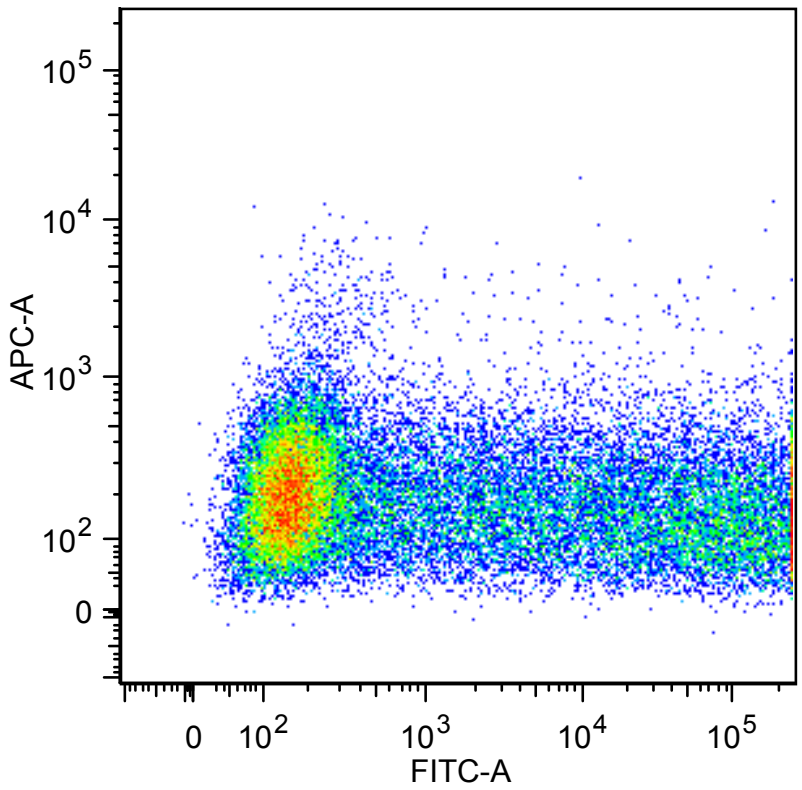
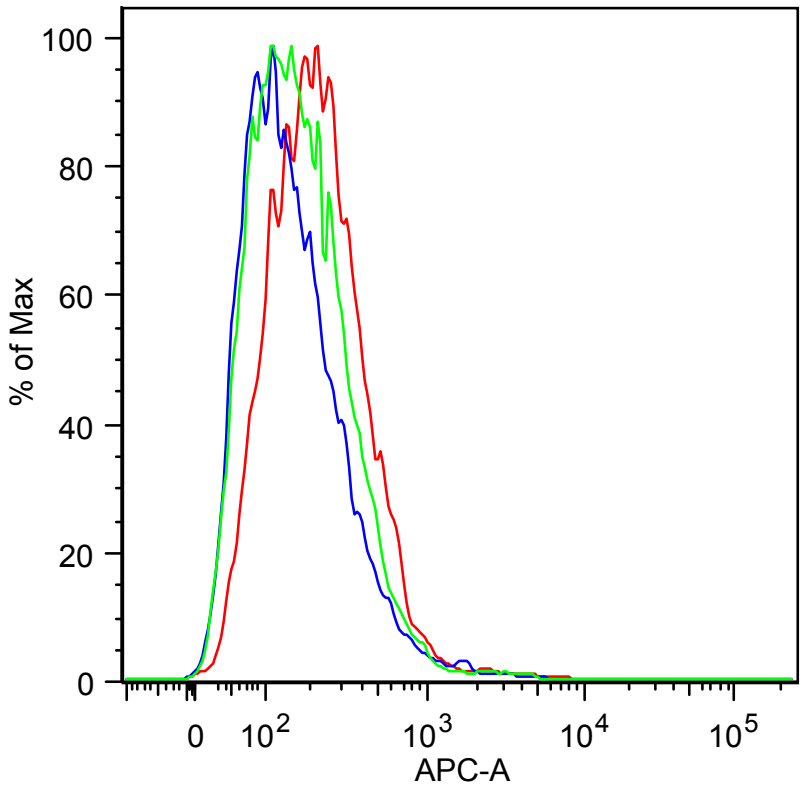


Figure 4.2. (cont'd)

4.2.1.d. MHC class I (histogram). Alexa-Fluor 633 fluorescence intensity histogram showing live, GFP+ cell populations stained with C6B12. Labels and geometric mean fluorescent intensities (GMFI) are noted in the box below.



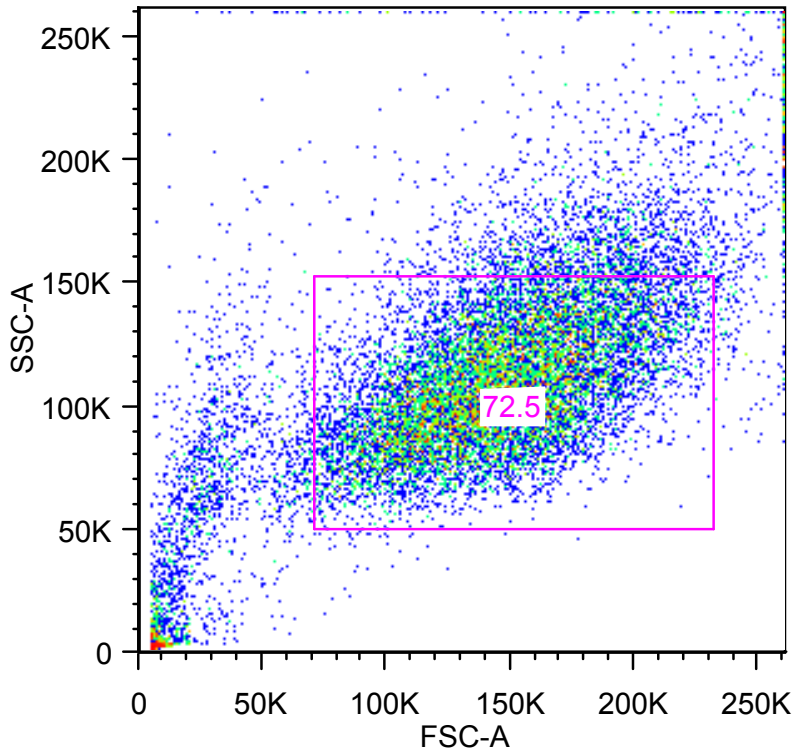
Sample:	GMFI:
CMV2	218
MDV012	154
MDV012ctg	169

Figure 4.2 (cont'd)

4.2.2. Experiment 2.

4.2.2.a. Live gate. Live cell gate shown on unstained control and isotype-control-stained untransfected cells.

4.2.2.a.i. Unstained:



4.2.2.a.ii. Isotype control:

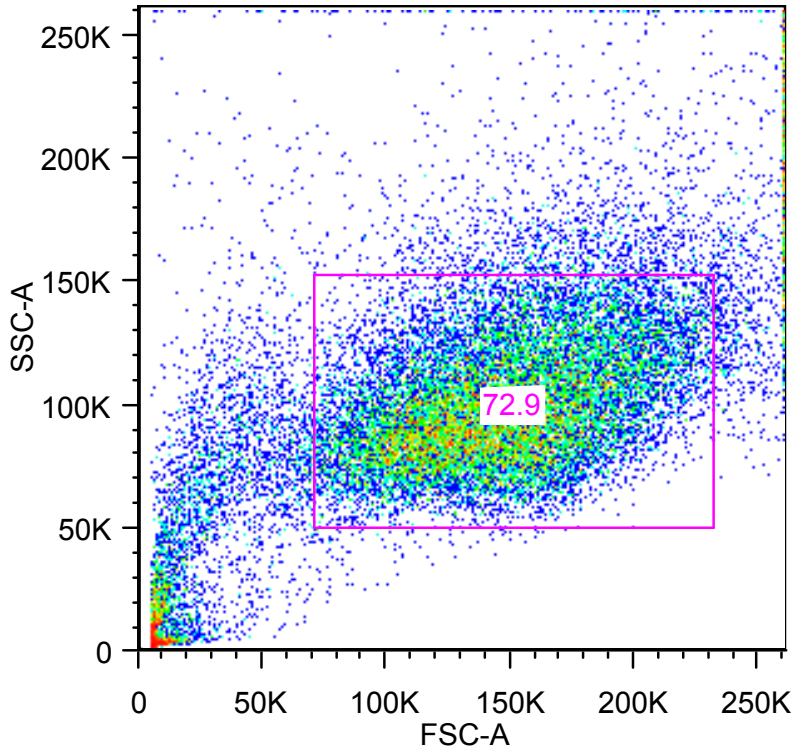
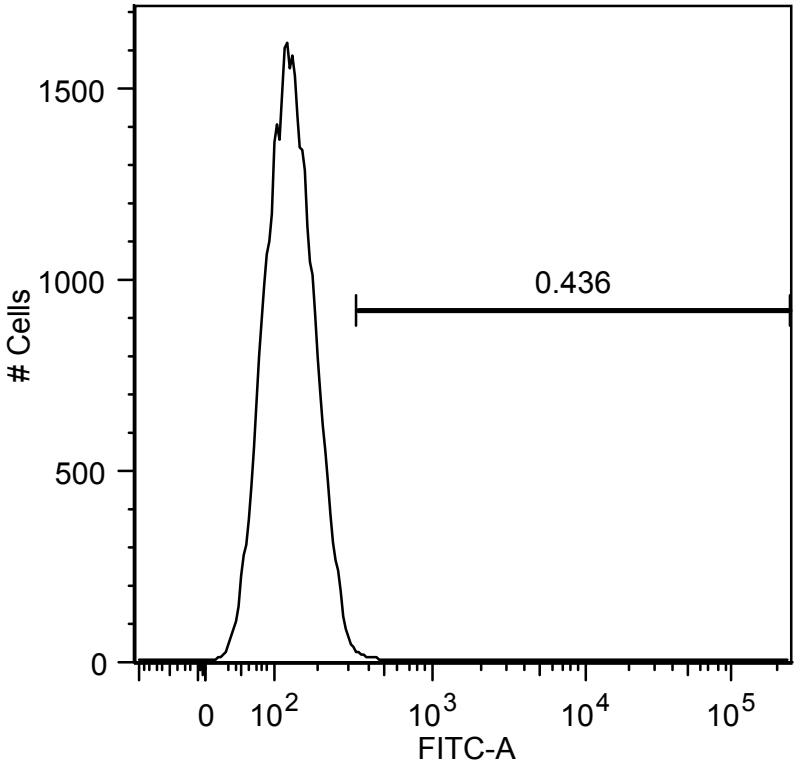


Figure 4.2. (cont'd)

4.2.2.b. Transfected gate. Set on non-transfected C6B12-stained cells (<5% positive), and applied to transfected samples. Transfection efficiencies are noted as percent GFP-positive.

4.2.2.b.i. C6B12:



4.2.2.b.ii. pTracer-CMV2:

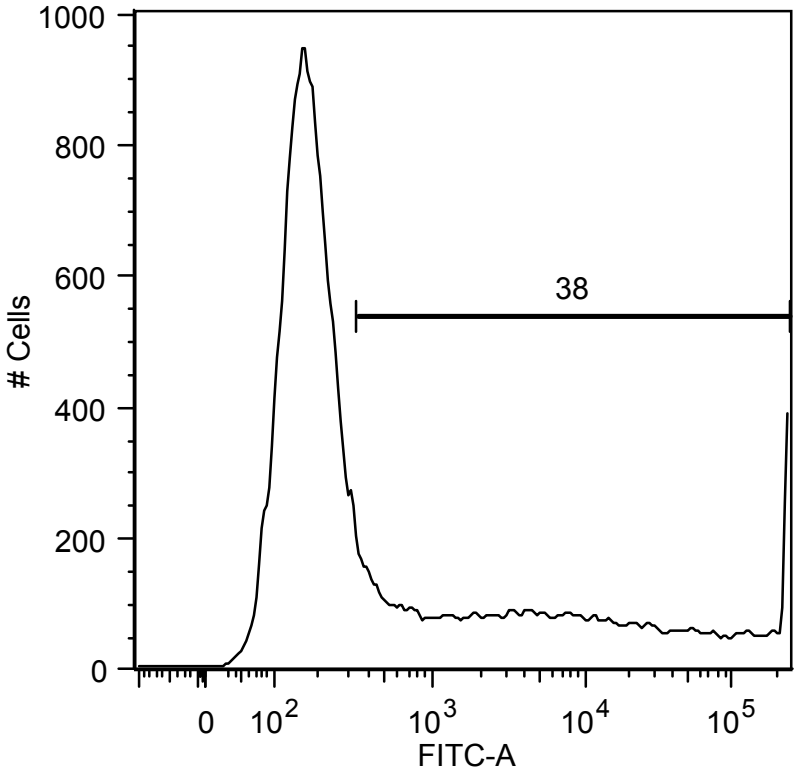
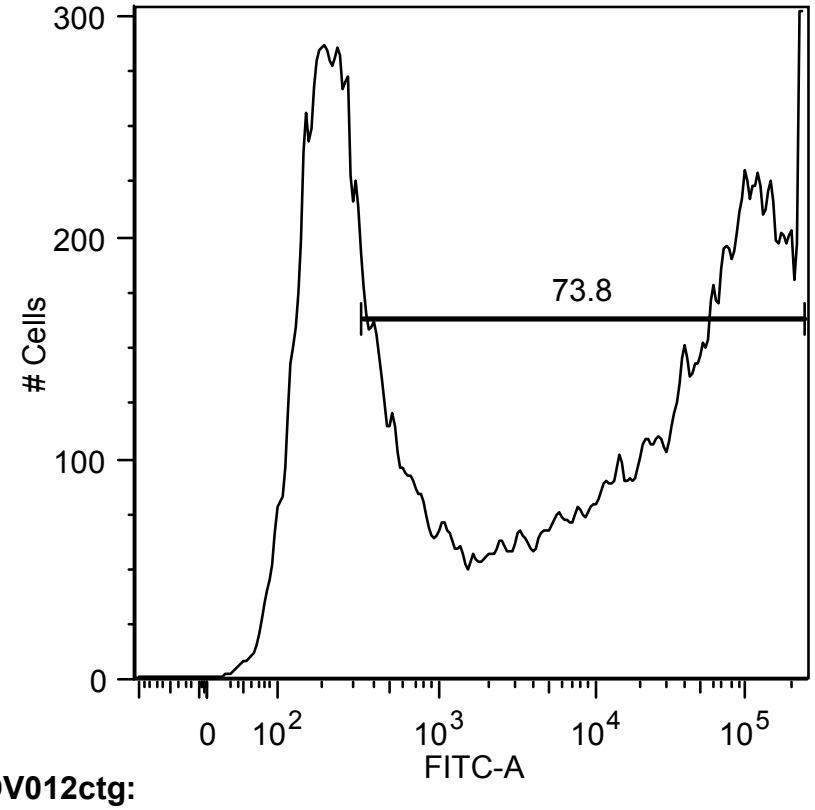


Figure 4.2. (cont'd)

4.2.2.b.iii. pTracer-CMV2-MDV012:



4.2.2.b.iv. pTracer-CMV2-MDV012ctg:

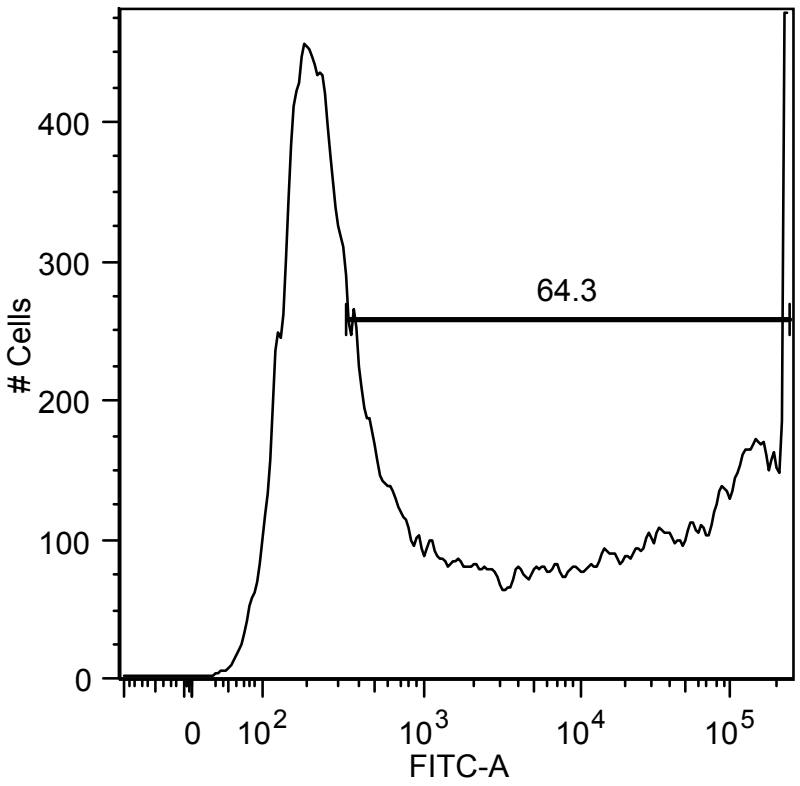
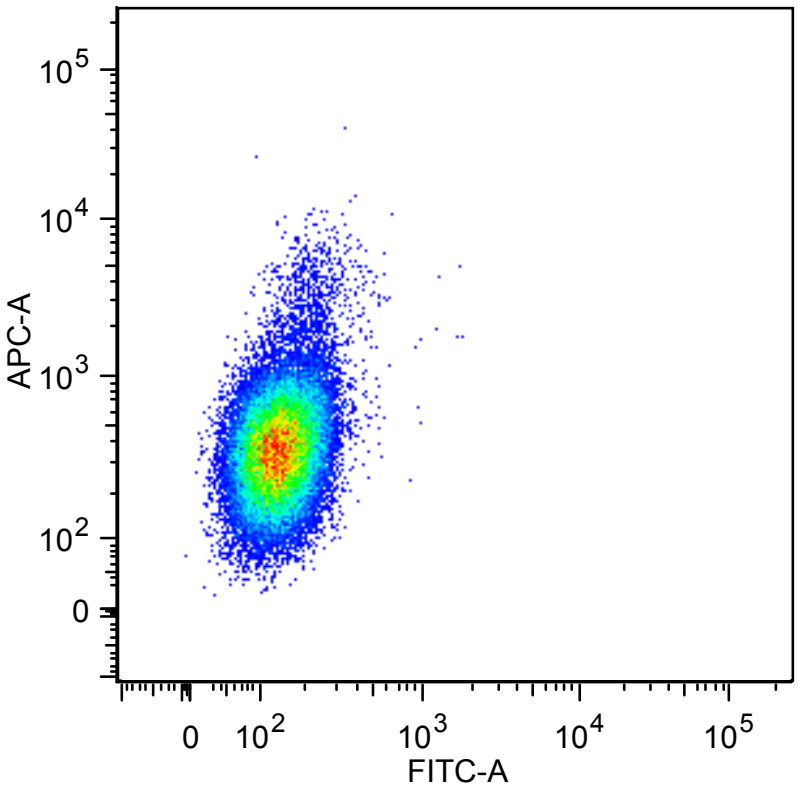


Figure 4.2. (cont'd)

4.2.2.c. GFP (FITC-A) vs. Alexa-Fluor 633 (APC-A). As in Experiment 1 (Figure 4.3.1.c.).

4.2.2.c.i. C6B12:



4.2.2.c.ii. pTracer-CMV2:

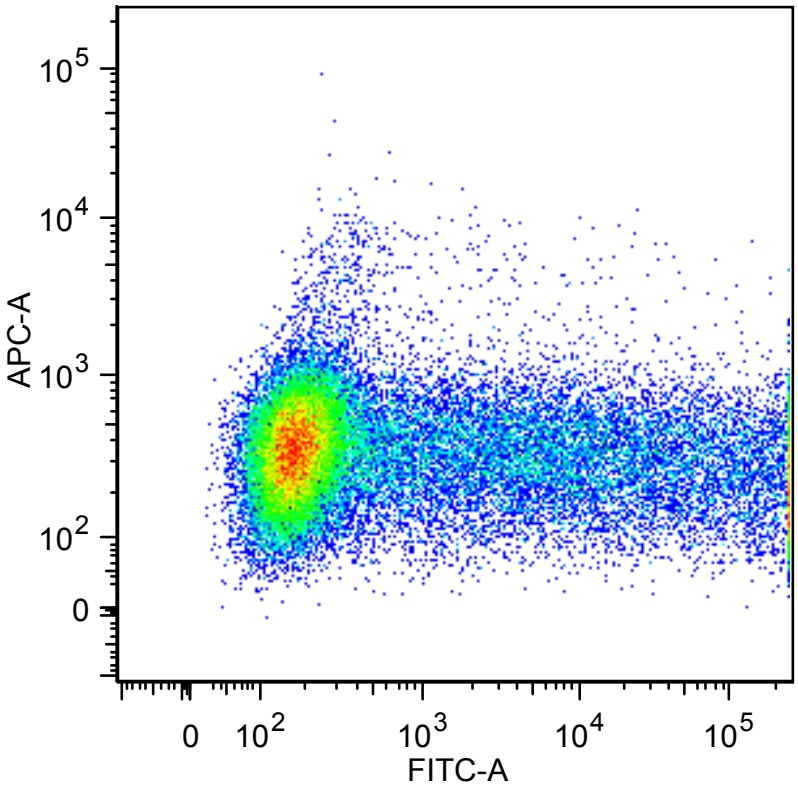
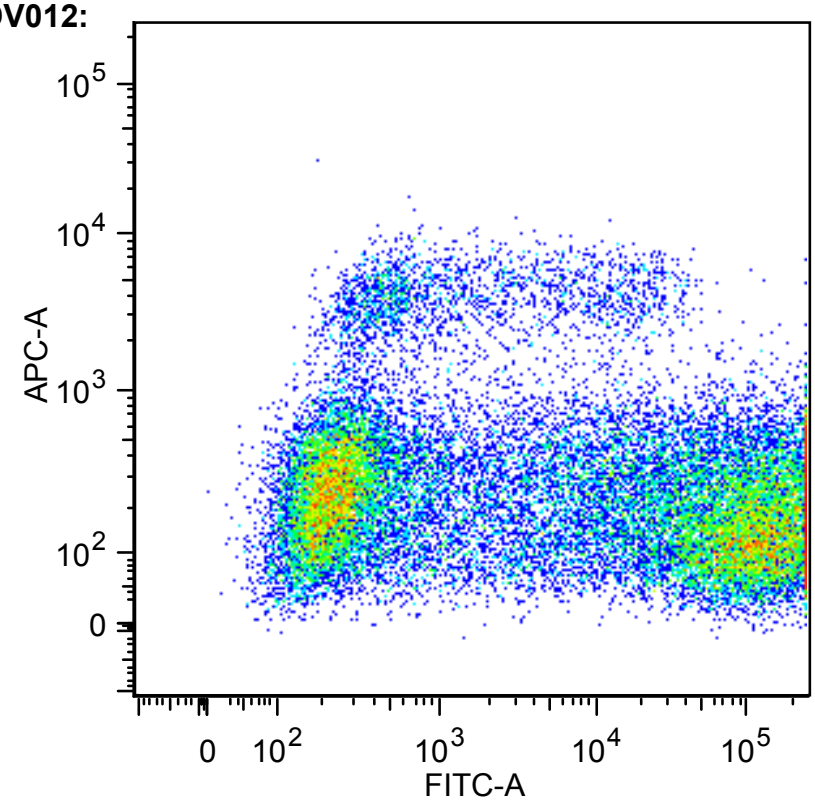


Figure 4.2. (cont'd)

4.2.2.c.iii. pTracer-CMV2-MDV012:



4.2.2.c.iv. pTracer-CMV2-MDV012ctg:

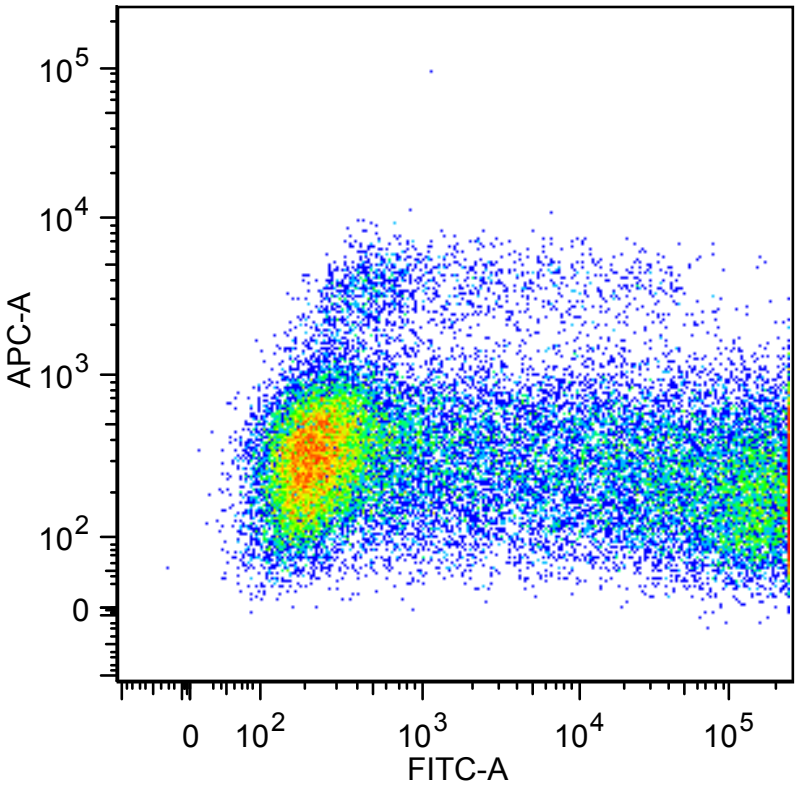
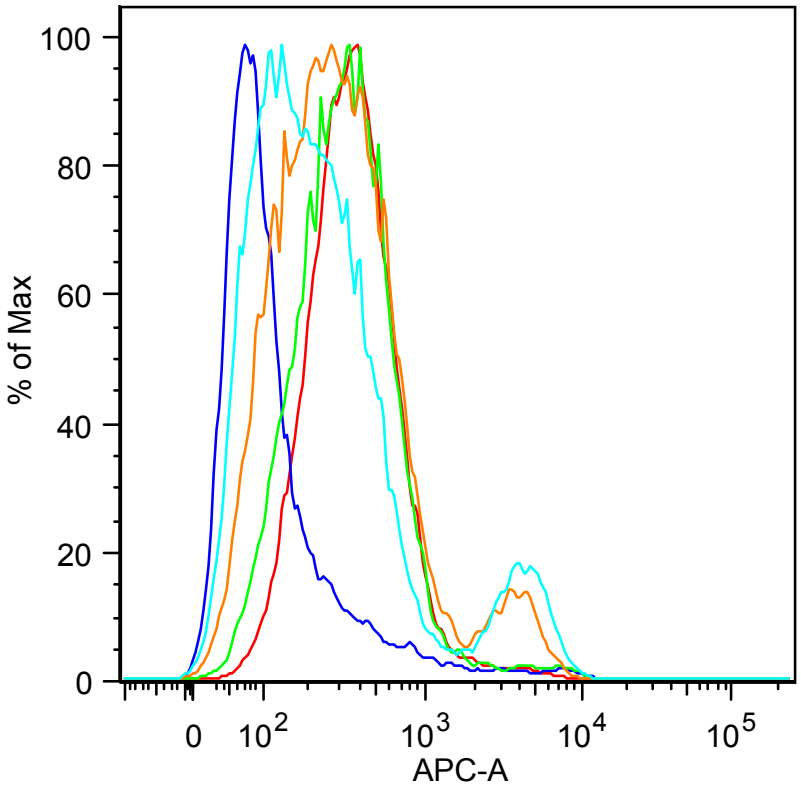


Figure 4.2. (cont'd)

4.2.2.d. MHC class I (histogram). C6B12/Alexa-Fluor 633 fluorescence intensity histogram showing live, transfected cell populations (live cells for untransfected isotype- and C6B12-stained controls). Labels and GMFIs are noted in the box below.

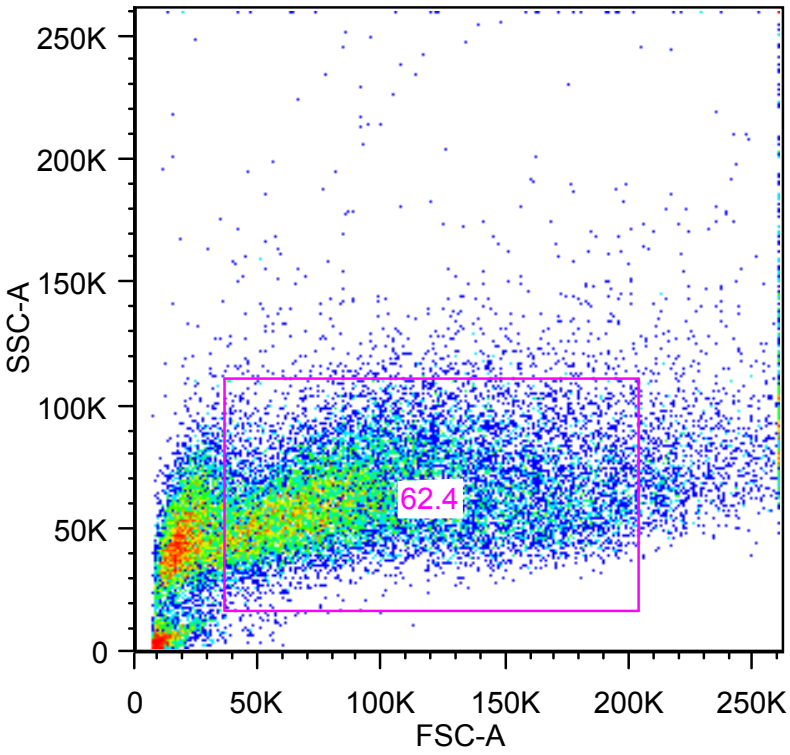


Sample	GMFI
Isotype control	123
C6B12	363
CMV2	328
MDV012	249
MDV012ctg	307

Figure 4.2 (cont'd)

4.2.3. Experiment 3.
4.2.3.a. Live gate. As in Experiment 2 (4.2.2.a.).

4.2.3.a.i. Unstained:



4.2.3.a.ii. Isotype control:

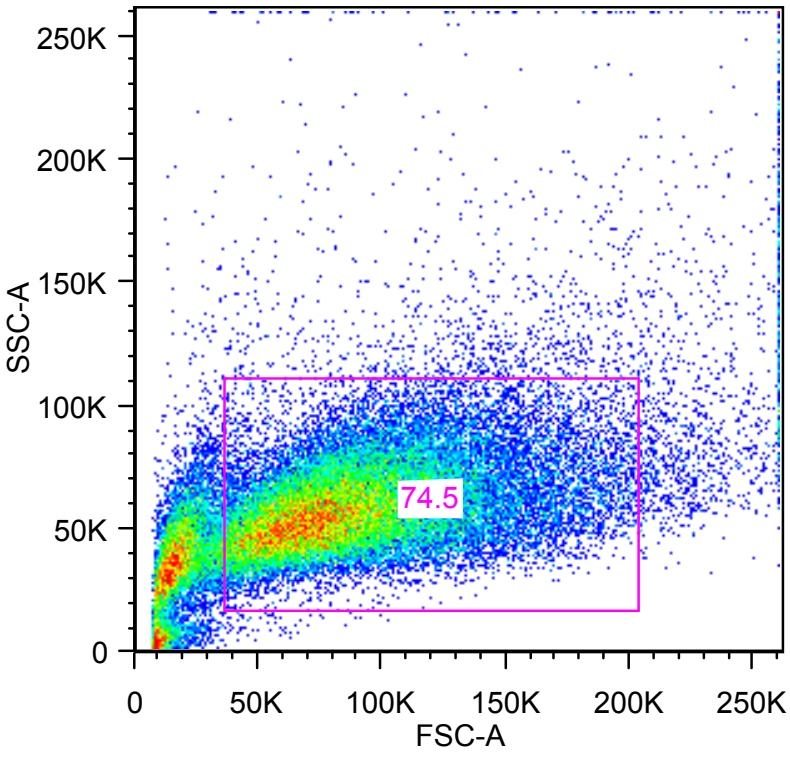
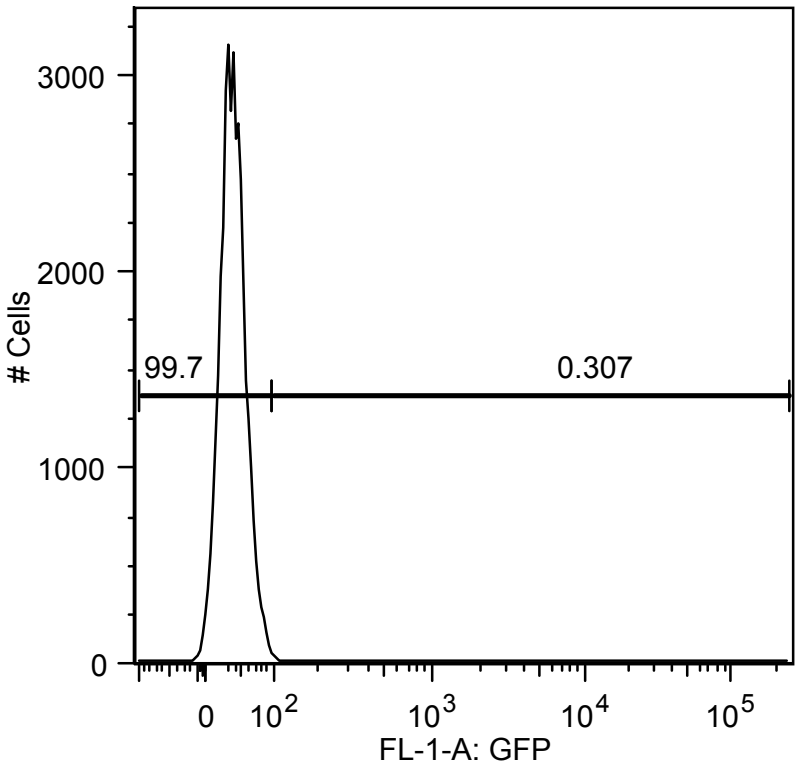


Figure 4.2. (cont'd)

4.2.3.b. Transfected gate. Set on non-transfected isotype-control-stained cells (<5% positive), and applied to transfected samples.

4.2.3.b.i. Isotype control:



4.2.3.b.ii. pTracer-CMV2:

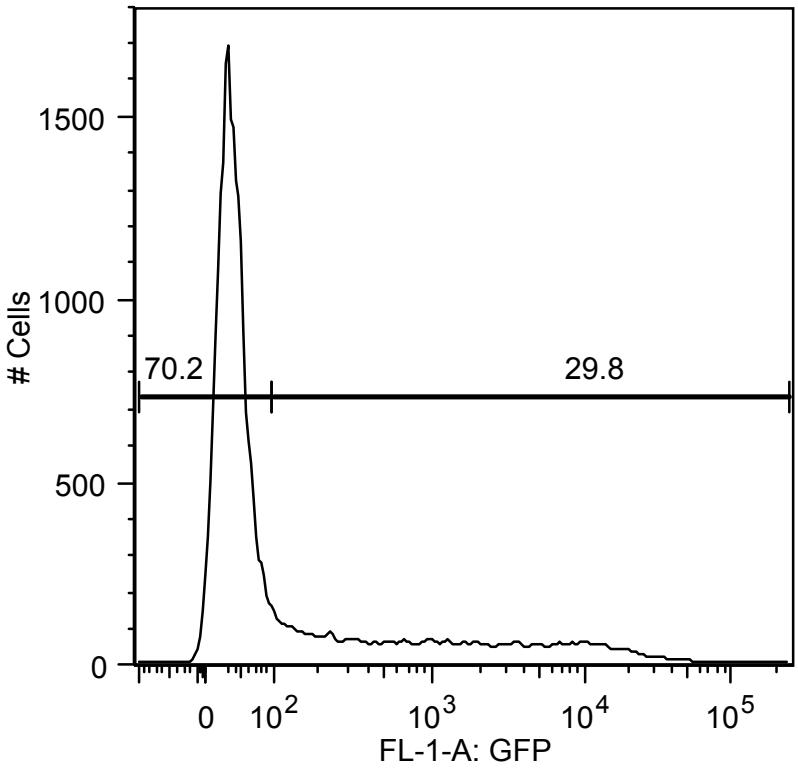
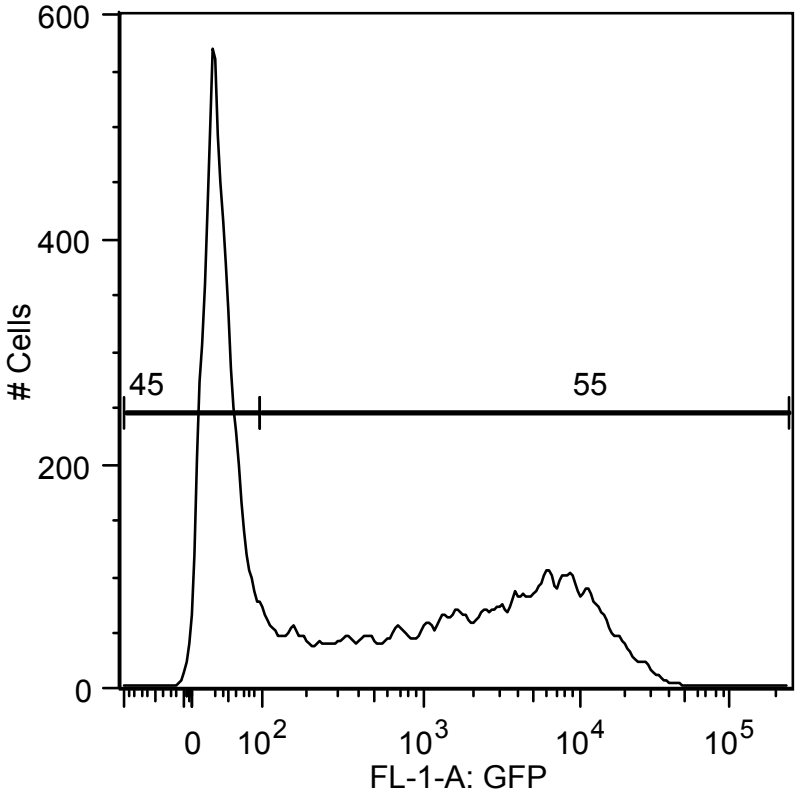


Figure 4.2. (cont'd)

4.2.3.b.iii. pTracer-CMV2-MDV012:



4.2.3.b.iv. pTracer-CMV2-MDV012ctg:

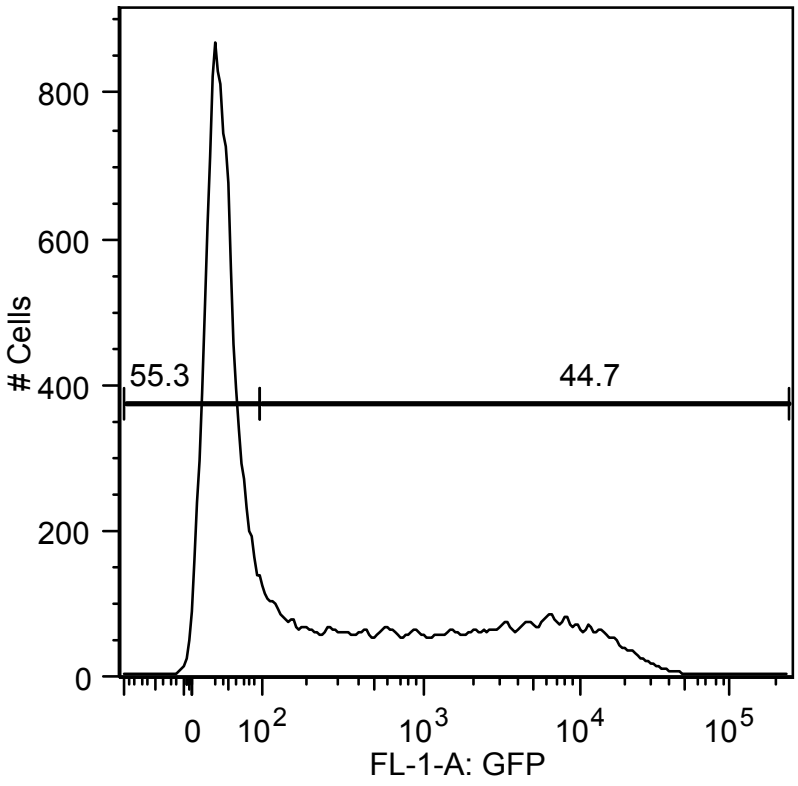
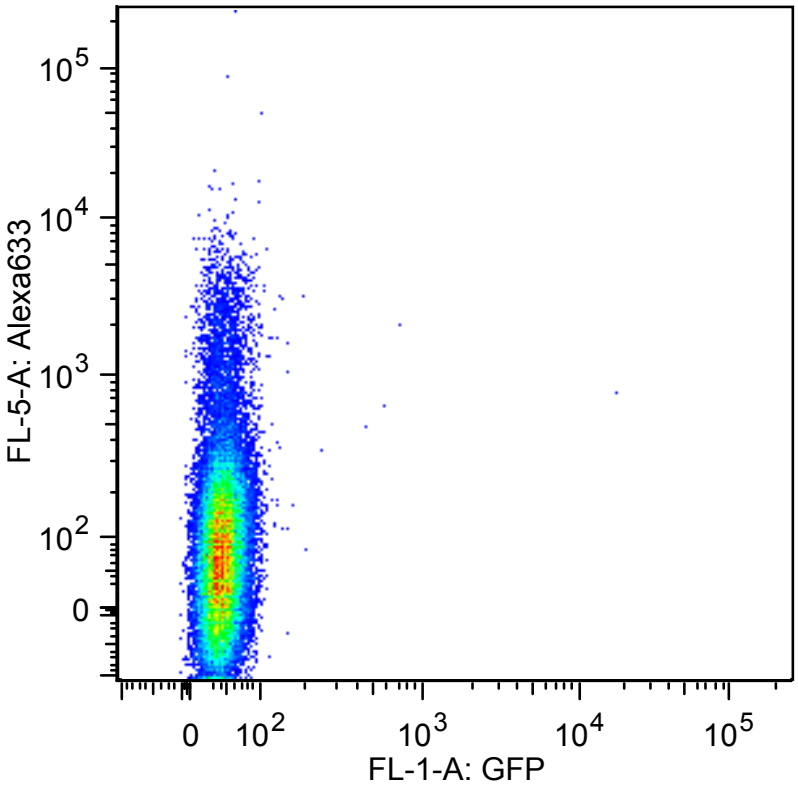


Figure 4.2. (cont'd)

4.2.3.c. GFP vs. Alexa-Fluor 633. As in Experiment 1 (4.2.1.c.).

4.2.3.c.i. Isotype control:



4.2.3.c.ii. pTracer-CMV2:

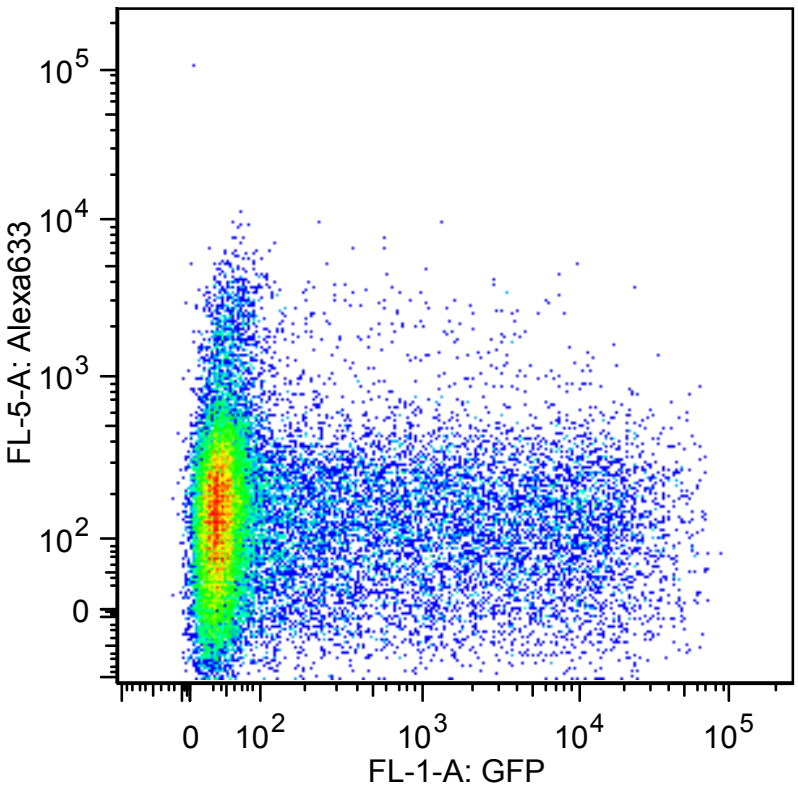
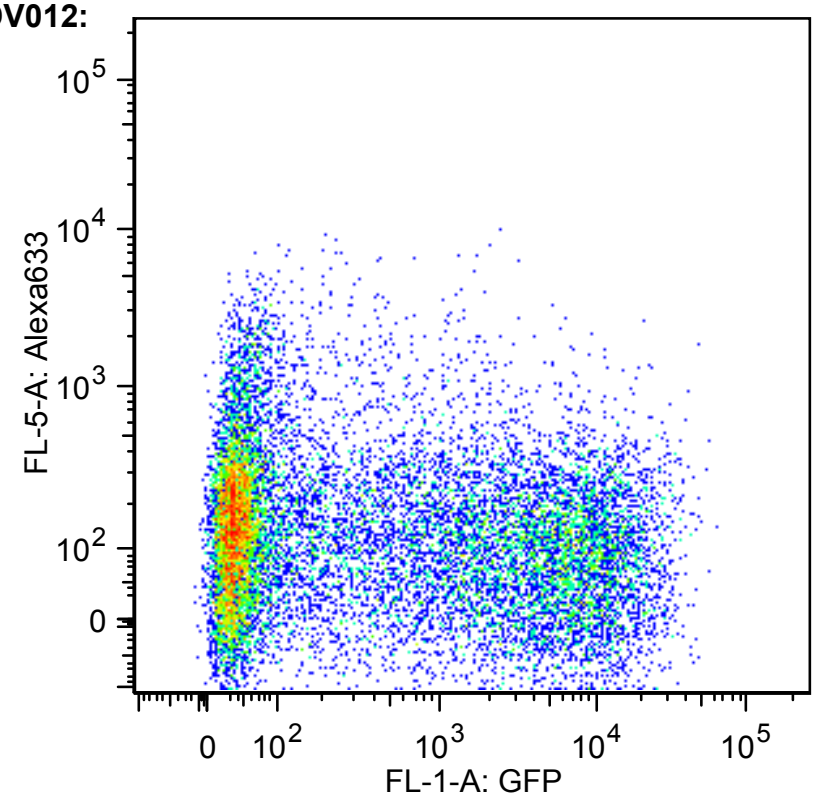


Figure 4.2. (cont'd)

4.2.3.c.iii. pTracer-CMV2-MDV012:



4.2.3.c.iv. pTracer-CMV2-MDV012ctg:

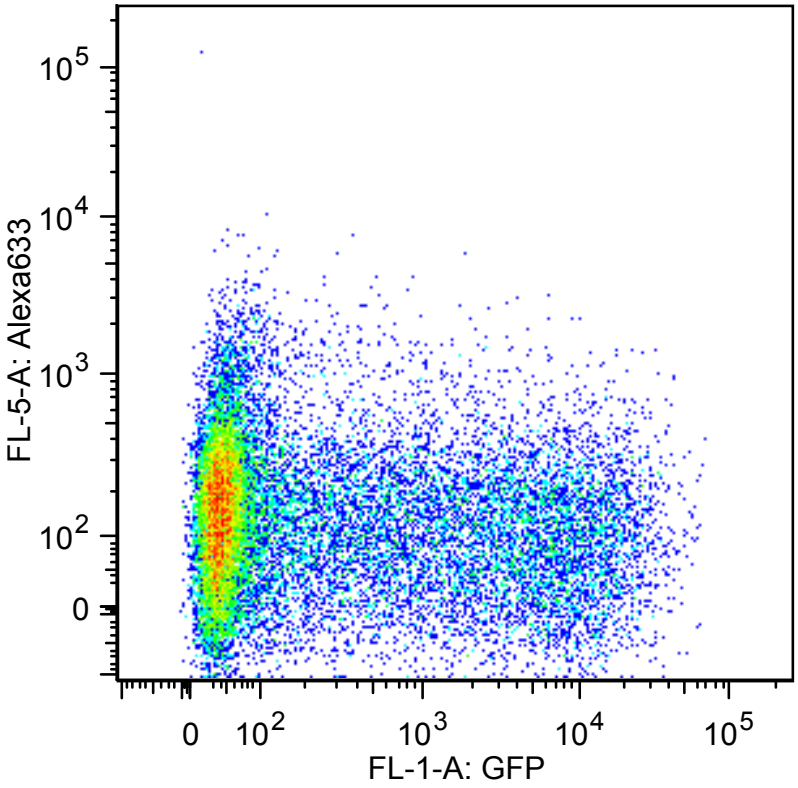
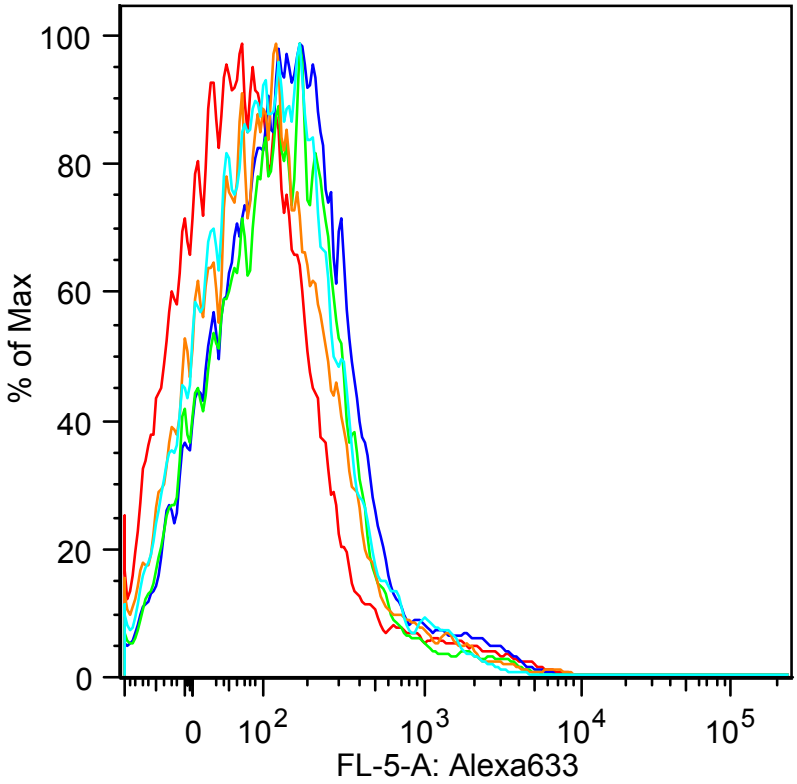


Figure 4.2. (cont'd)

4.2.3.d. MHC class I (histogram). C6B12/Alexa-Fluor 633 fluorescence intensity histogram of live, transfected cells (live cells for untransfected controls). GFP(-) cells from the pTracer-CMV2-transfected sample are included as a C6B12-stained, untransfected control. Labels and GMFIs are noted in the box below.

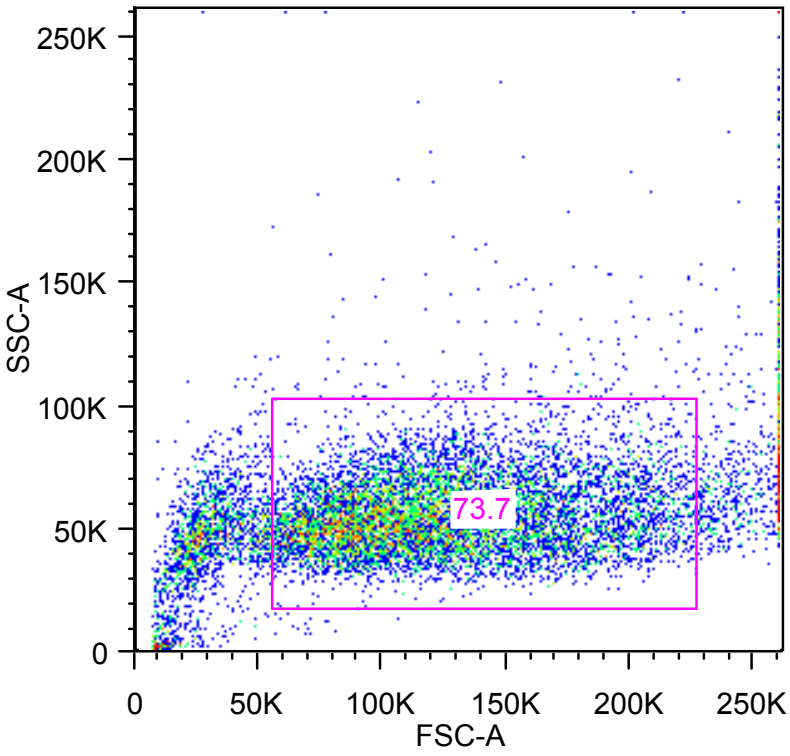


Sample:	GMFI:
Isotype control	80
CMV2 GFP(-)	143
CMV2 GFP(+)	125
MDV012	107
MDV012ctg	114

Figure 4.2 (cont'd)

4.2.4. Experiment 4.
4.2.4.a. Live gate. As in Experiment 2 (4.2.2.a.).

4.2.4.a.i. Unstained:



4.2.4.a.ii. Isotype control:

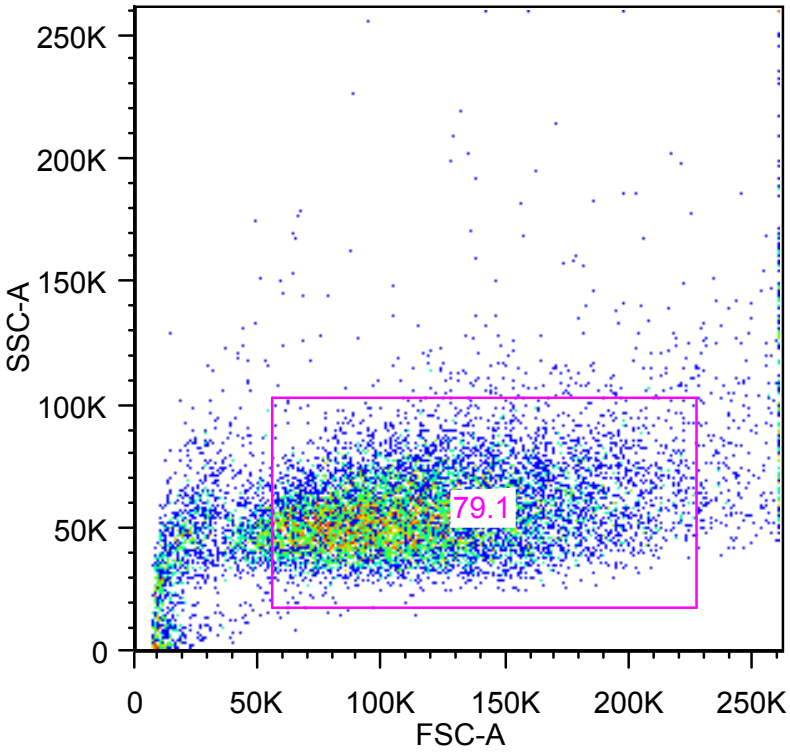
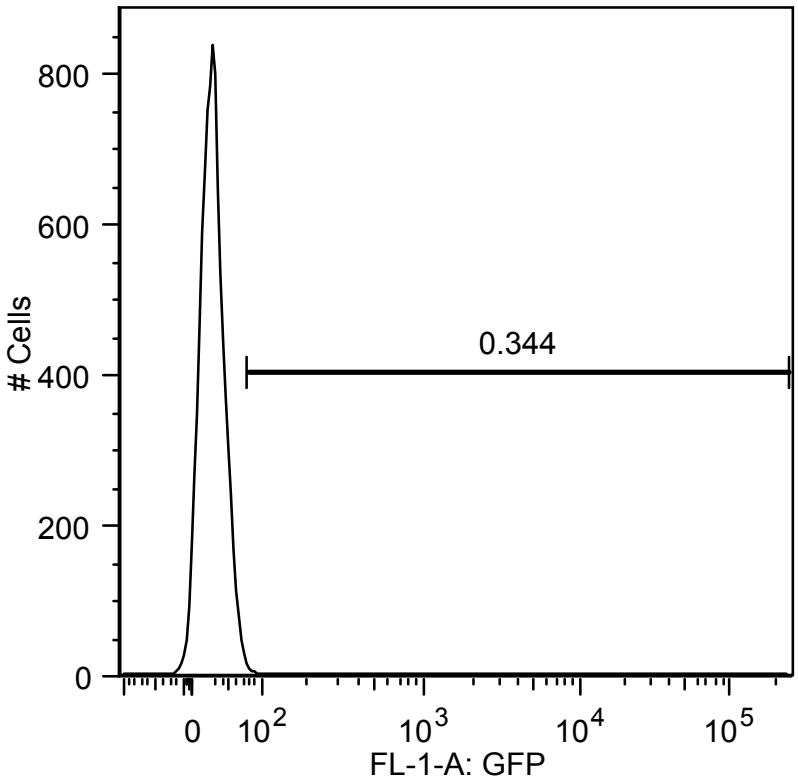


Figure 4.2. (cont'd)

4.2.4.b. Transfected gate. As in Experiment 2 (Fig. 4.2.2.b.).

4.2.4.b.i. C6B12:



4.2.4.b.ii. pTracer-CMV2:

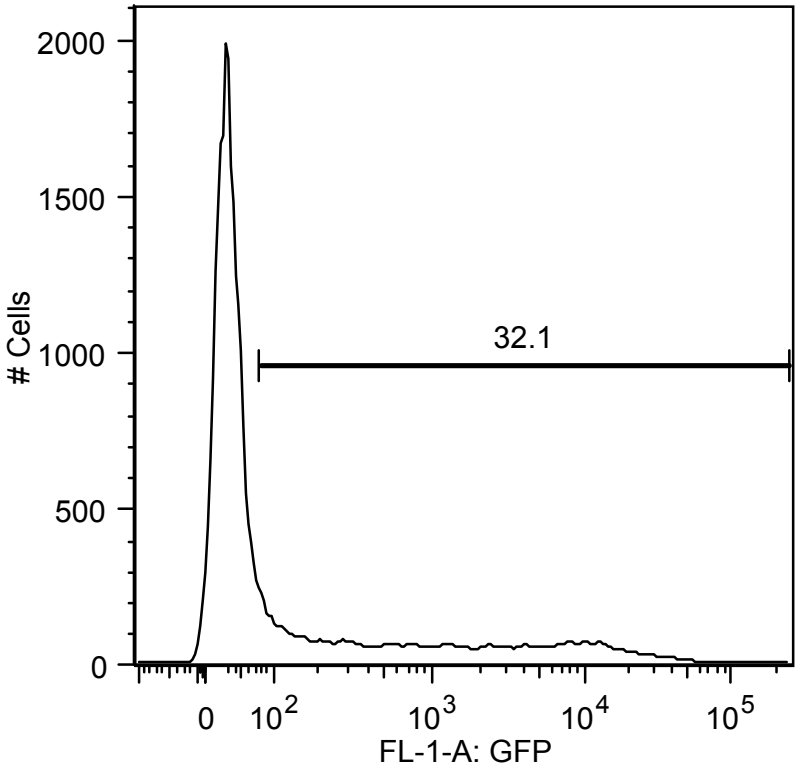
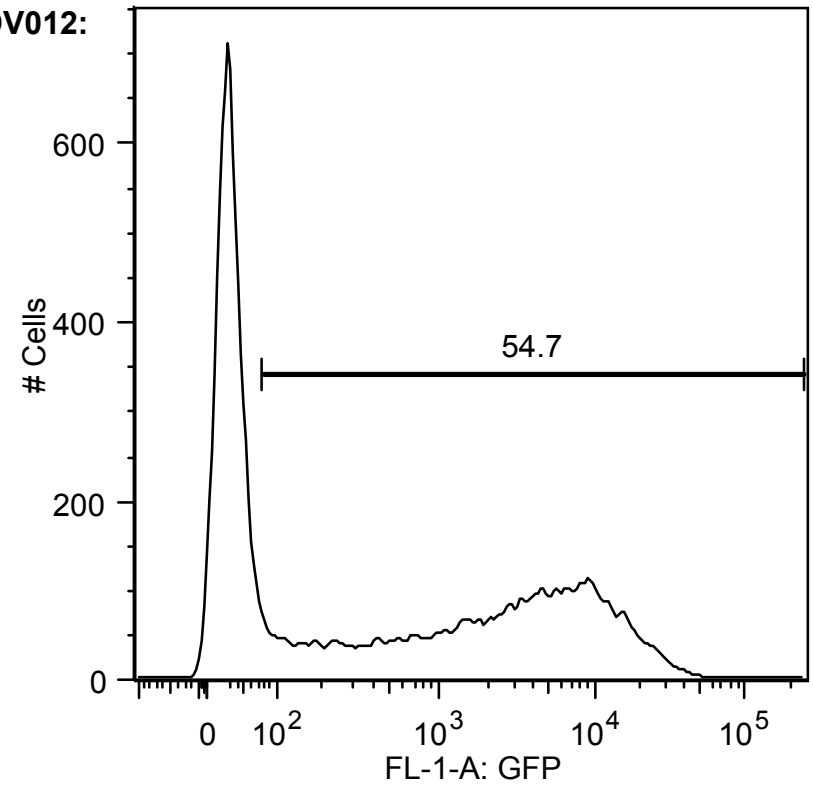


Figure 4.2. (cont'd)

4.2.4.b.iii. pTracer-CMV2-MDV012:



4.2.4.b.iv. pTracer-CMV2-MDV012ctg:

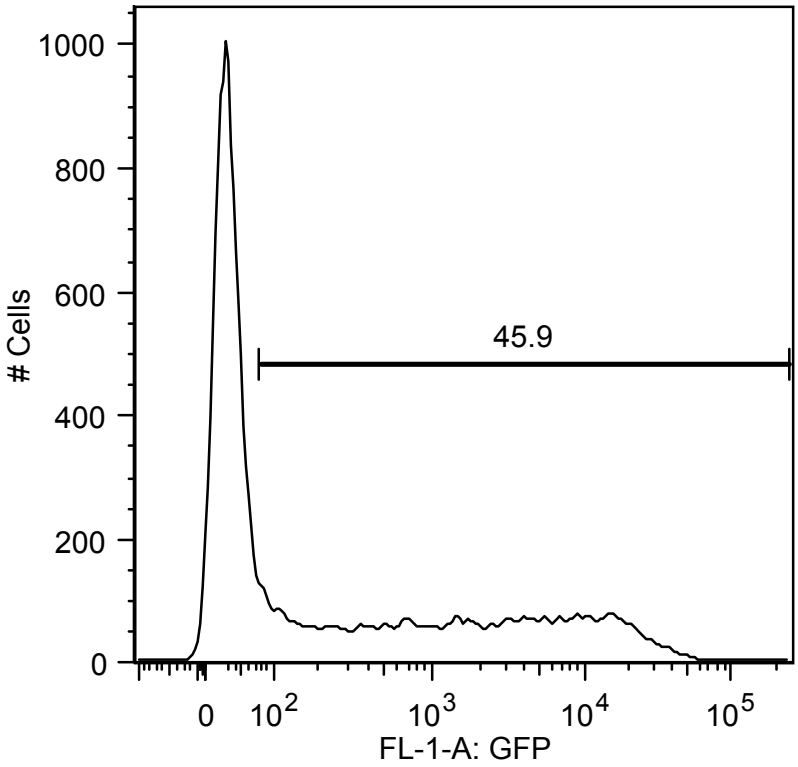
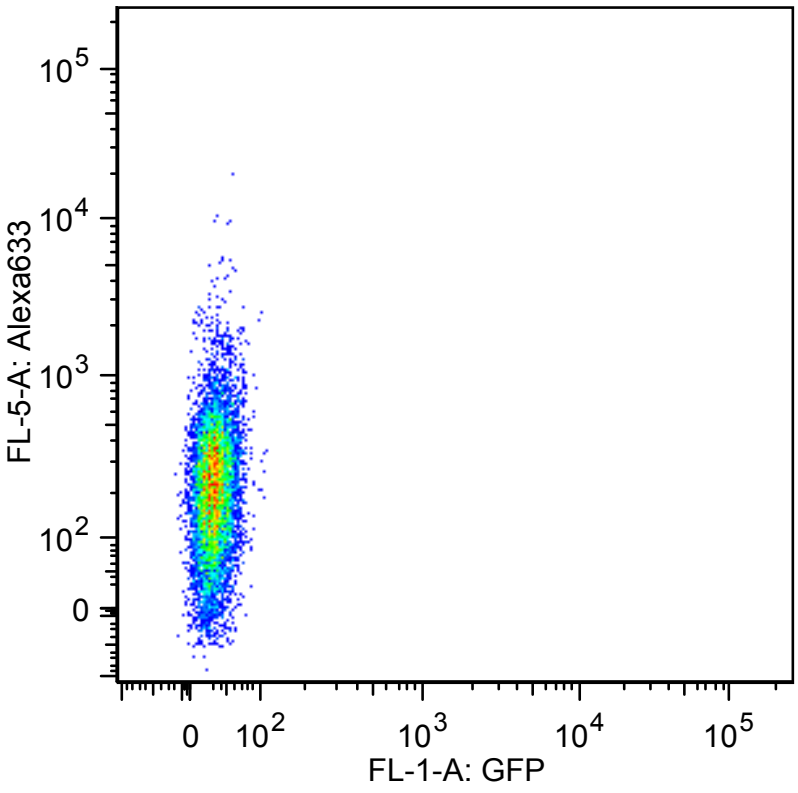


Figure 4.2. (cont'd)

4.2.4.c. GFP vs. Alexa-Fluor 633. As in Experiment 2 (Fig. 4.2.2.c.).

4.2.4.c.i. C6B12:



4.2.4.c.ii. pTracer-CMV2:

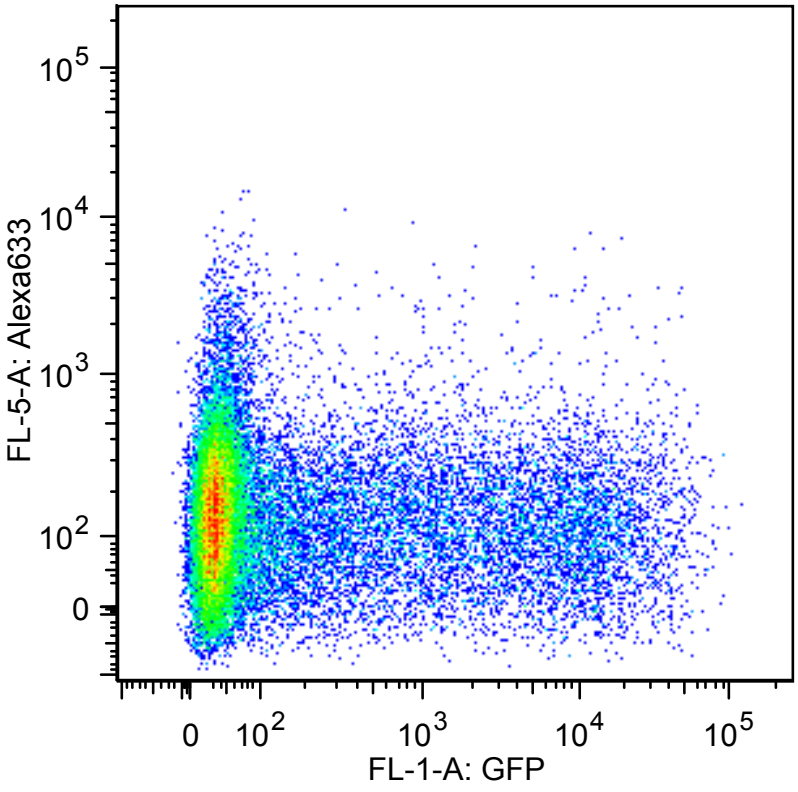
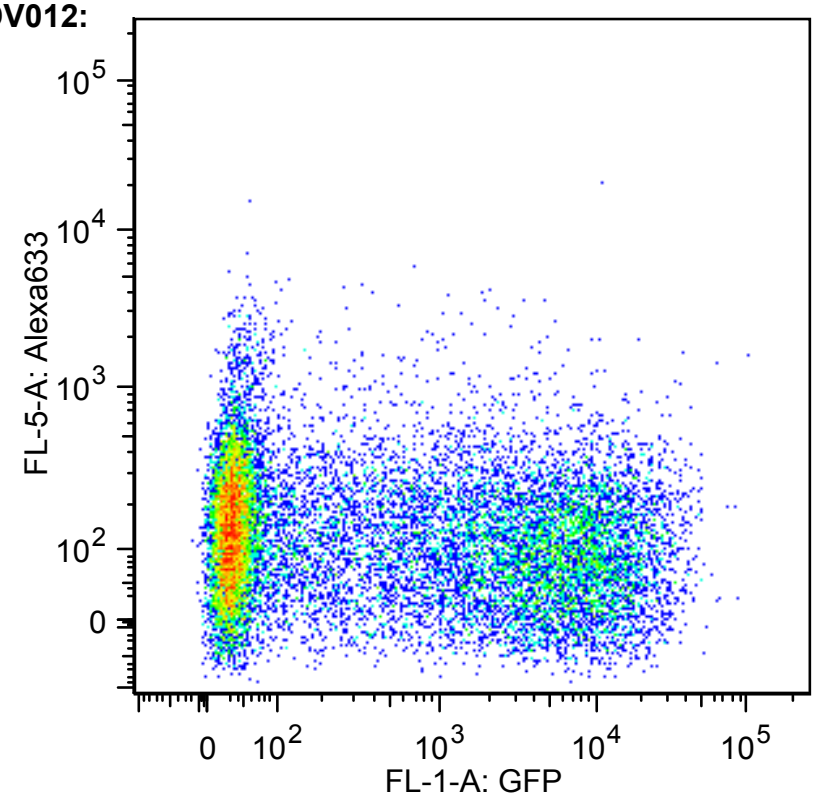


Figure 4.2. (cont'd)

4.2.4.c.iii. pTracer-CMV2-MDV012:



4.2.4.c.iv. pTracer-CMV2-MDV012ctg:

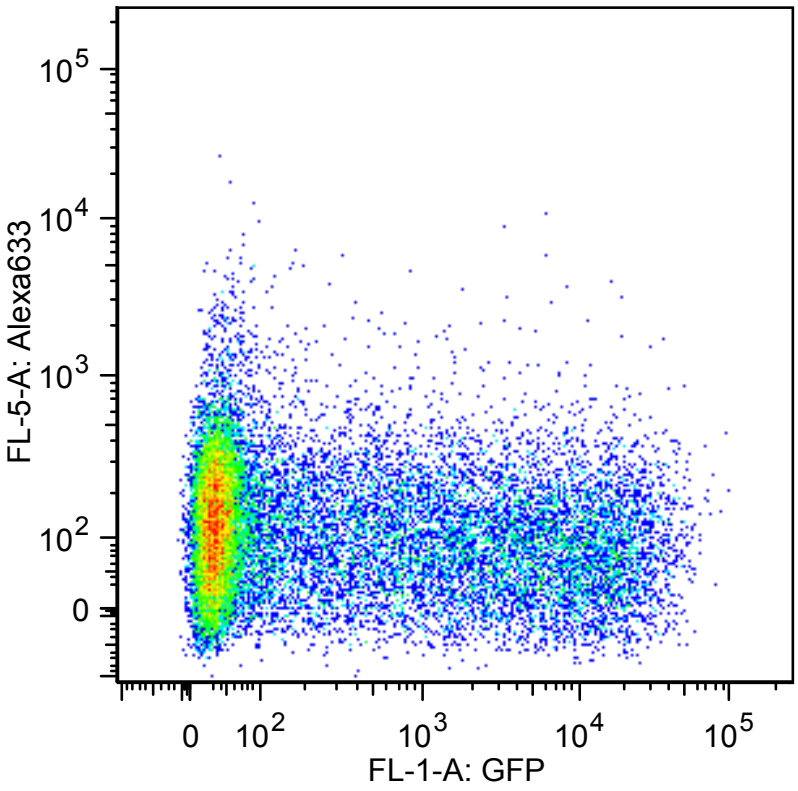
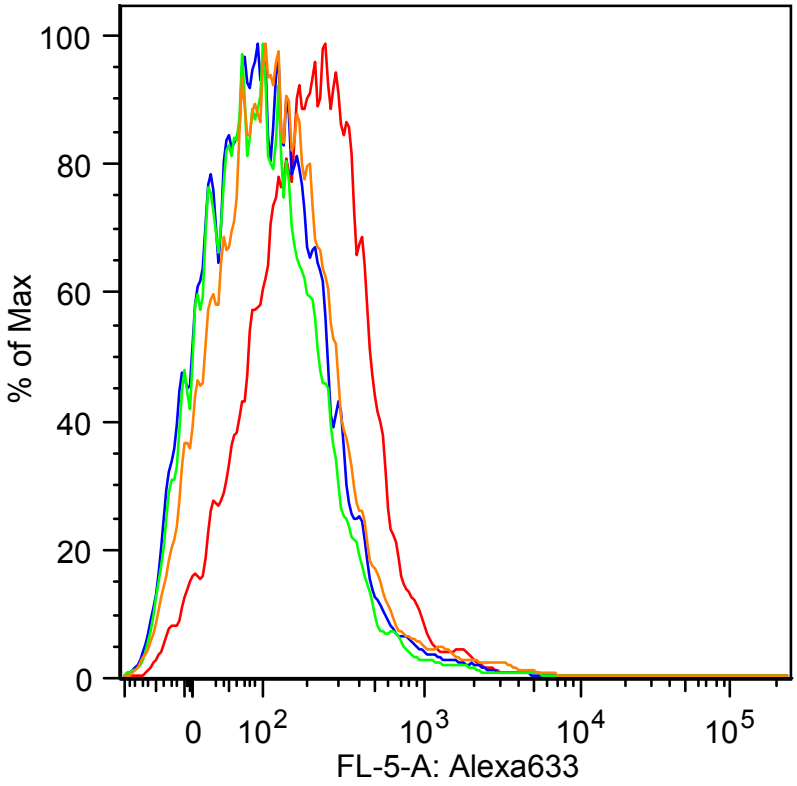


Figure 4.2. (cont'd)

4.2.4.d. MHC class I (histogram). C6B12/Alexa-Fluor 633 fluorescence intensity histogram of live, transfected cells (live cells for untransfected controls). GFP(-) cells from the pTracer-CMV2-transfected sample are included as a C6B12-stained, untransfected control. Labels and GMFIs are noted in the box below.



Sample:	GMFI:
C6B12	198
CMV2	125
MDV012	105
MDV012ctg	98.9

Figure 4.2. (cont'd)

4.2.5. Summary of the above 4 experiments. Staining with C6B12, as measured by geometric mean fluorescence intensity of Alexa-Fluor 633 secondary antibody on GFP+ cells. Average of Experiments 1-4, normalized to vector control (pTracer-CMV2). Error bars represent +/- standard deviation.

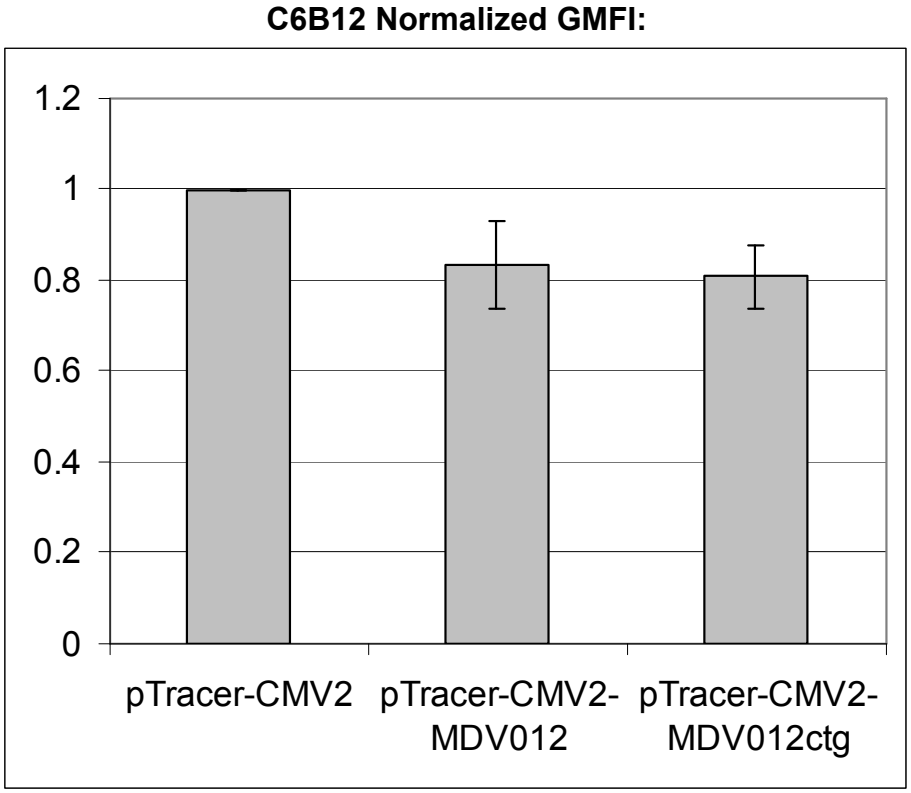


Figure 4.3. DF-1 cells transiently transfected with MDV012 plasmids did not show consistently decreased MHC class I major allele surface expression. DF-1 cells were transfected with pTracer-CMV2, pTracer-CMV2-MDV012, or pTracer-CMV2-MDV012ctg plasmids and stained with H1222-RP9BFIV21m78 (chicken anti-chicken BF2*21 MHC class I serum), then with Alexa-Fluor 633-conjugated anti-chicken IgG. Cells were analyzed by flow cytometry, gated on live and transfected cell (GFP+) populations, and GFP+ cells were compared for H1222-RP9BFIV21m78 staining as measured by Alexa-Fluor 633 intensity. Four experiments are shown.

4.3.1. Experiment 1.

4.3.1.a. Live gate. Live cell gate shown on isotype control (same gate shown in Figure 2.2.1).

4.3.1.a.i. Isotype control:

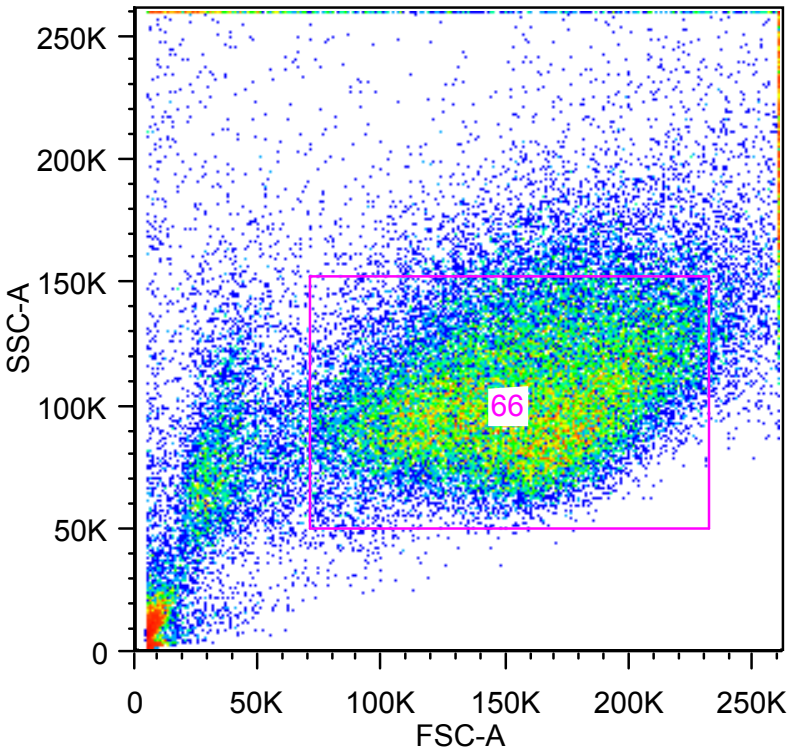
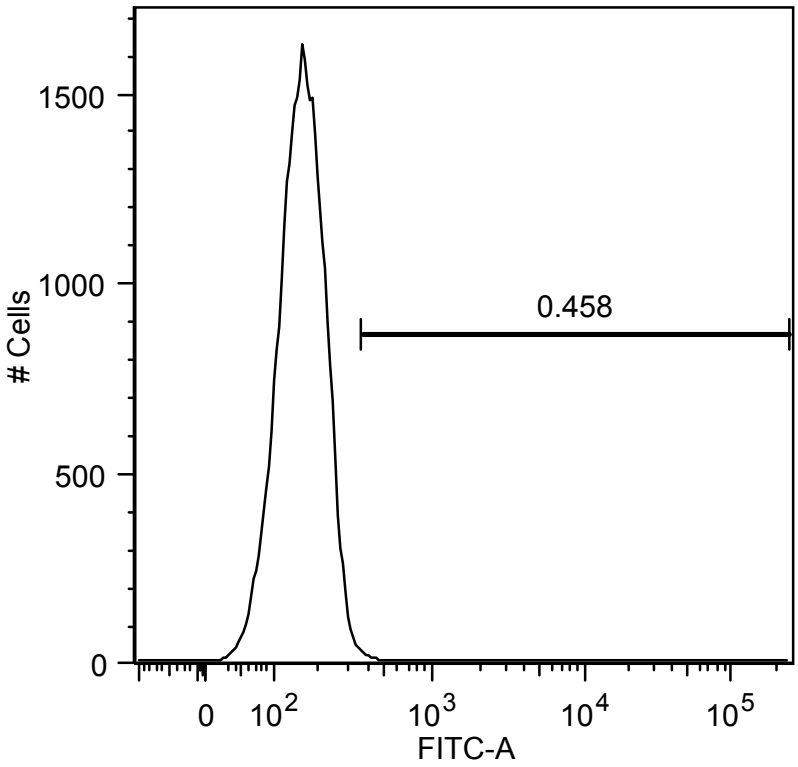


Figure 4.3. (cont'd)

4.3.1.b. Transfected gate. Set on non-transfected anti-BF2*21-stained cells (<5% positive) and applied to transfected samples.

4.3.1.b.i. Anti-BF2*21:



4.3.1.b.ii. pTracer-CMV2:

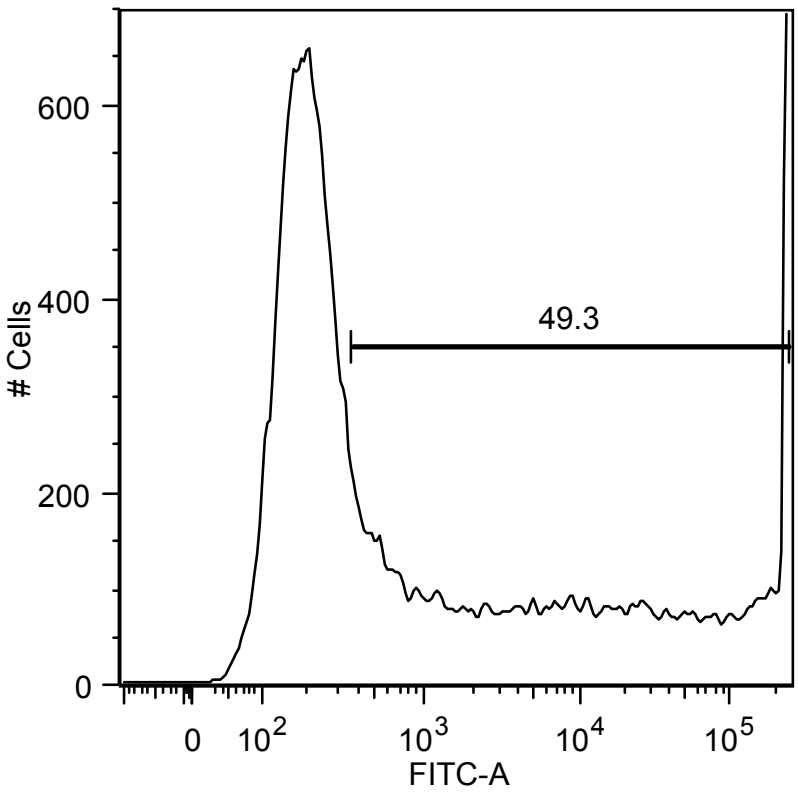
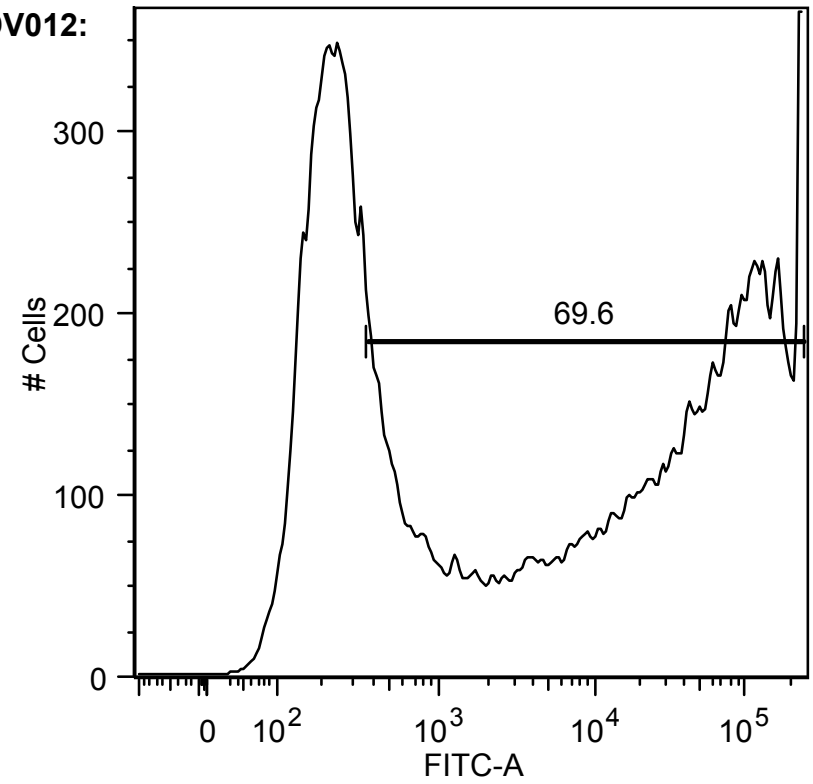


Figure 4.3. (cont'd)

4.3.1.b.iii. pTracer-CMV2-MDV012:



4.3.1.b.iv. pTracer-CMV2-MDV012ctg:

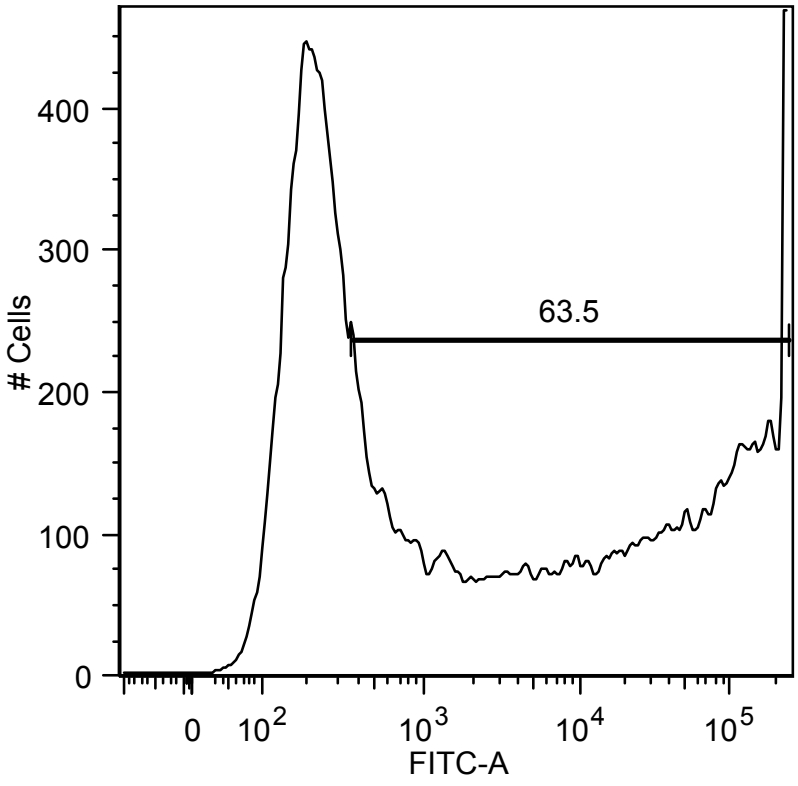
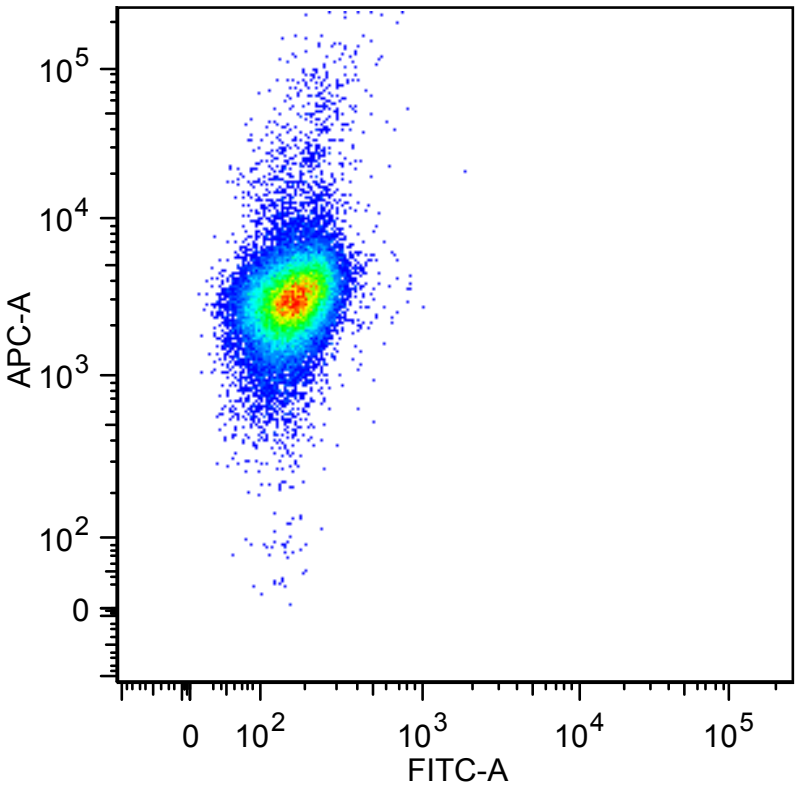


Figure 4.3. (cont'd)

4.3.1.c. GFP (FITC-A) vs. Alexa-Fluor 633 (APC-A). Live population.

4.3.1.c.i. Anti-BF2*21:



4.3.1.c.ii. pTracer-CMV2:

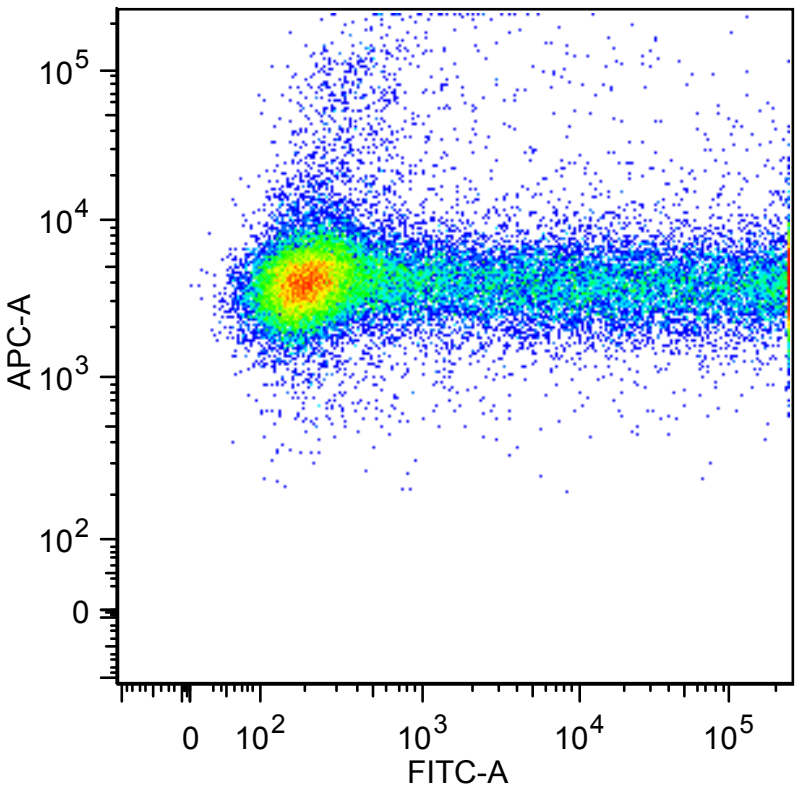
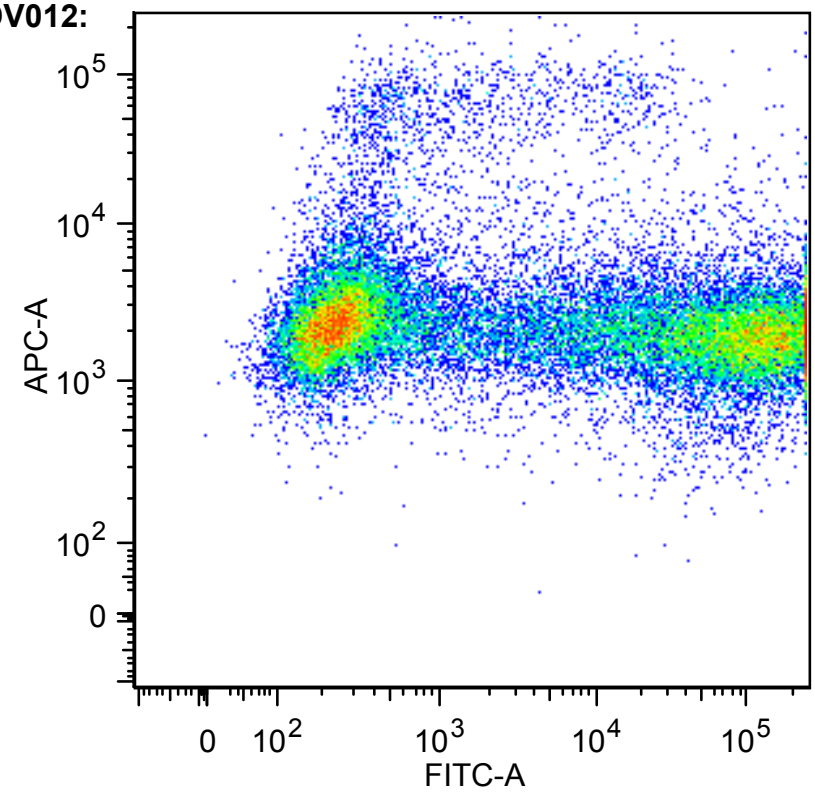


Figure 4.3. (cont'd)

4.3.1.c.iii. pTracer-CMV2-MDV012:



4.3.1.c.iv. pTracer-CVM2-MDV012:

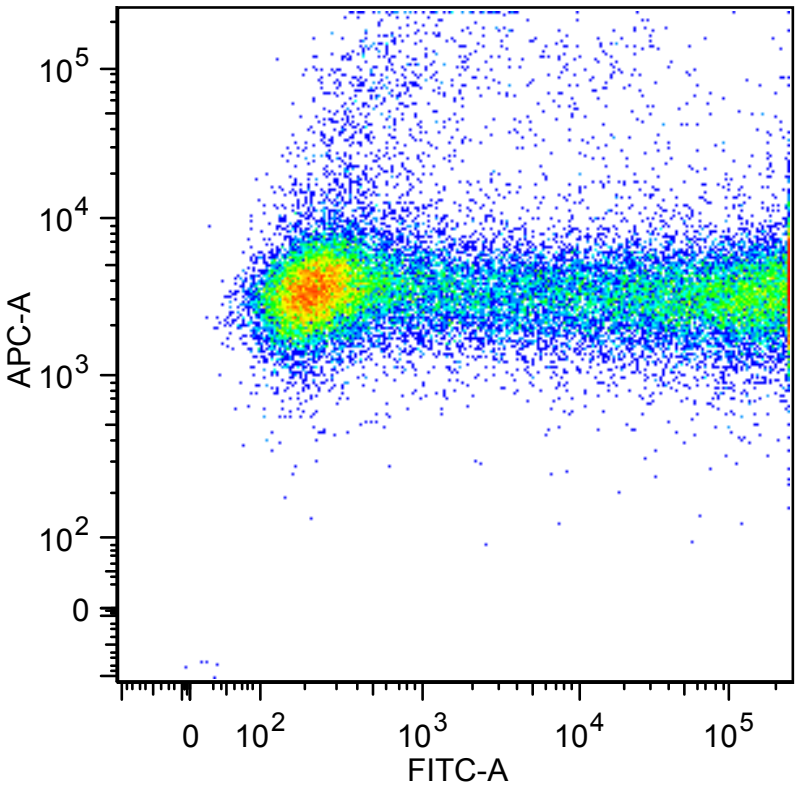
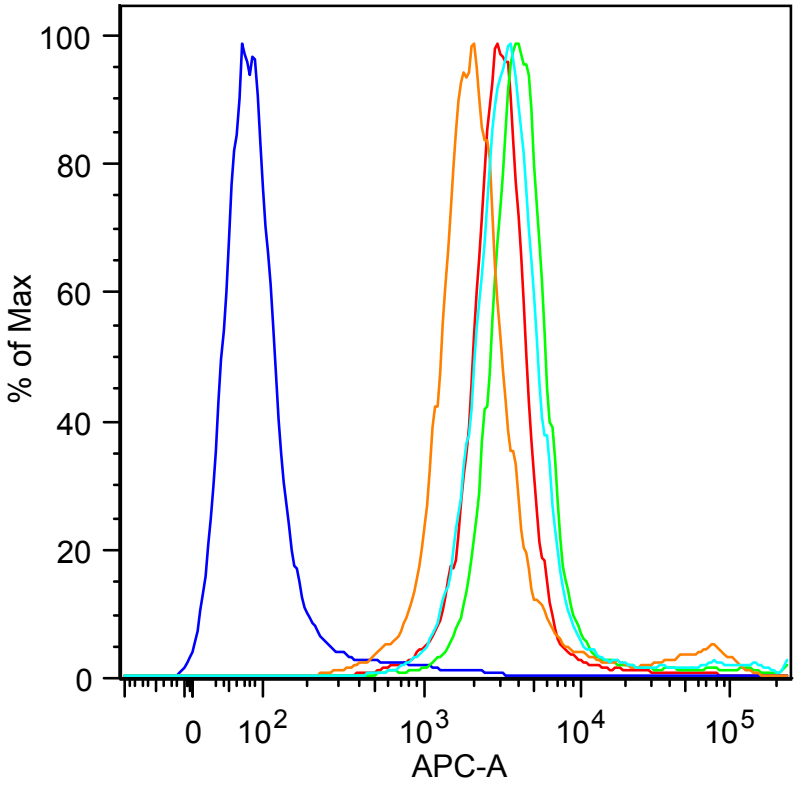


Figure 4.3. (cont'd)

4.3.1.d. MHC class I major (histogram). Anti-BF2*21/Alexa-Fluor 633 fluorescence intensity histogram of live, transfected cell populations (live cells for untransfected isotype- and anti-BF2*21-stained controls). Labels and geometric mean fluorescent intensities (GMFI) are noted in the box below.



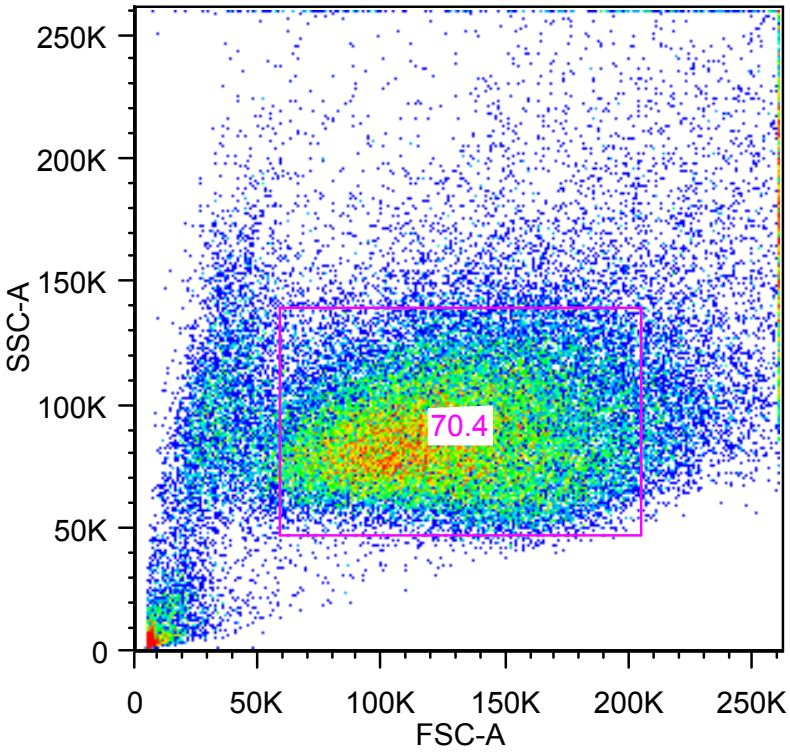
Sample:	GMFI:
Isotype control	93
Anti-BF2*21	3050
CMV2	4327
MDV012	2426
MDV012ctg	3766

Figure 4.3 (cont'd)

4.3.2. Experiment 2.

4.3.2.a. Live gate. Live cell gate shown on unstained and isotype control-stained untransfected cells.

4.3.2.a.i. Unstained:



4.3.2.a.ii. Isotype control:

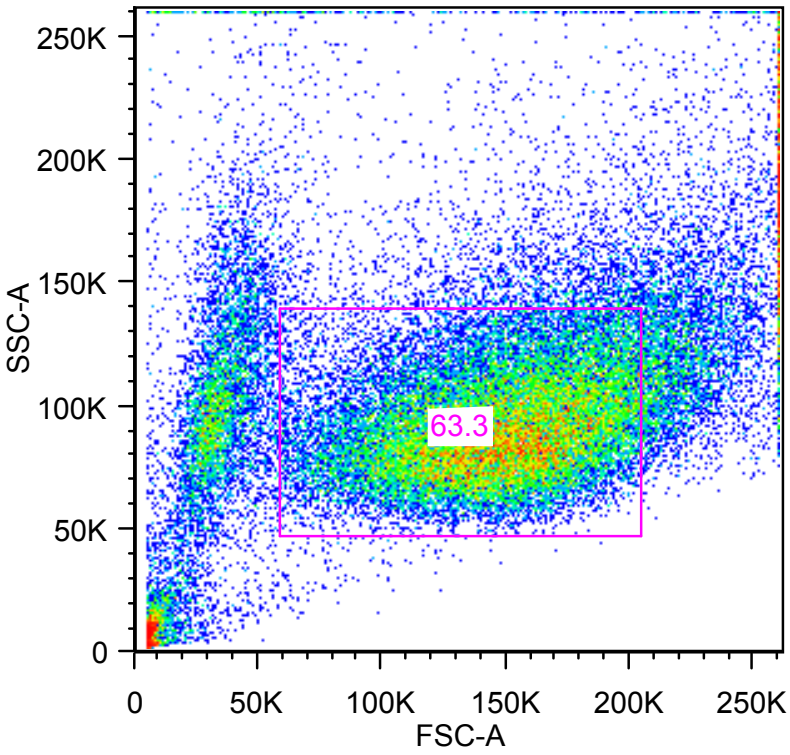
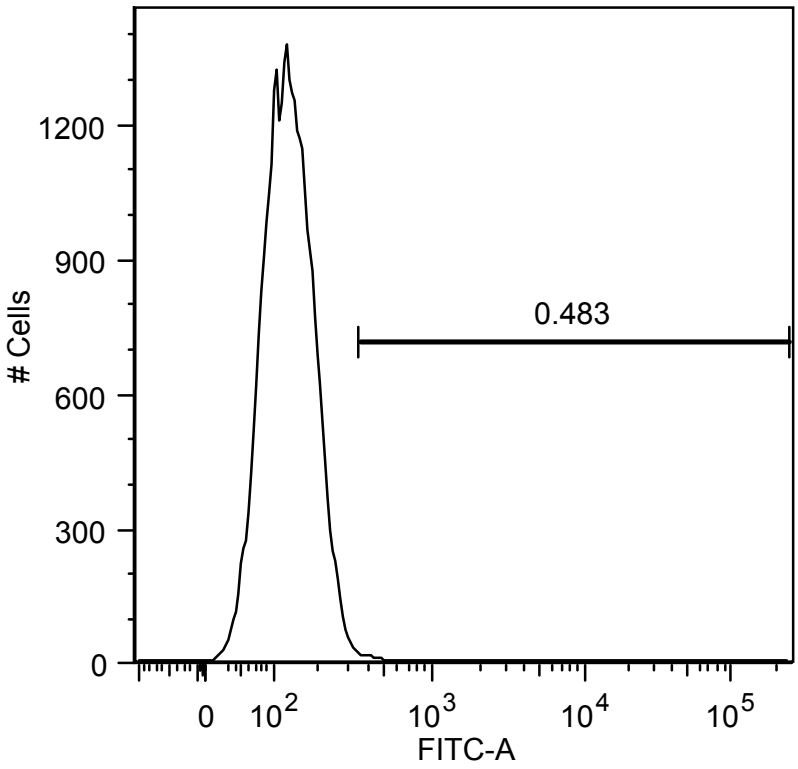


Figure 4.3. (cont'd)

4.3.2.b. Transfected gate. As in Experiment 1 (4.3.1.b.).

4.3.2.b.i. Anti-BF2*21:



4.3.2.b.ii. pTracer-CMV2:

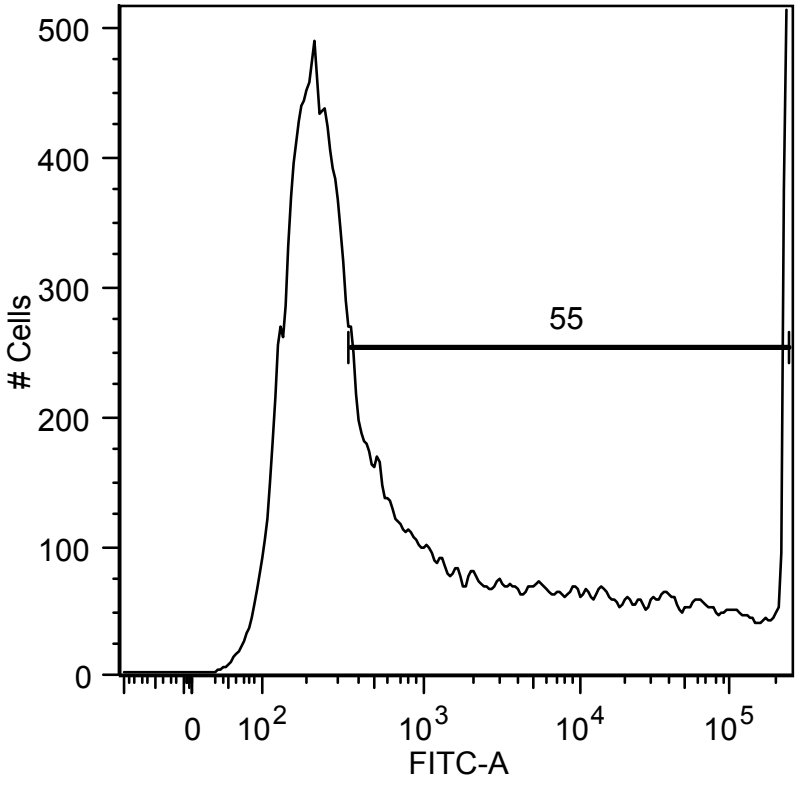
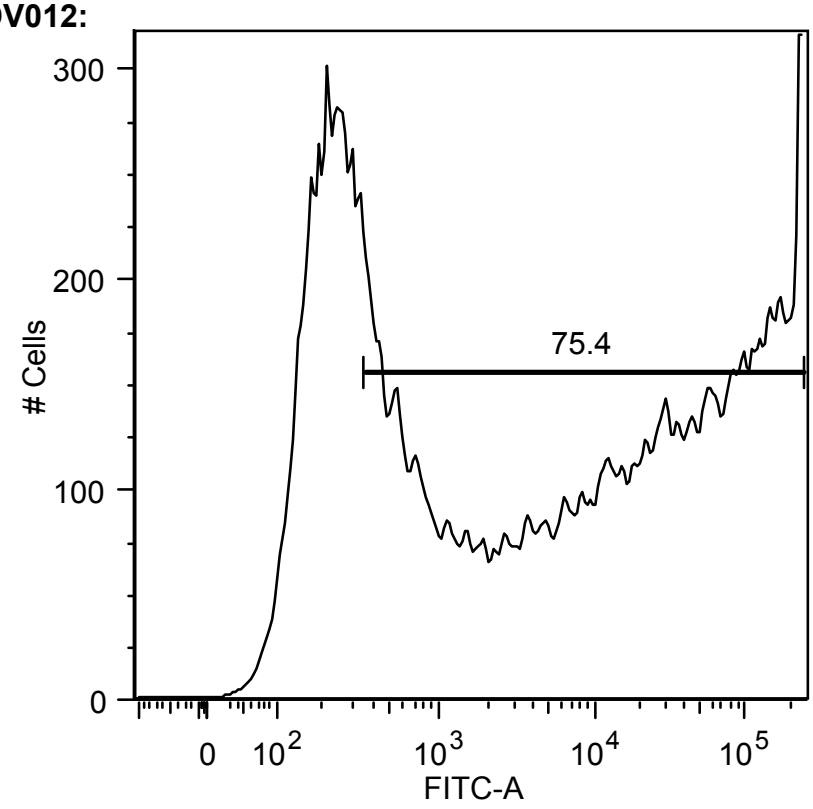


Figure 4.3. (cont'd)

4.3.2.b.iii. pTracer-CMV2-MDV012:



4.3.2.b.iv. pTracer-CMV2-MDV012ctg:

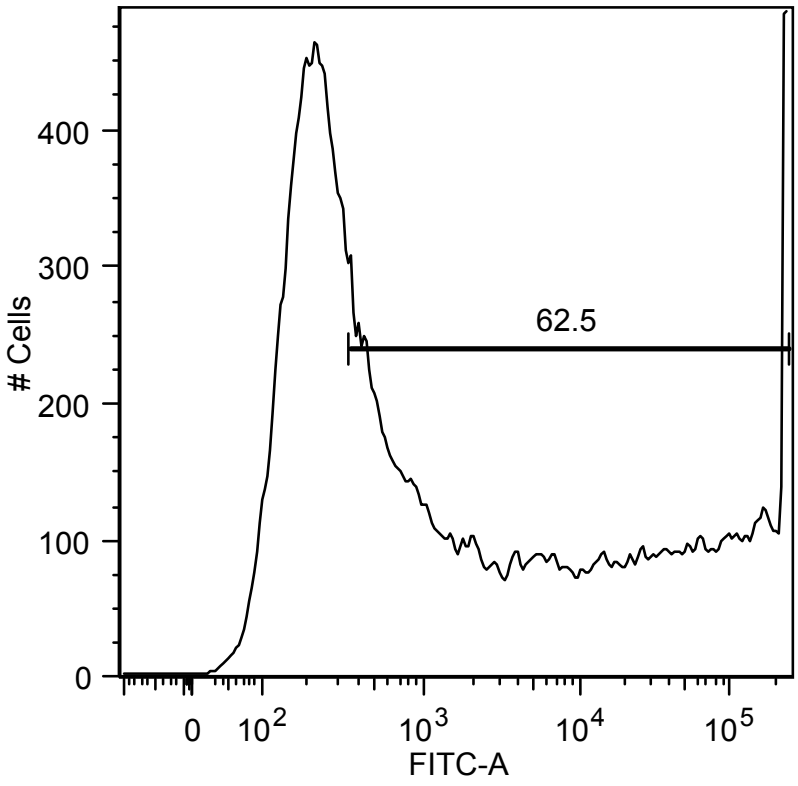
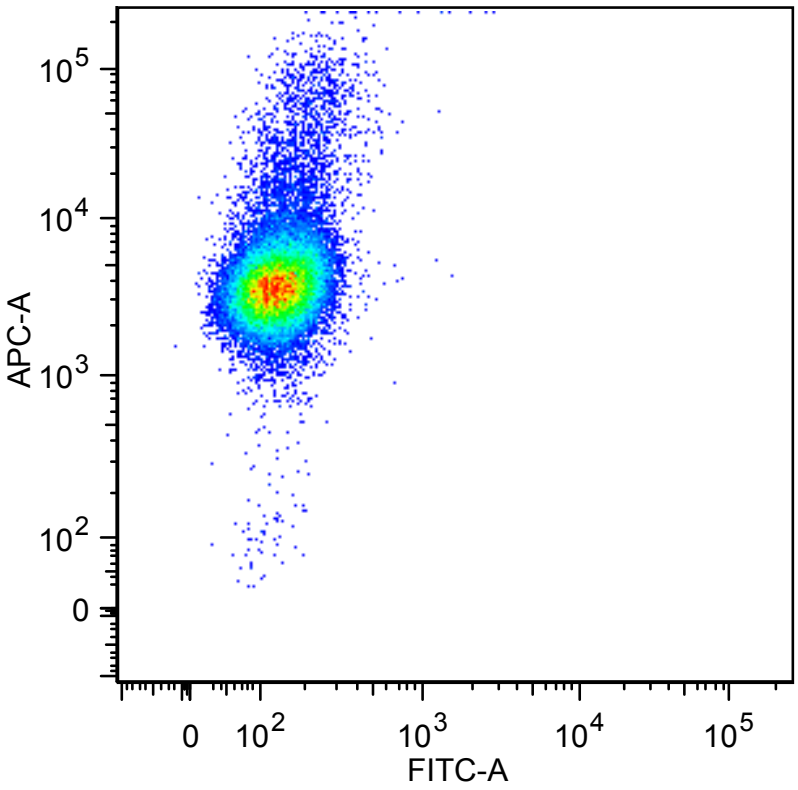


Figure 4.3. (cont'd)

4.3.2.c. GFP (FITC-A) vs. Alexa-Fluor 633 (APC-A).

4.3.2.c.i. Anti-BF2*21:



4.3.2.c.ii. pTracer-CMV2:

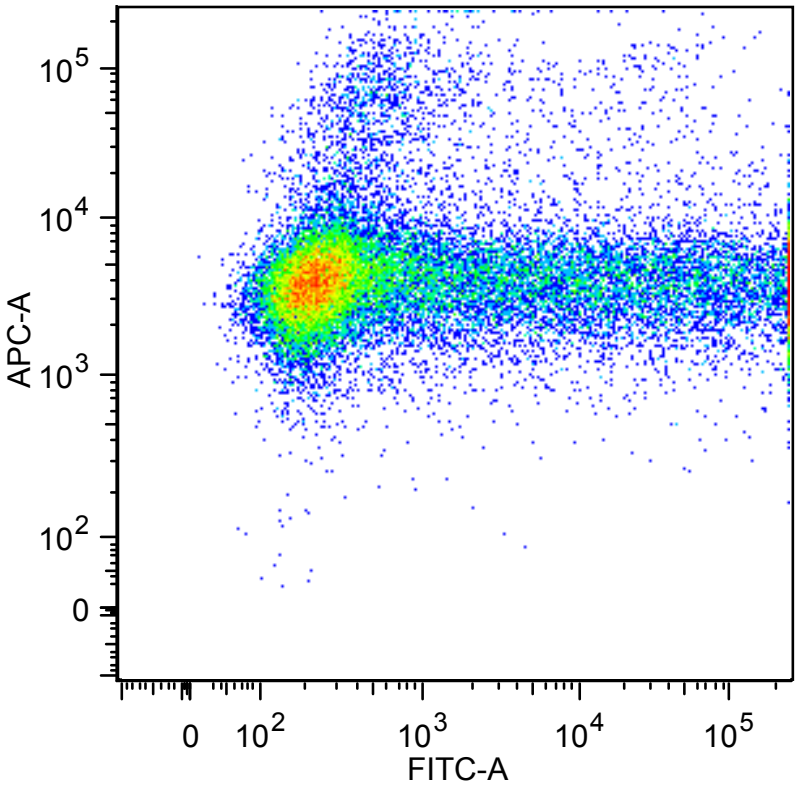
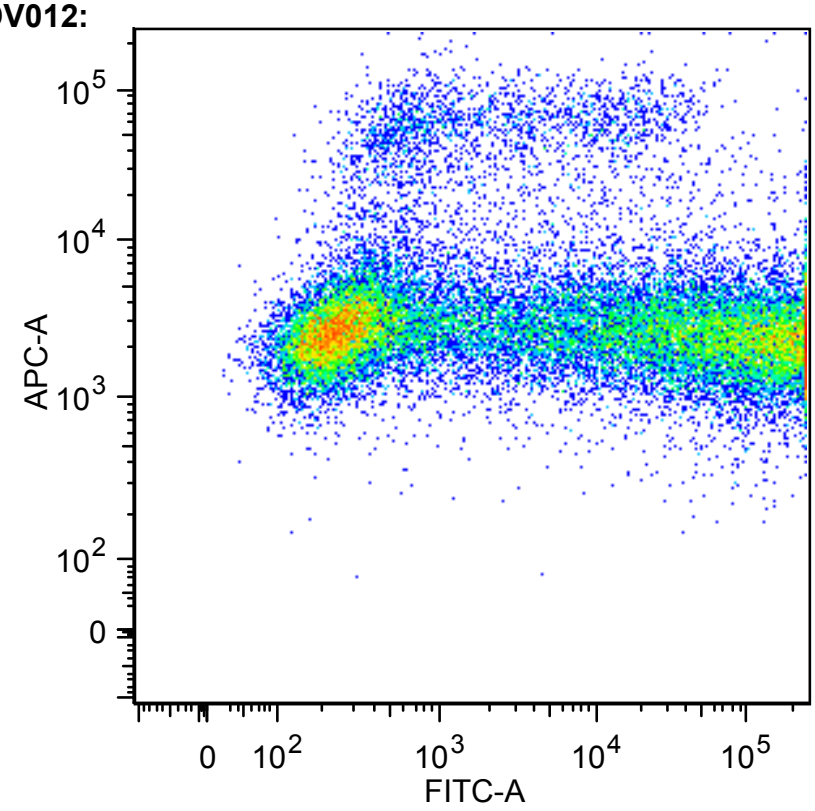


Figure 4.3. (cont'd)

4.3.2.c.iii. pTracer-CMV2-MDV012:



4.3.2.c.iv. pTracer-CMV2-MDV012ctg:

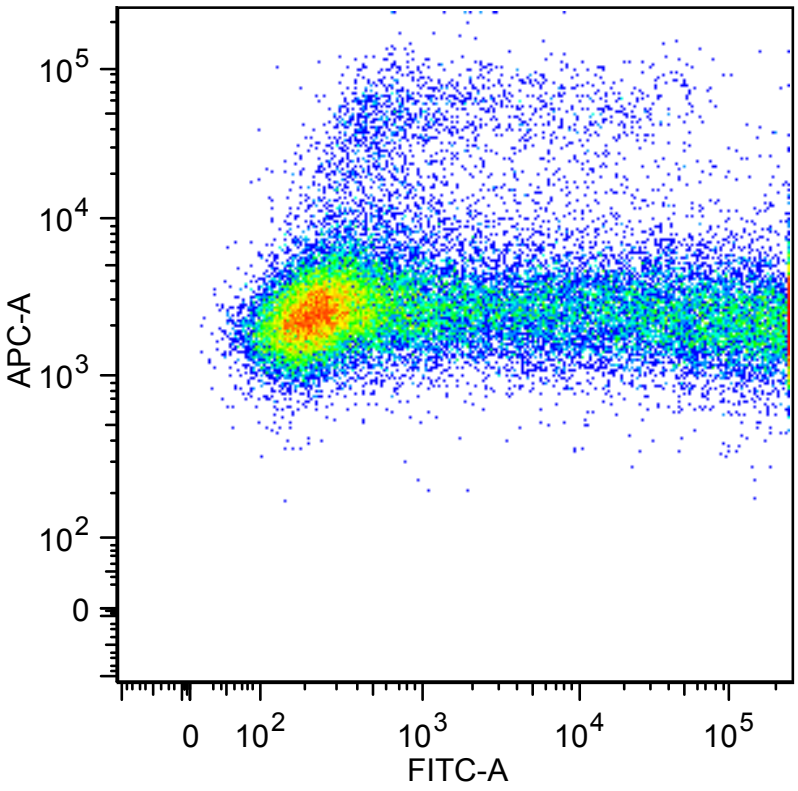
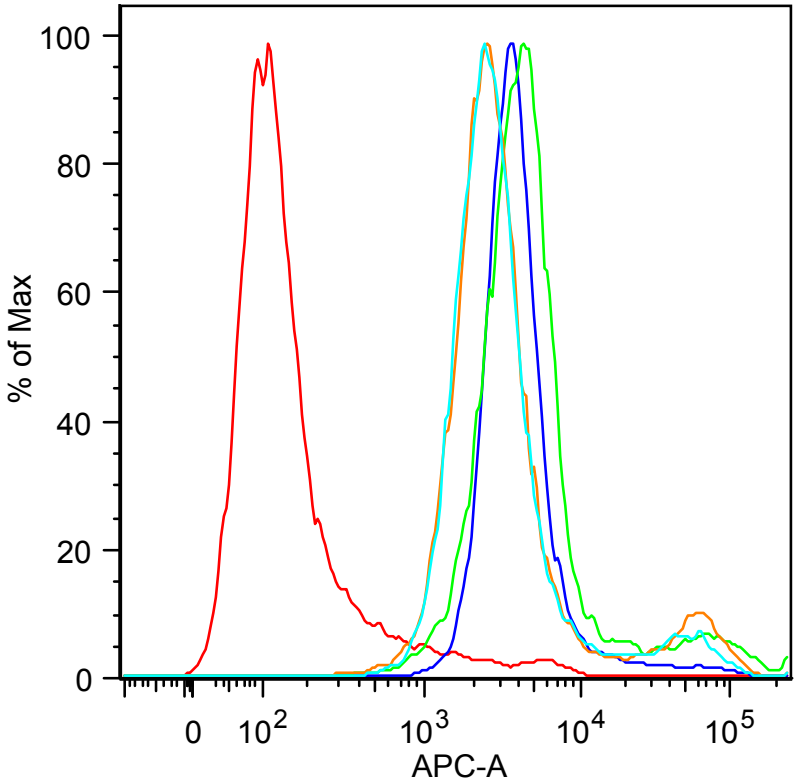


Figure 4.3. (cont'd)

4.3.2.d. MHC class I major (histogram). Anti-BF2*21/Alexa-Fluor 633 fluorescence intensity histogram of live, transfected cell populations (live cells for untransfected isotype- and anti-BF2*21-stained controls). Labels and geometric mean fluorescent intensities (GMFI) are noted in the box below.



Sample:	GMFI:
Isotype control	155
Anti-BF2*21	4069
CMV2	4966
MDV012	3407
MDV012ctg	3212

Figure 4.3. (cont'd)

4.3.3. Experiment 3.

4.3.3.a. Live gate. Shown on isotype control (same gate shown in Fig. 4.2.3.a.).

4.3.3.a.i. Isotype control:

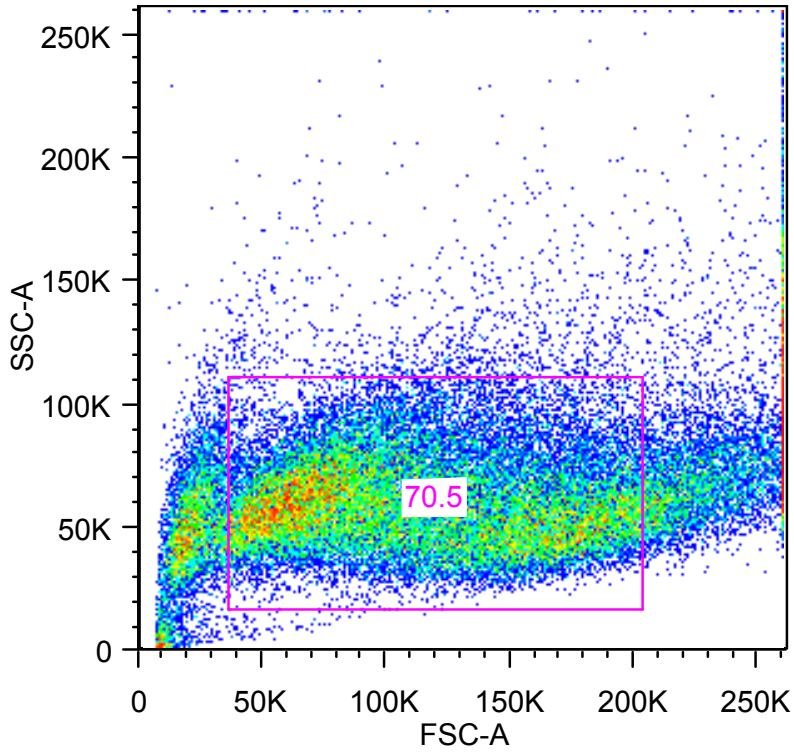
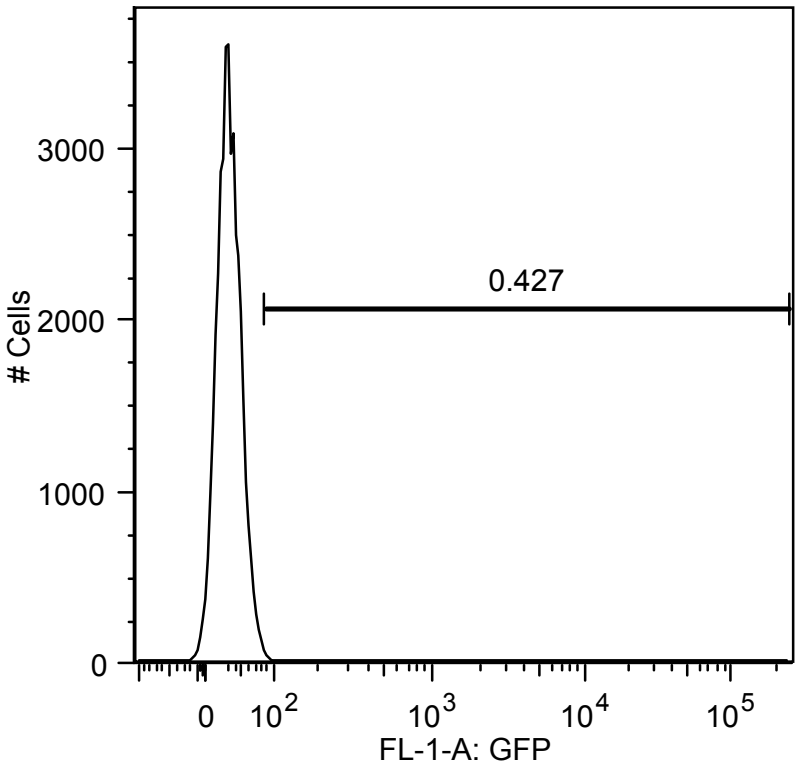


Figure 4.3. (cont'd)

4.3.3.b. Transfected gate. As in Experiment 1 (4.3.1.).

4.3.3.b.i. Anti-BF2*B21:



4.3.3.b.ii. pTracer-CMV2:

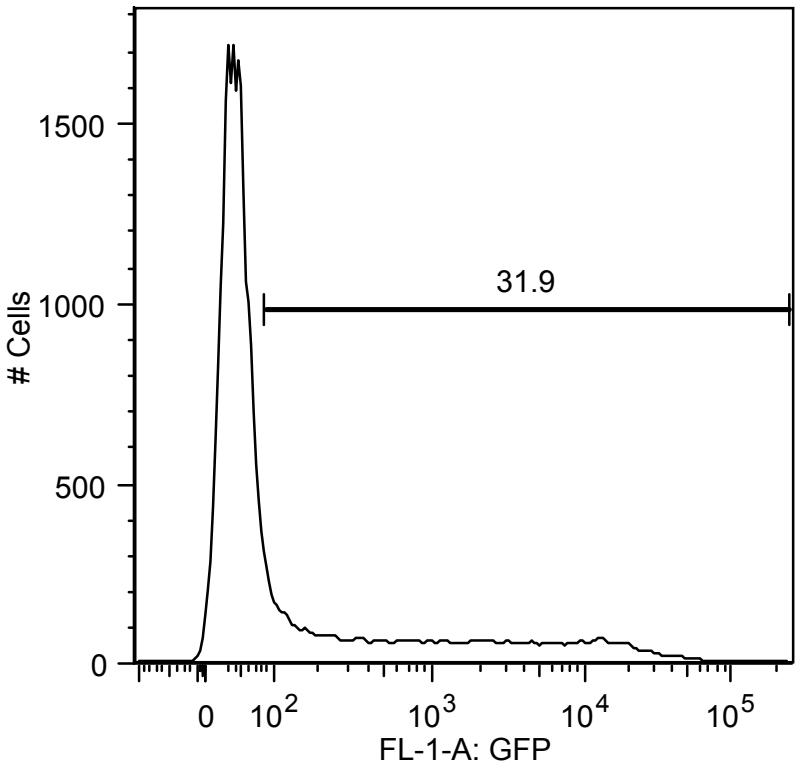
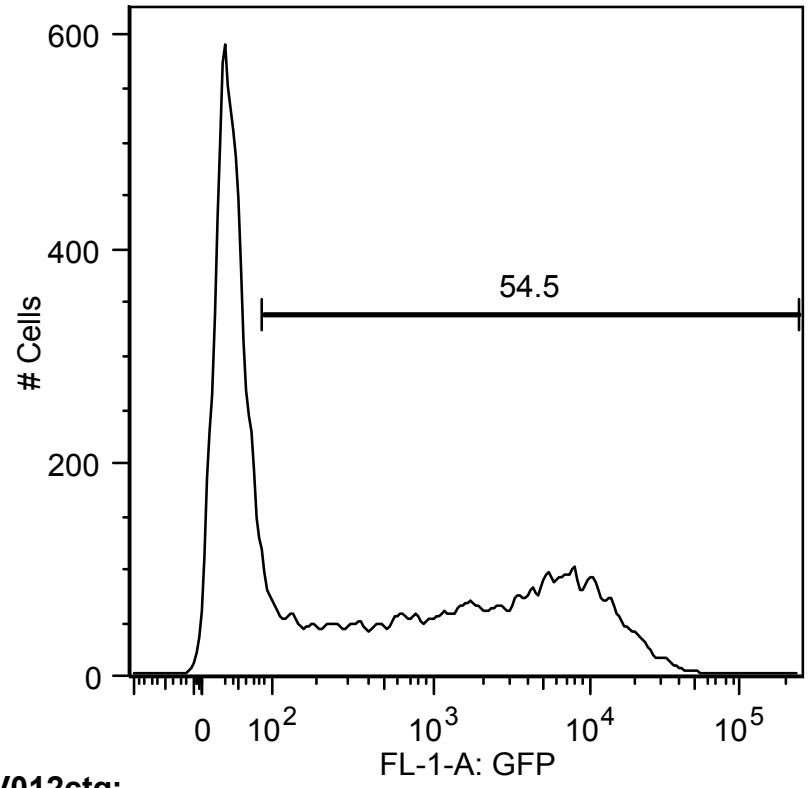


Figure 4.3. (cont'd)

4.3.3.b.iii. pTracer-CMV2-MDV012:



4.3.3.b.iv. pTracer-CMV2-MDV012ctg:

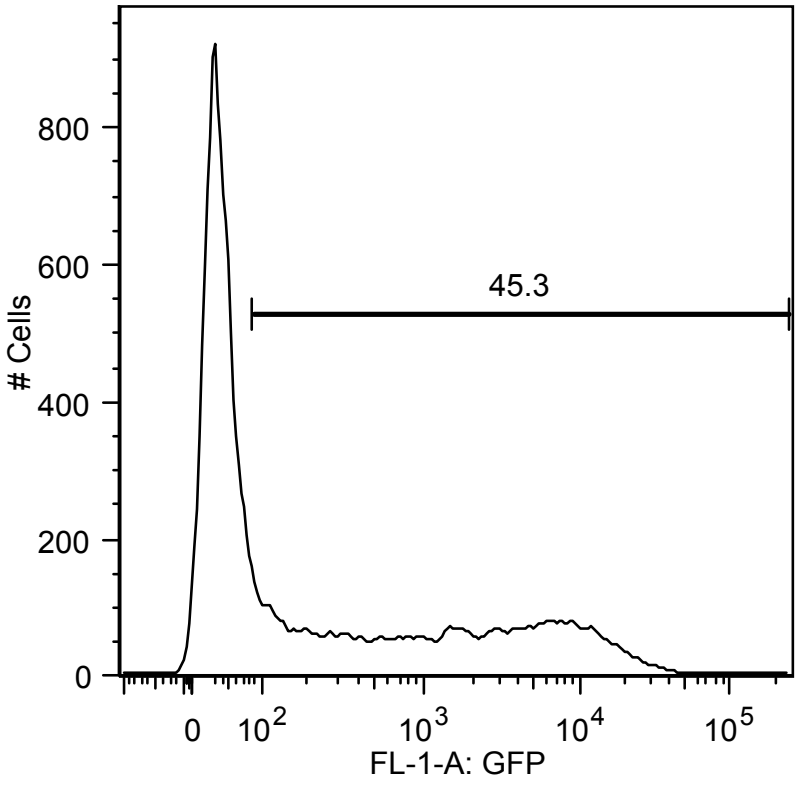
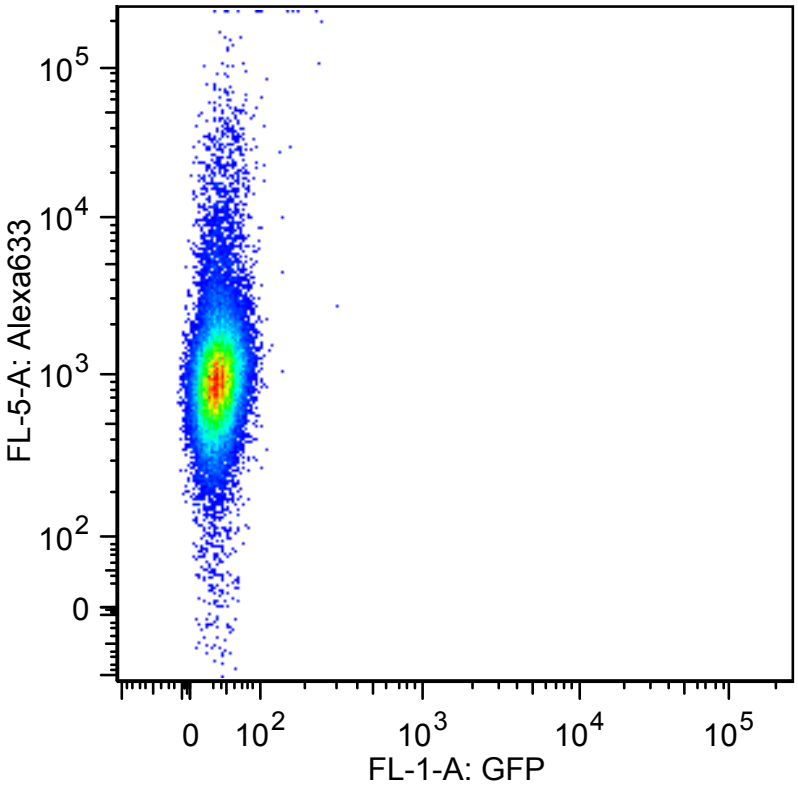


Figure 4.3. (cont'd)

4.3.3.c. GFP vs. Alexa-Fluor 633.

4.3.3.c.i. Anti-BF2*21:



4.3.3.c.ii. pTracer-CMV2:

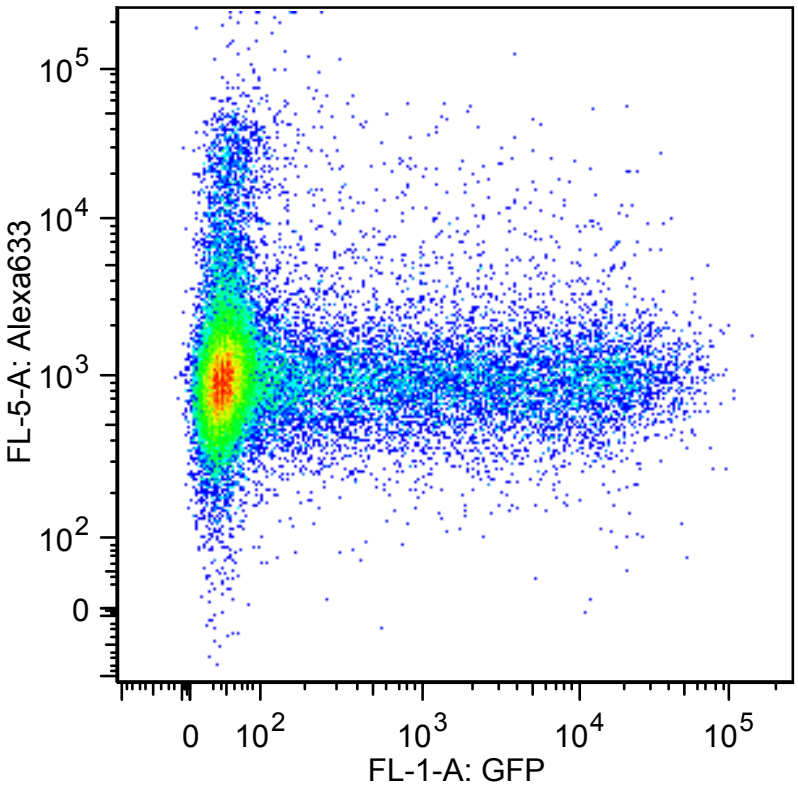
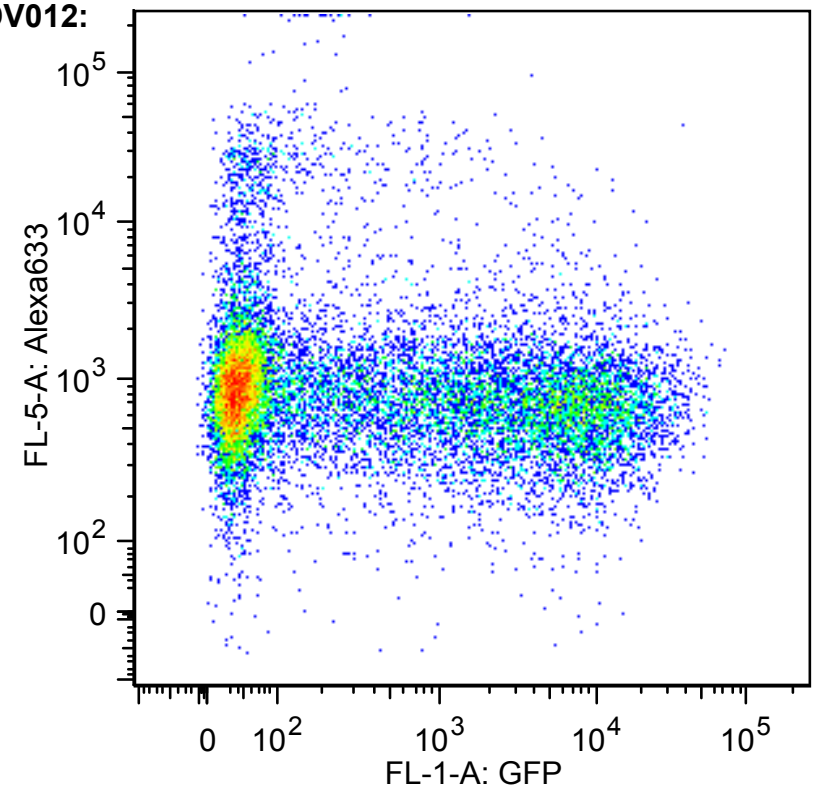


Figure 4.3. (cont'd)

4.3.3.c.iii. pTracer-CMV2-MDV012:



4.3.3.c.iv. pTracer-CMV2-MDV012ctg:

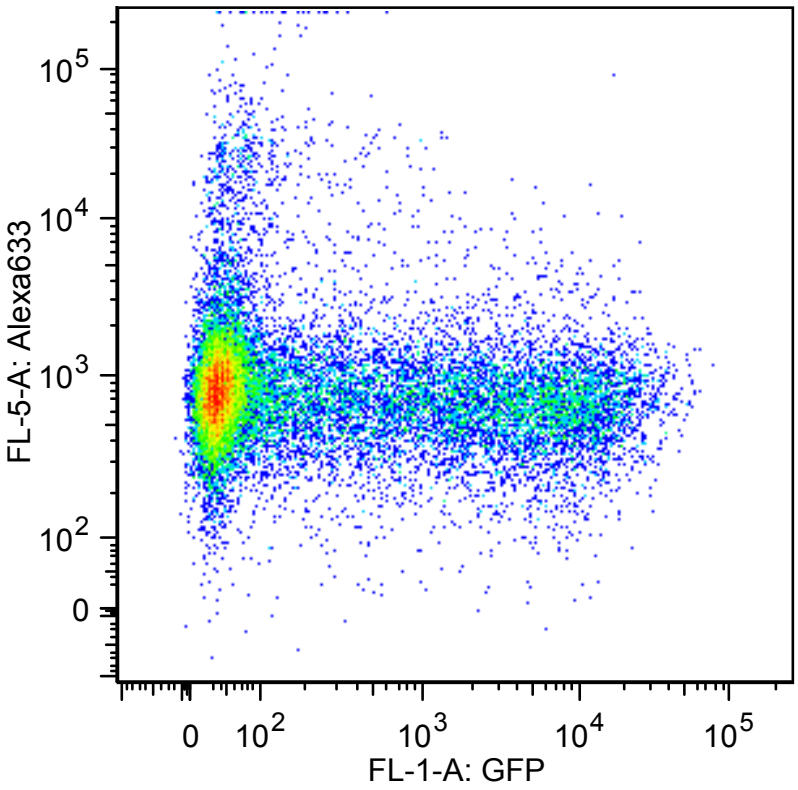
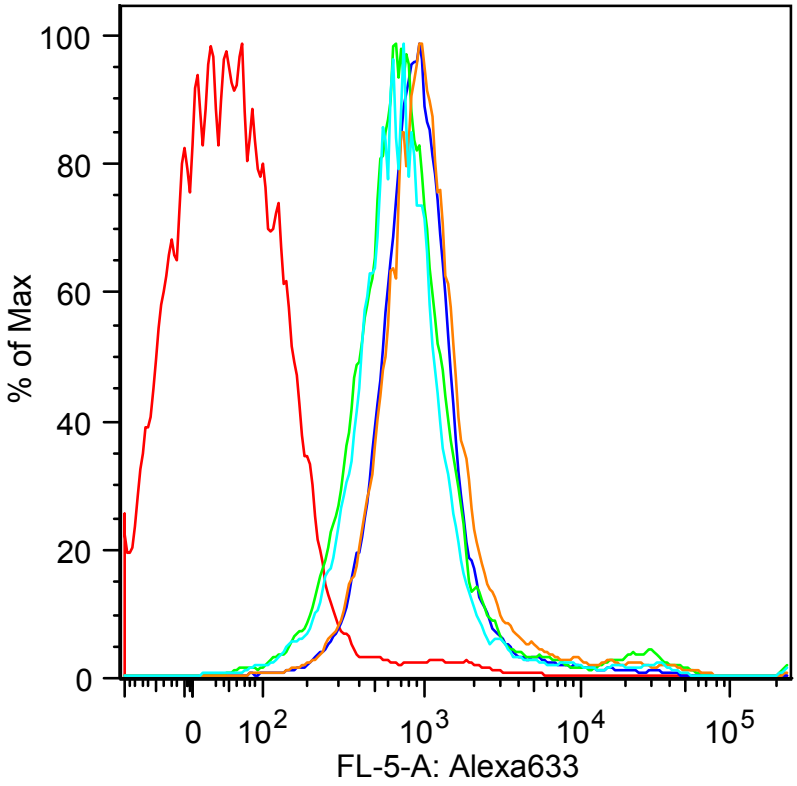


Figure 4.3. (cont'd)

4.3.3.d. MHC class I major (histogram). Anti-BF2*21/Alexa-Fluor 633 fluorescence intensity histogram of live, transfected cell populations (live cells for untransfected isotype- and anti-BF2*21-stained controls). Labels and geometric mean fluorescent intensities (GMFI) are noted in the box below.



Sample:	GMFI
Isotype contol	50.5
Anti-BF2*21	931
CMV2	1063
MDV012	791
MDV012ctg	747

Figure 4.3. (cont'd)

4.3.4. Experiment 4.
4.3.4.a. Live gate. Shown on isotype control (same gate shown in Fig. 4.2.4.a.).

4.3.4.a.i. Isotype control:

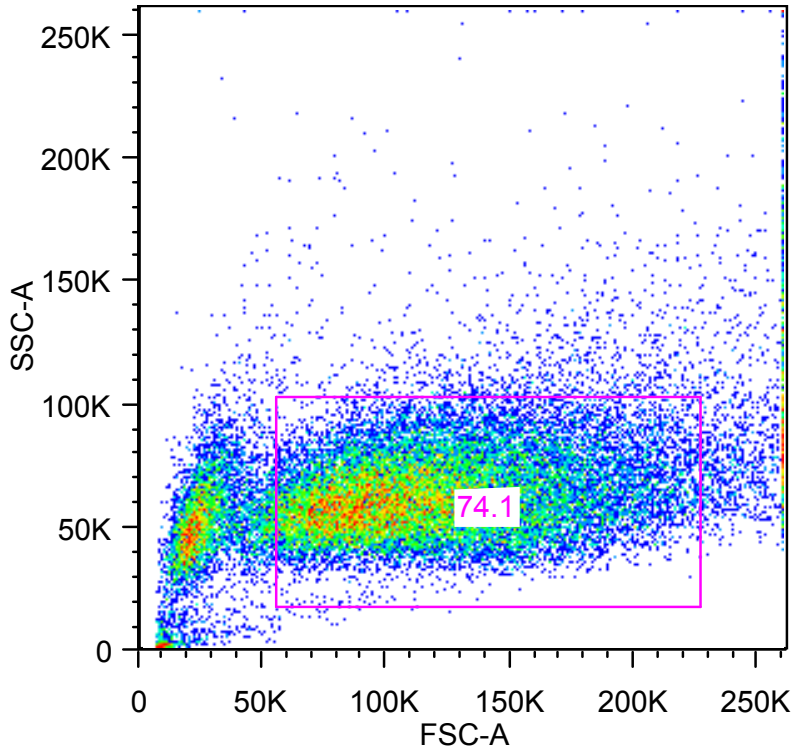
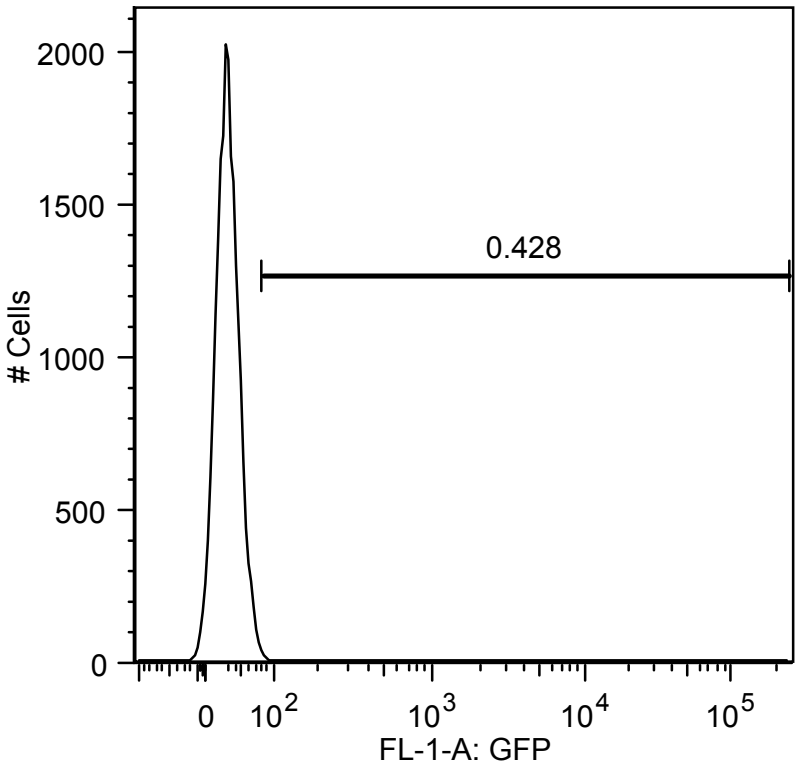


Figure 4.3. (cont'd)

4.3.4.b. Transfected gate. As in Experiment 1 (Fig. 4.3.1.b.).

4.3.4.b.i. Anti-BF2*21:



4.3.4.b.ii. pTracer-CMV2:

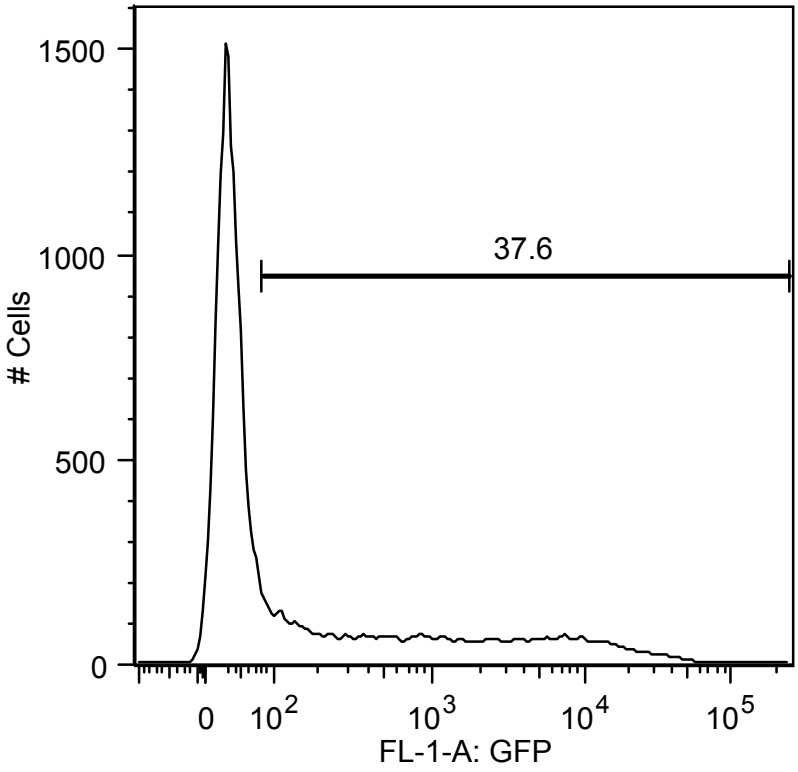
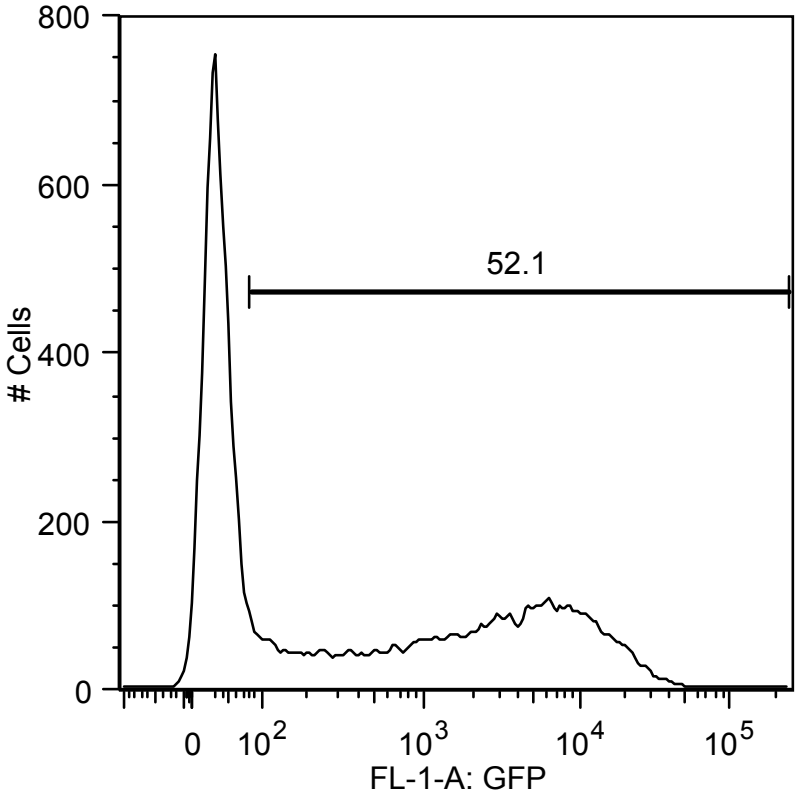


Figure 4.3. (cont'd)

4.3.4.b.iii. pTracer-CMV2-MDV012:



4.3.4.b.iv. pTracer-CMV2-MDV012ctg:

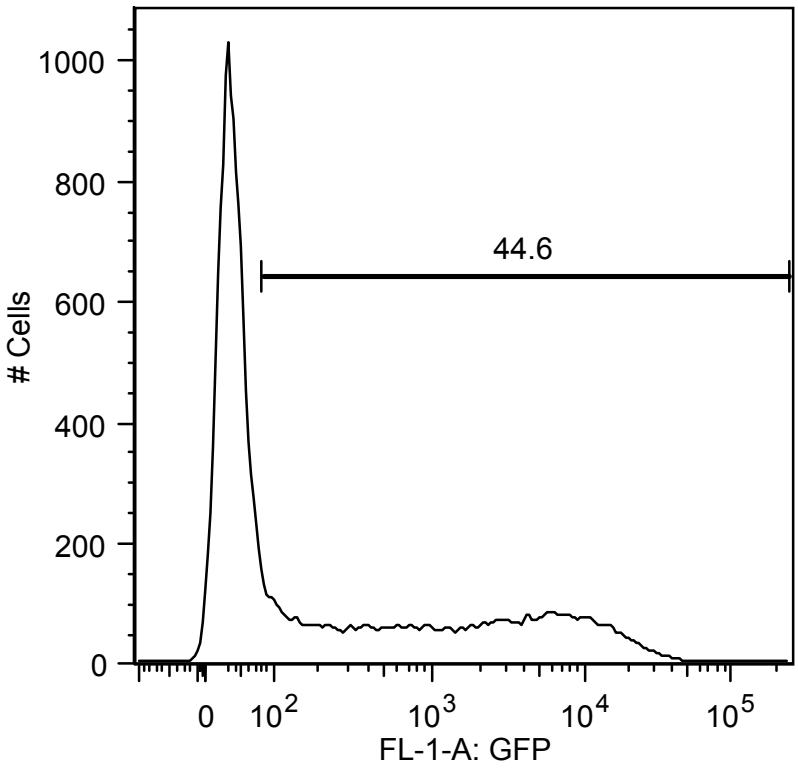
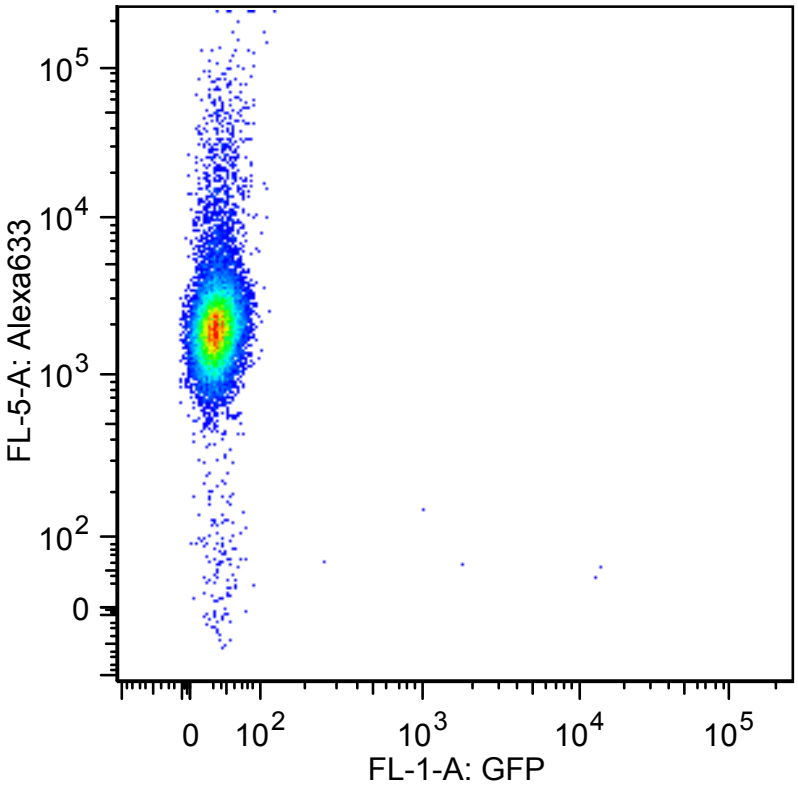


Figure 4.3 (cont'd)

4.3.4.c. GFP vs. Alexa-Fluor 633.

4.3.4.c.i. Anti-BF2*21:



4.3.4.c.ii. pTracer-CMV2:

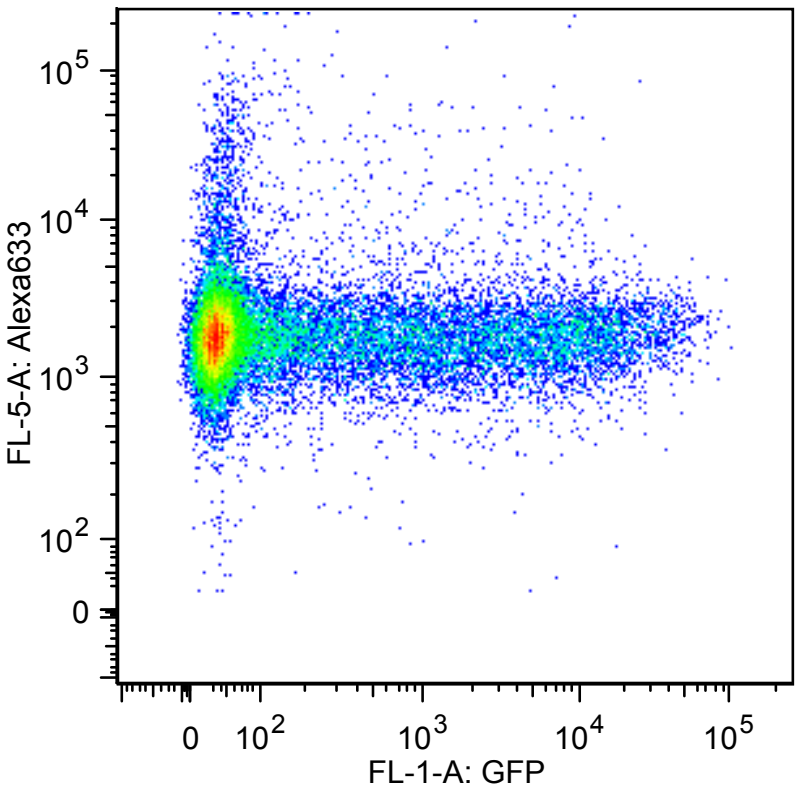
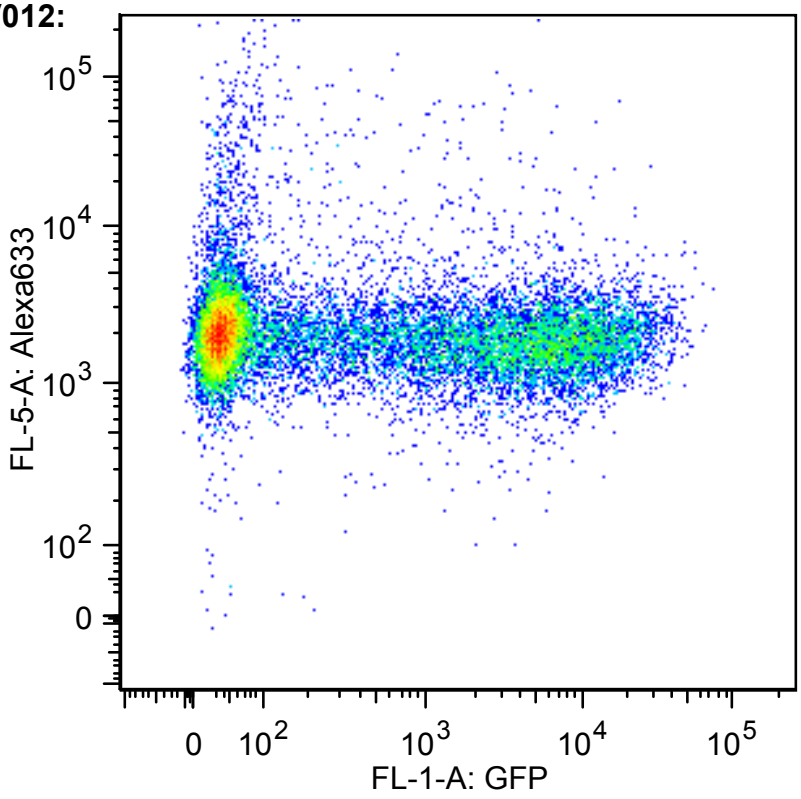


Figure 4.3. (cont'd)

4.3.4.c.iii. pTracer-CMV2-MDV012:



4.3.4.c.iv. pTracer-CMV2-MDV012ctg:

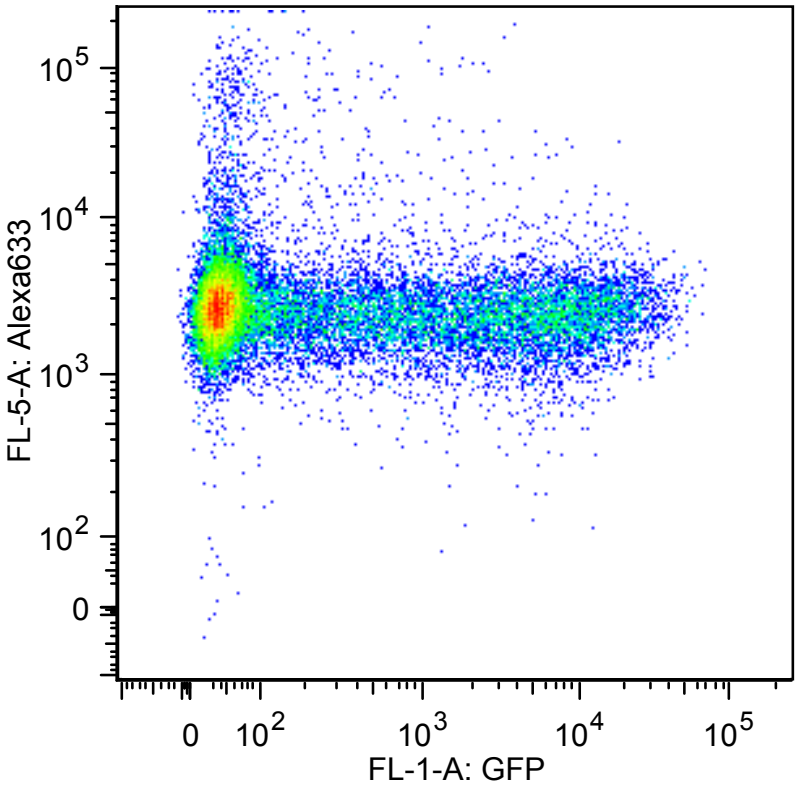
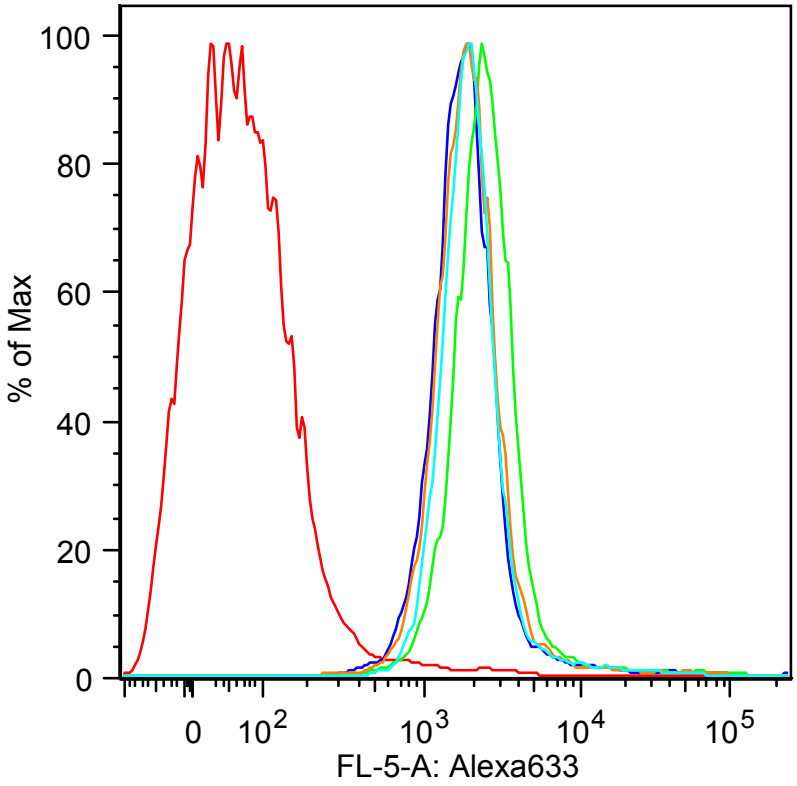


Figure 4.3. (cont'd)

4.3.4.d. MHC class I major (histogram). Anti-BF2*21/Alexa-Fluor 633 fluorescence intensity histogram of live, transfected cell populations (live cells for untransfected isotype- and anti-BF2*21-stained controls). Labels and geometric mean fluorescent intensities (GMFI) are noted in the box below.



Sample:	GMFI:
Isotype control	66
Anti-BF2*21	2016
CMV2	1853
MDV012	1969
MDV012ctg	2515

Figure 4.3. (cont'd)

4.3.5. Summary of the above 4 experiments. Staining with anti-BF2*21, as measured by geometric mean fluorescence intensity of Alexa-Fluor 633 secondary antibody on GFP+ cells. Average of Experiments 1-4, normalized to vector control (pTracer-CMV2). Error bars represent +/- standard deviation.

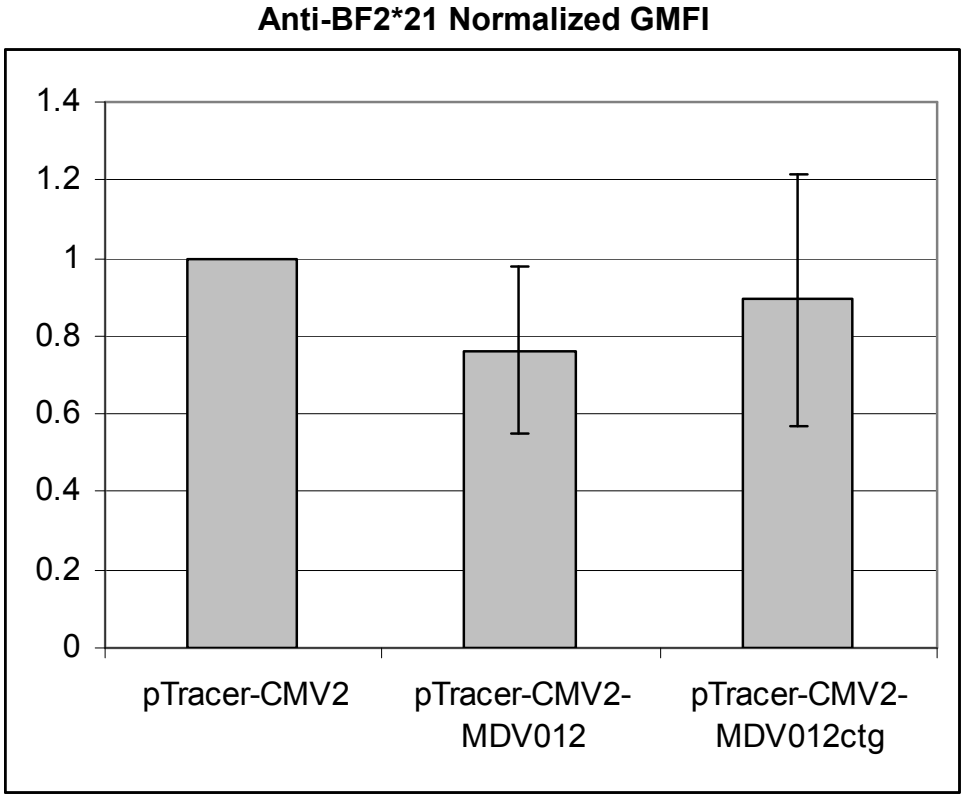


Figure 4.4. DF-1 cells transiently transfected with MDV012 plasmids express similar amounts of MHC class I minor allele to vector controls. DF-1 cells were transfected with pTracer-CMV2, pTracer-CMV2-MDV012, or pTracer-CMV2-MDV012ctg plasmids and stained with E6338 (chicken anti-chicken BF1*21 MHC class I serum), then with Alexa-Fluor 633-conjugated anti-mouse IgG. Cells were analyzed by flow cytometry, gated on live and transfected cell (GFP+) populations, and GFP+ cells were compared for anti-BF1*21 staining as measured by Alexa-Fluor 633 intensity. Three experiments shown.

4.4.1. Experiment 1. Live cell gating was the same as in sub-figure 4.3.1.a.
4.4.1.a. Transfected gate. Set on non-transfected anti-BF1*21-stained cells (<5% positive) and applied to transfected samples.

4.4.1.a.i. Anti-BF1*21:

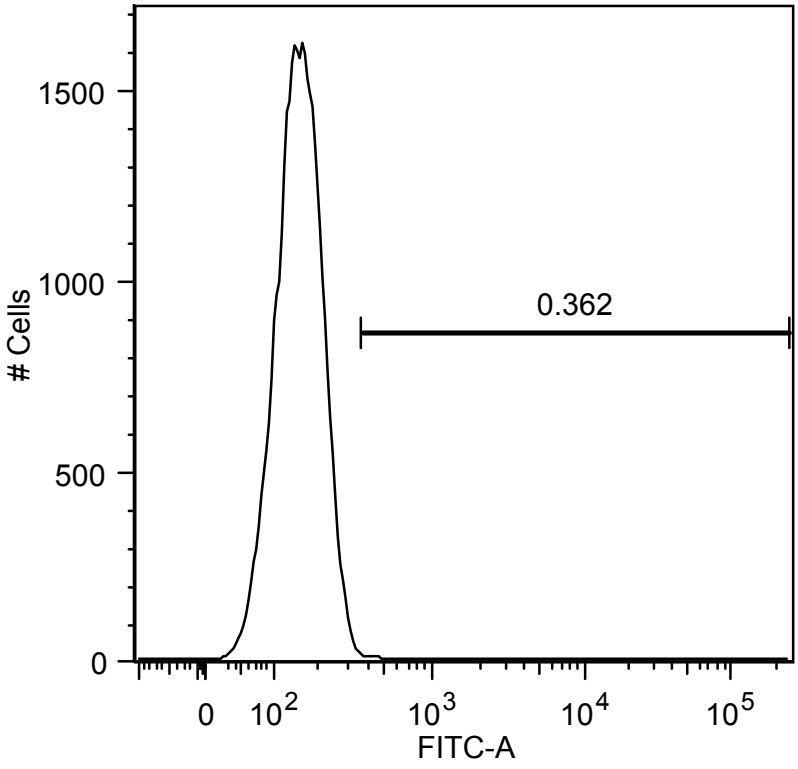
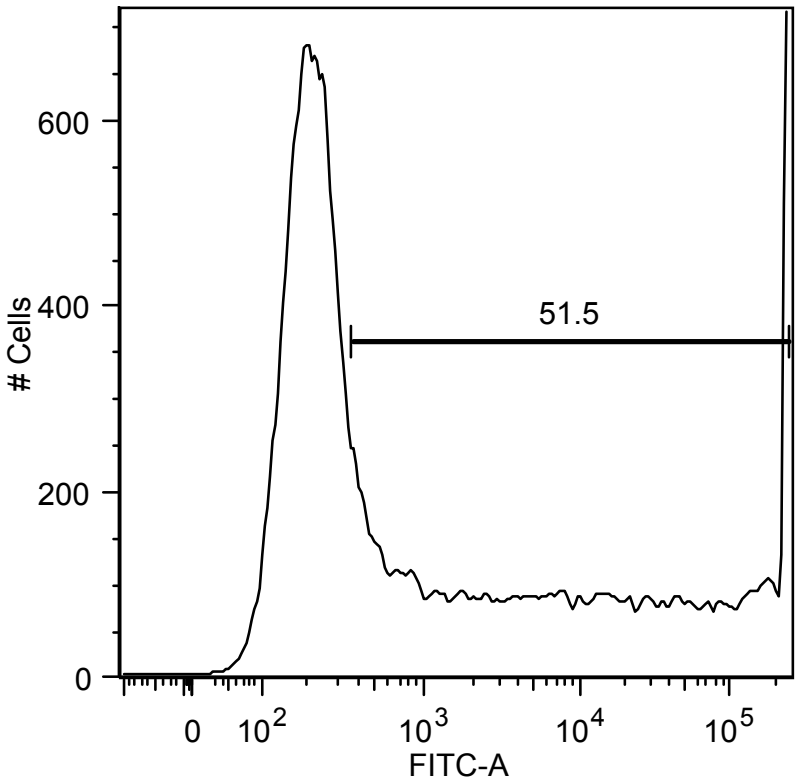


Figure 4.4. (cont'd)

4.4.1.a.ii. pTracer-CMV2:



4.4.1.a.iii. pTracer-CMV2-MDV012:

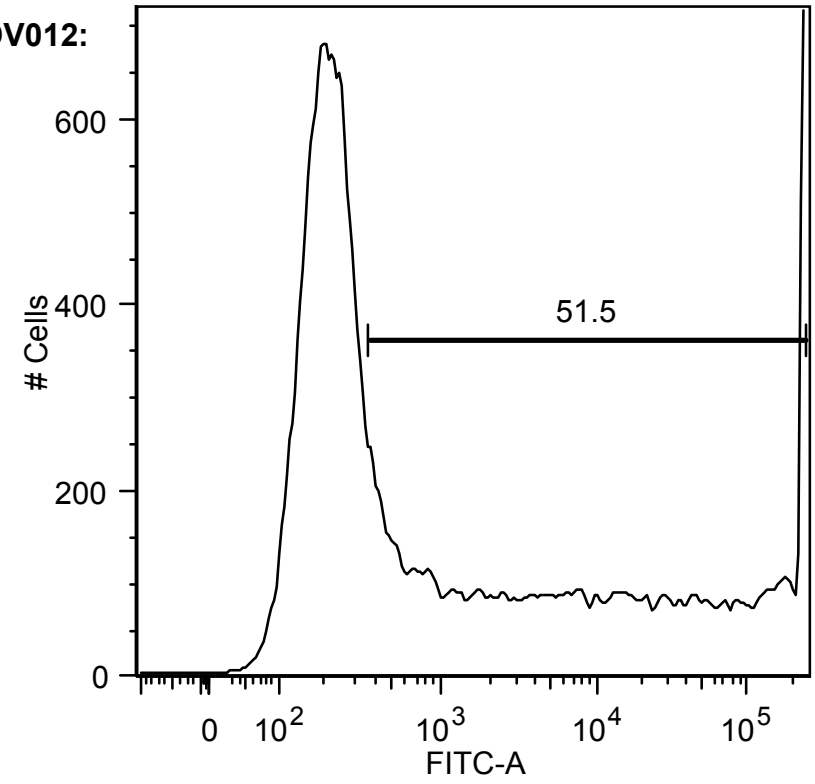


Figure 4.4. (cont'd)

4.4.1.a.iv. pTracer-CMV2-MDV012ctg:

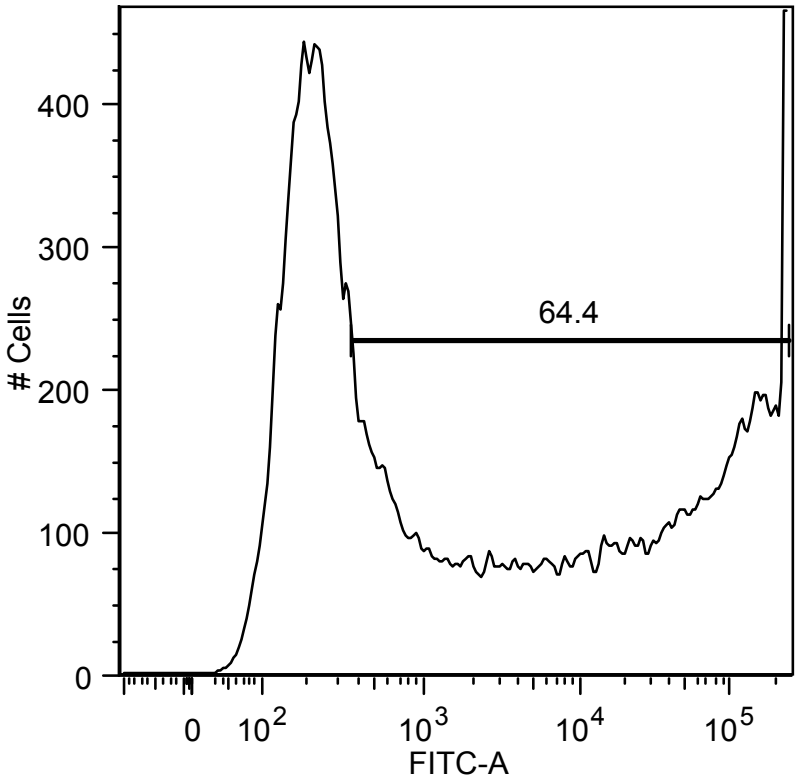
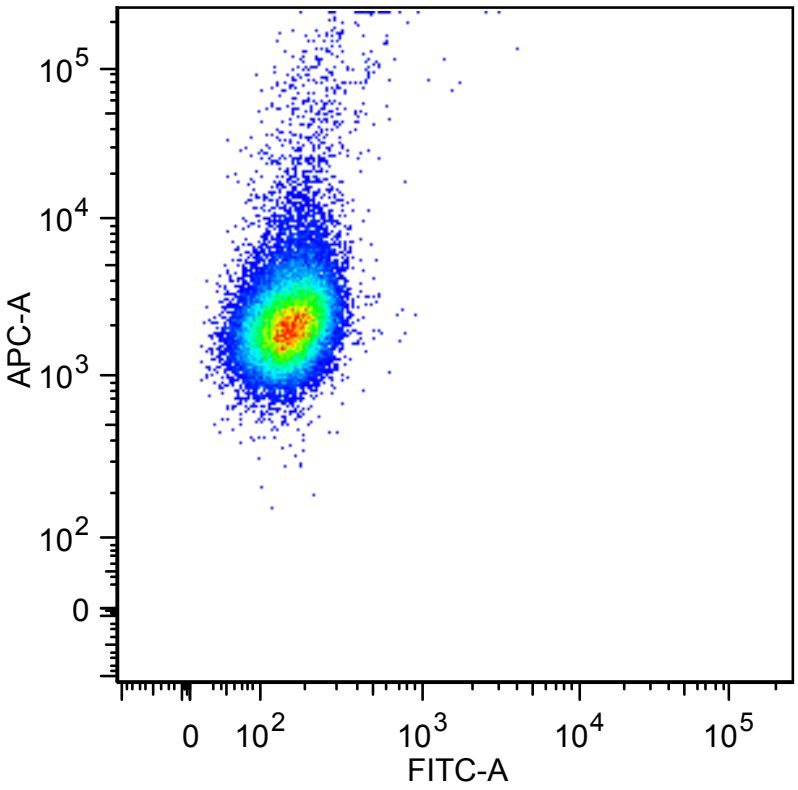


Figure 4.4. (cont'd)

4.4.1.b. GFP (FITC-A) vs. Alexa-Fluor 633 (APC-A). Live cell populations.

4.4.1.b.i. Anti-BF1*21:



4.4.1.b.ii. pTracer-CMV2:

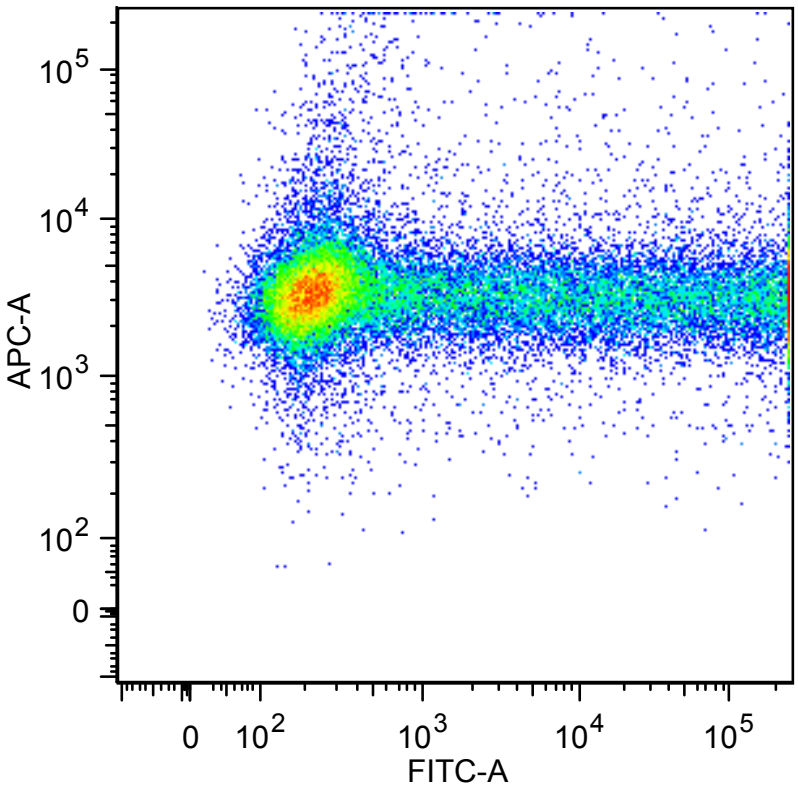
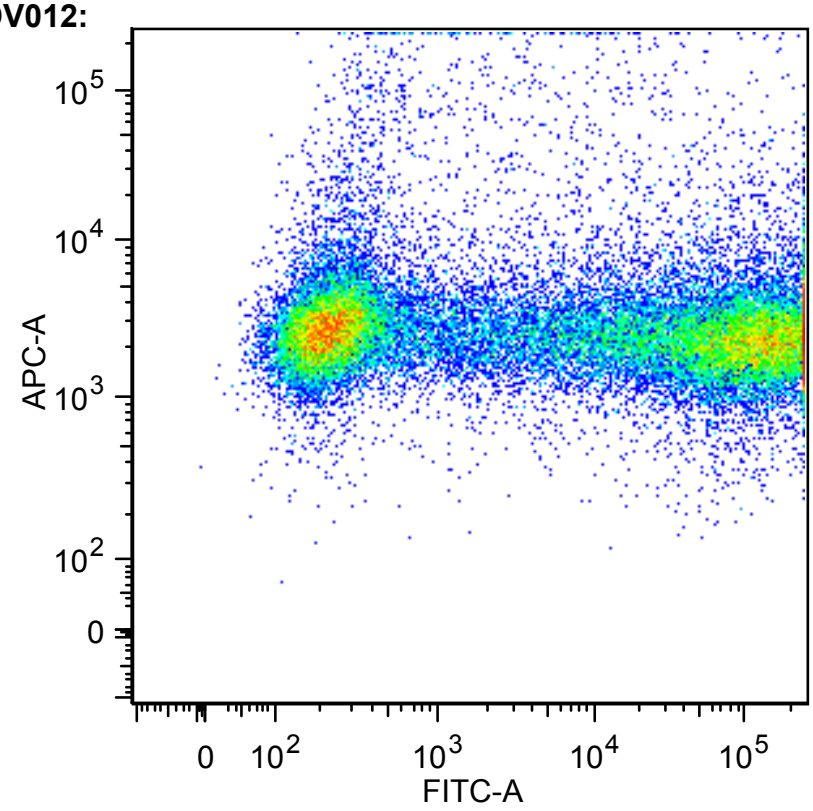


Figure 4.4. (cont'd)

4.4.1.b.iii. pTracer-CMV2-MDV012:



4.4.1.b.iv. pTracer-CMV2-MDV012ctg:

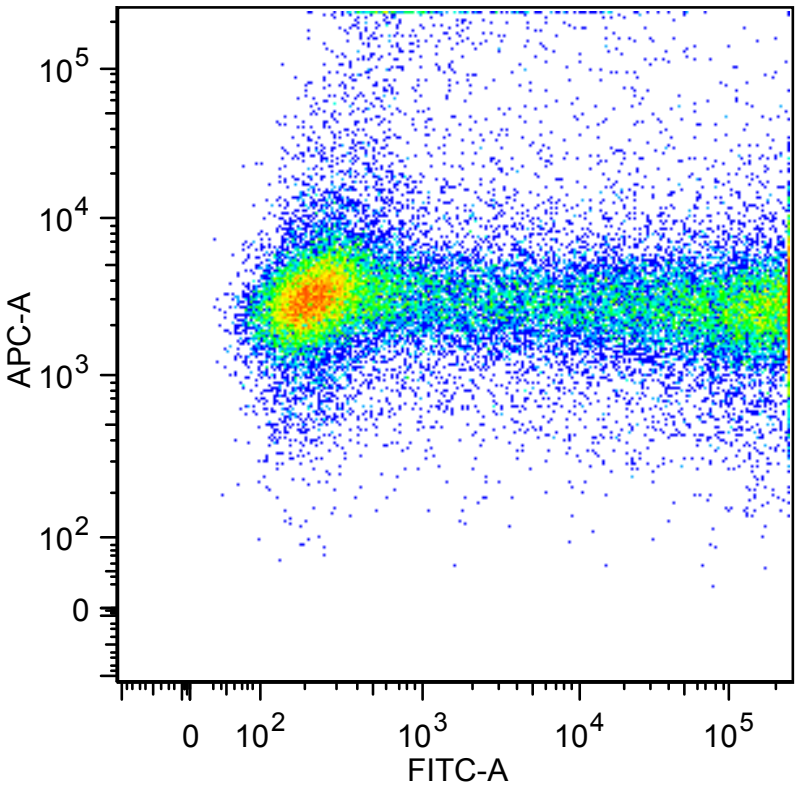
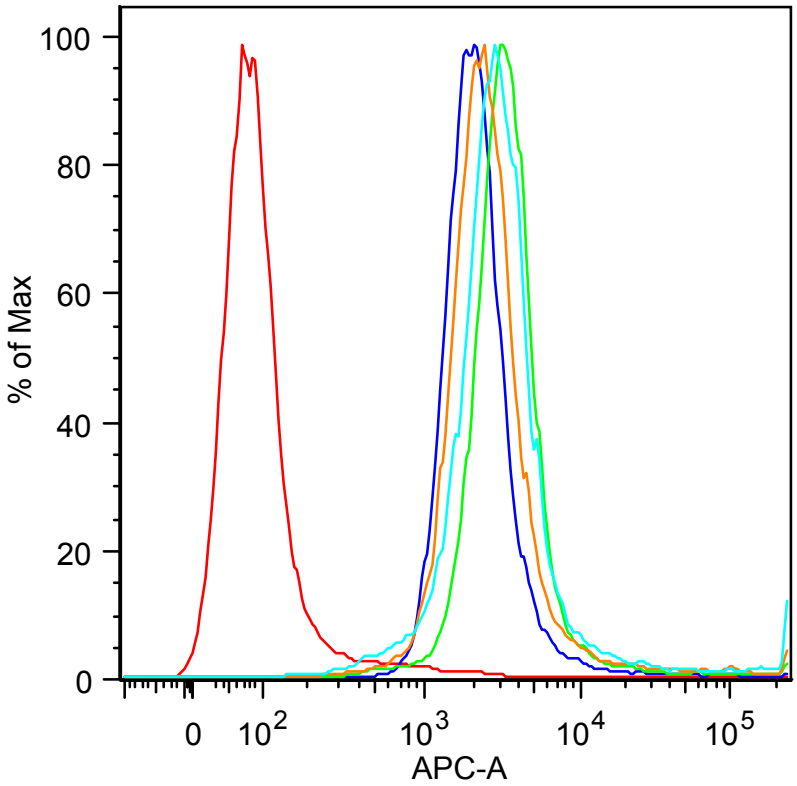


Figure 4.4. (cont'd)

4.4.1.c. MHC class I minor (histogram). Anti-BF1*21/Alexa-Fluor 633 fluorescence intensity histogram of live, transfected cell populations (live cells for untransfected isotype- and anti-BF1*21-stained controls). Labels and GMFIs are noted in the box below.

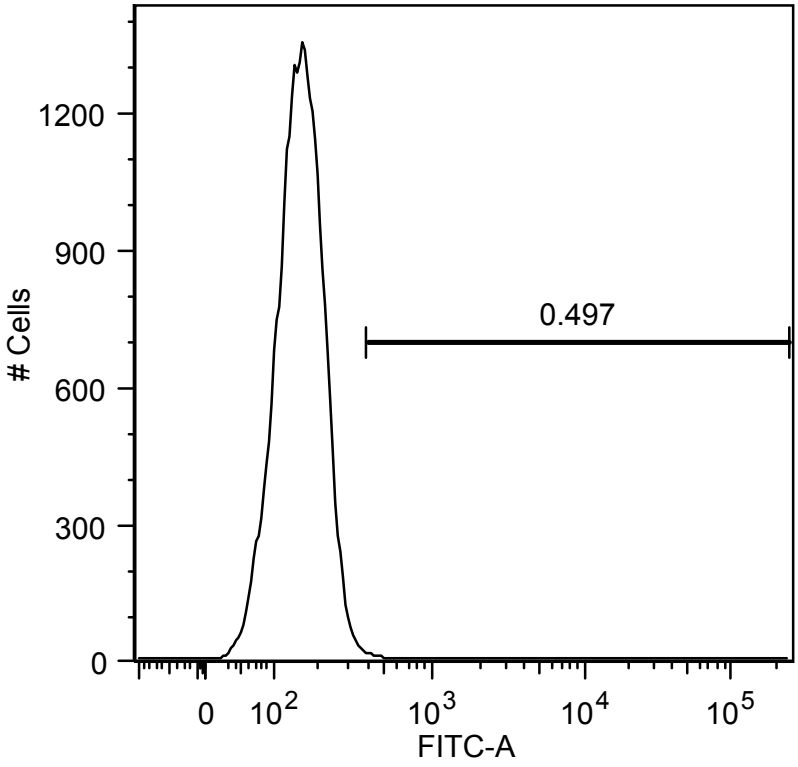


Sample:	GMFI:
Isotype control	93
Anti-BF1*21	2243
CMV2	3433
MDV012	2711
MDV012ctg	3153

Figure 4.4. (cont'd)

4.4.2. Experiment 2. Live cell gating was the same as in sub-figure 4.3.2.a.
4.4.2.a Transfected gate. As in Experiment 1 (Fig. 4.4.1.a.)

4.4.2.a.i. Anti-BF1*21:



4.4.2.a.ii. pTracer-CMV2:

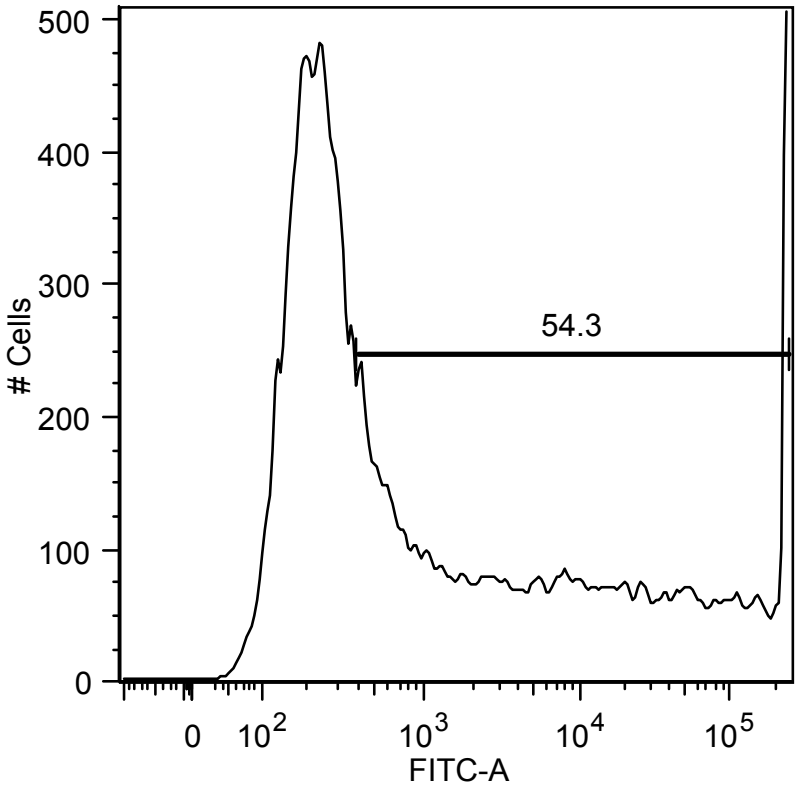
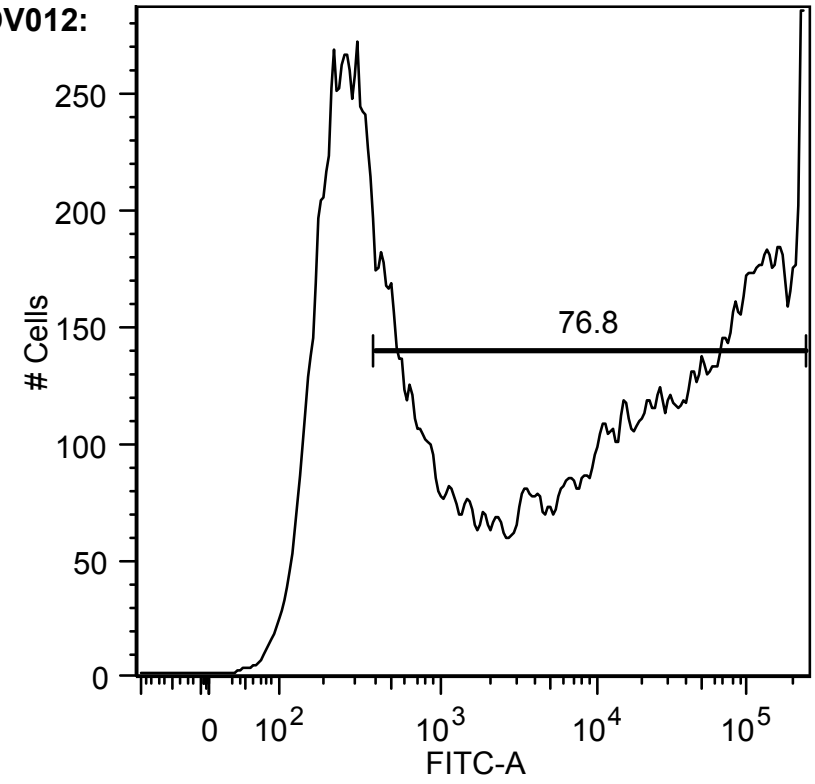


Figure 4.4. (cont'd)

4.4.2.a.iii. pTracer-CMV2-MDV012:



4.4.2.a.iv. pTracer-CMV2-MDV012ctg:

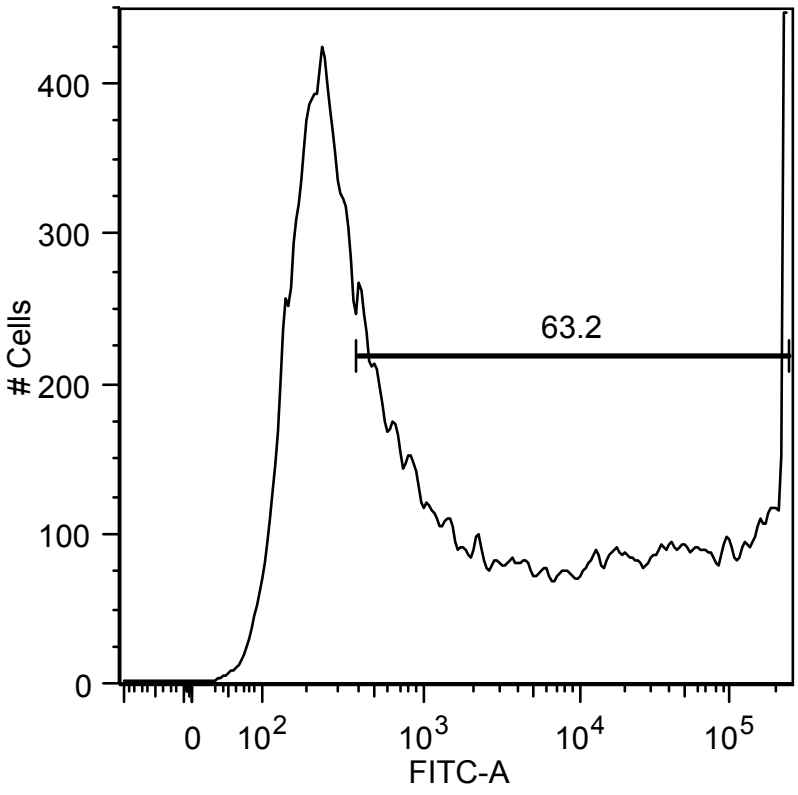
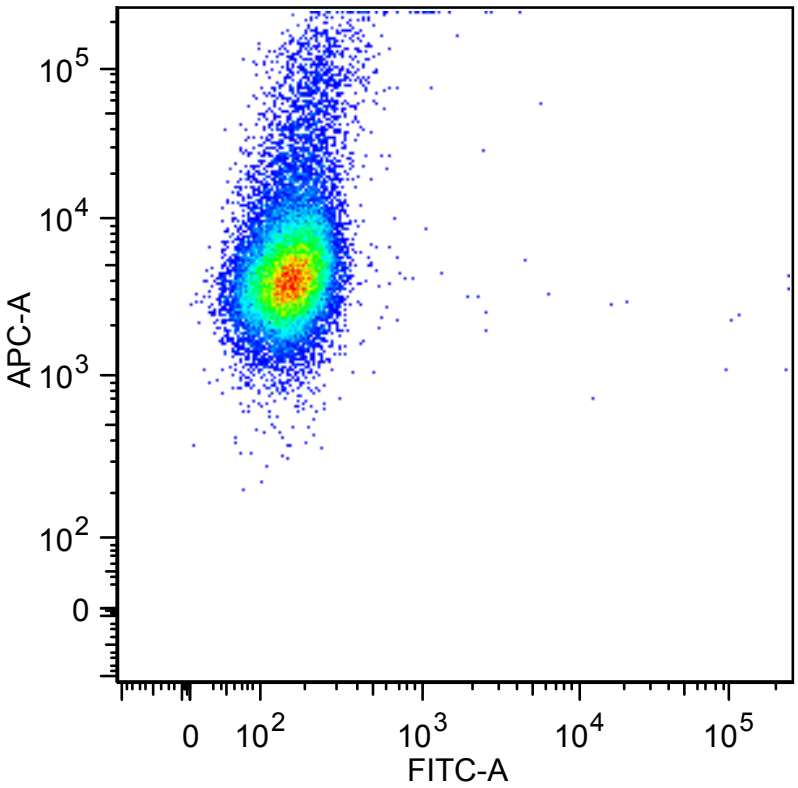


Figure 4.4. (cont'd)

4.4.2.b. GFP (FITC-A) vs. Alexa-Fluor 633 (APC-A).

4.4.2.b.i. Anti-BF1*21:



4.4.2.b.ii. pTracer-CMV2:

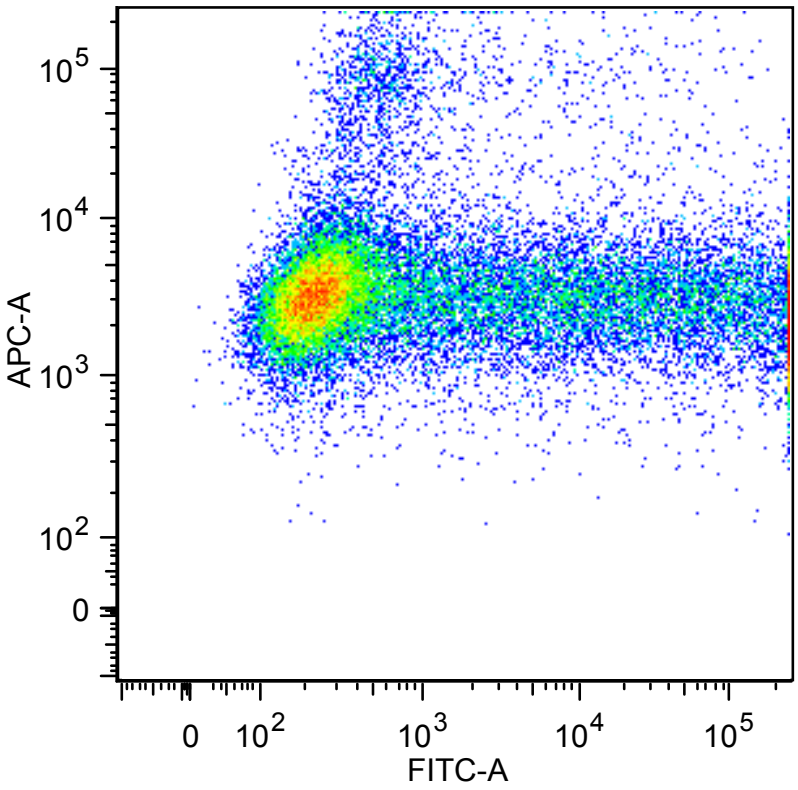
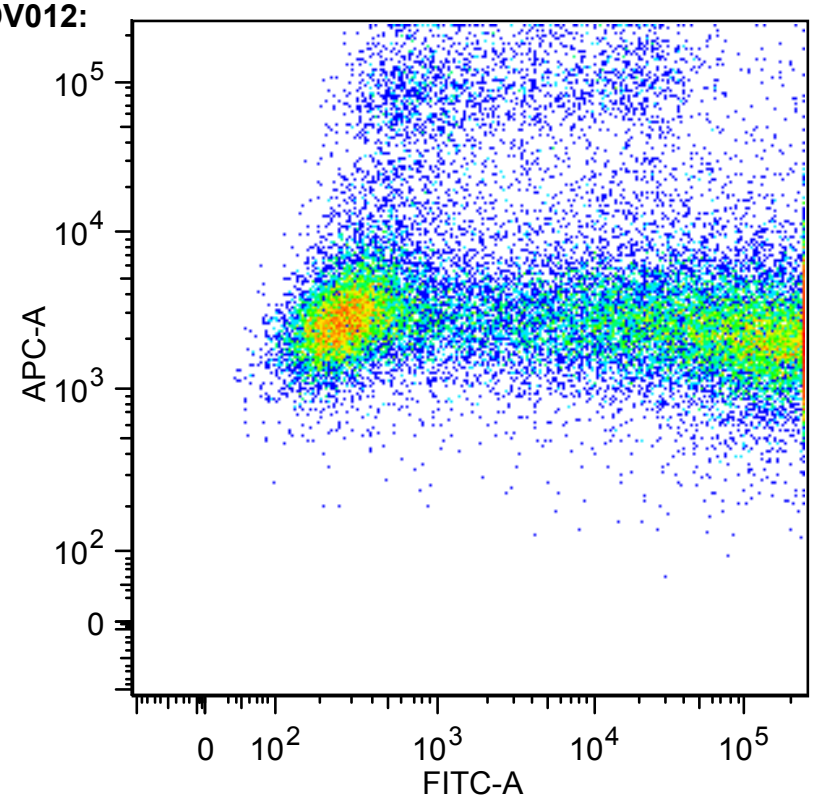


Figure 4.4. (cont'd)

4.4.2.b.iii. pTracer-CMV2-MDV012:



4.4.2.b.iv. pTracer-CMV2-MDV012ctg:

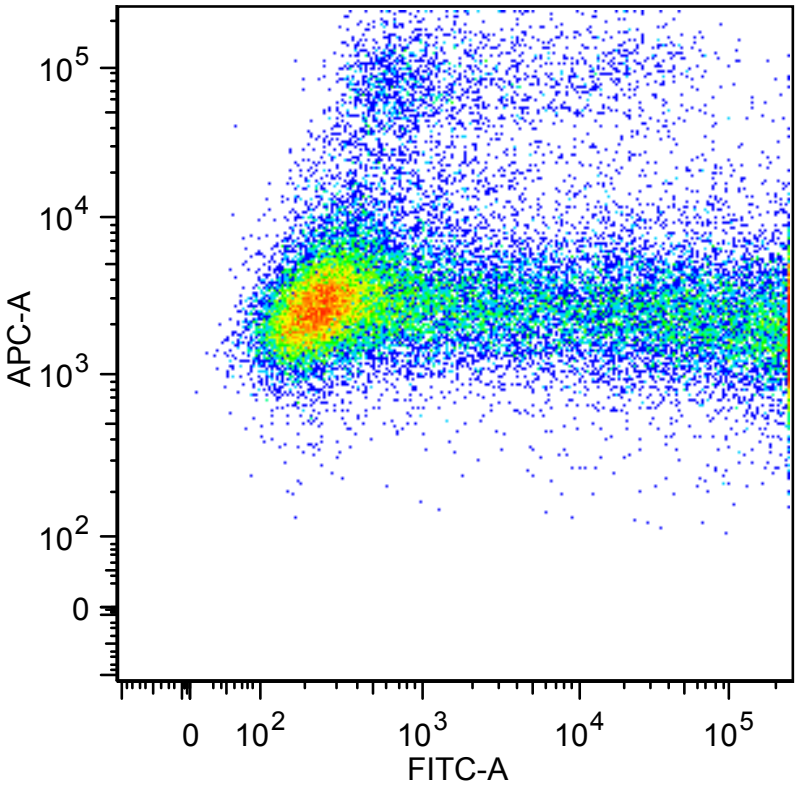
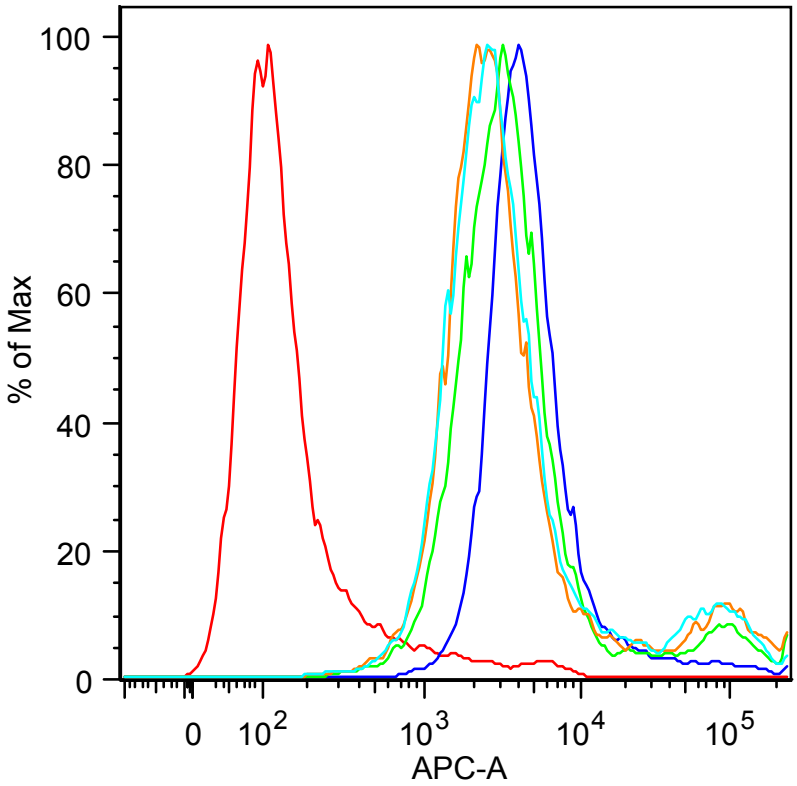


Figure 4.4. (cont'd)

4.4.2.c. MHC class I minor (histogram). Anti-BF1*21/Alexa-Fluor 633 fluorescence intensity histogram of live, transfected cell populations (live cells for untransfected isotype- and anti-BF1*21-stained controls). Labels and GMFIs are noted in the box below.



Sample:	GMFI:
Isotype control	3786
Anti-BF1*21	3743
CMV2	3986
MDV012	14020
MDV012ctg	12697

Figure 4.4. (cont'd)

4.4.3. Experiment 3. Live gating was the same as in sub-figure 4.3.3.a.
4.4.3.a. Transfected gate. Gating on GFP+ transfected cells was performed using the gate from sub-figure 4.2.3.b. (collected in the same flow cyotmetry run), as there was an unexpectedly large GFP+ population present in the untransfected, anti-BF1*21 sample (see 4.4.3.a.i. and pseudo-color plot in 4.4.3.b.i.).

4.4.3.a.i. Anti-BF1*21:

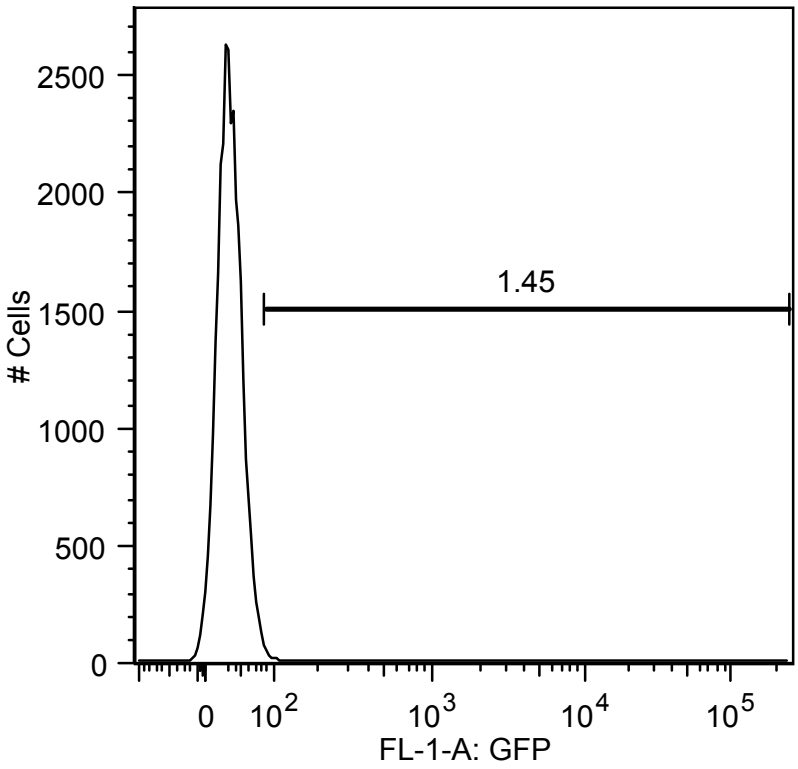
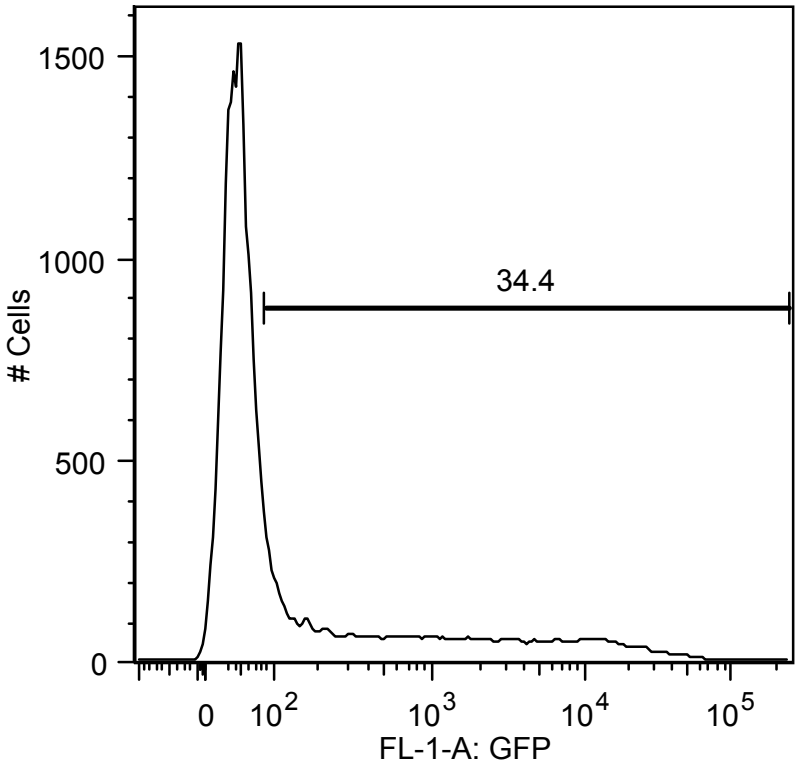


Figure 4.4. (cont'd)

4.4.3.a.ii. pTracer-CMV2:



4.4.3.a.iii. pTracer-CMV2-MDV012:

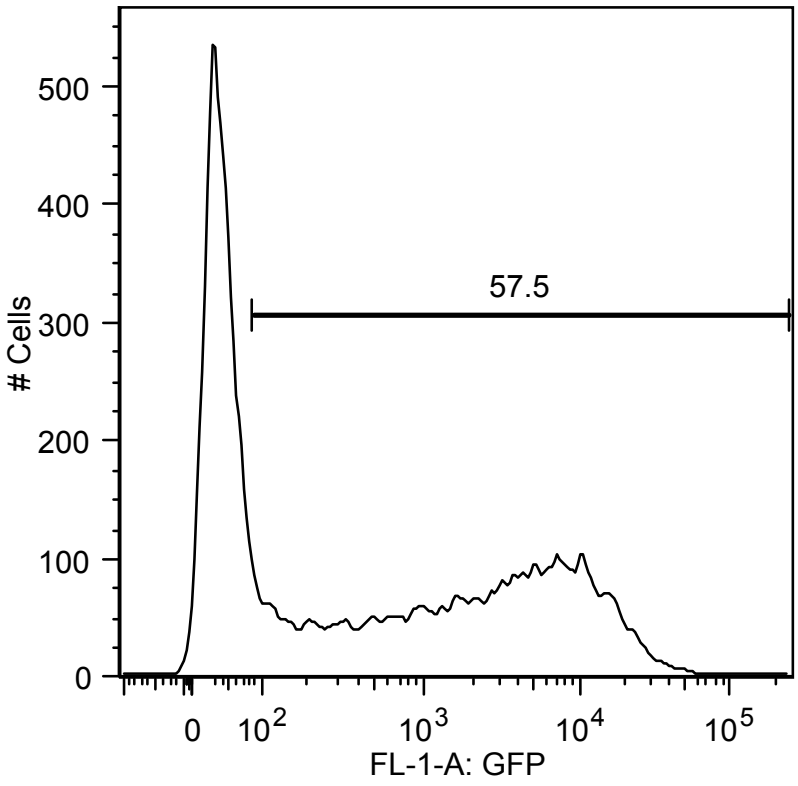


Figure 4.4. (cont'd)

4.4.3.a.iv. pTracer-CMV2-MDV012ctg:

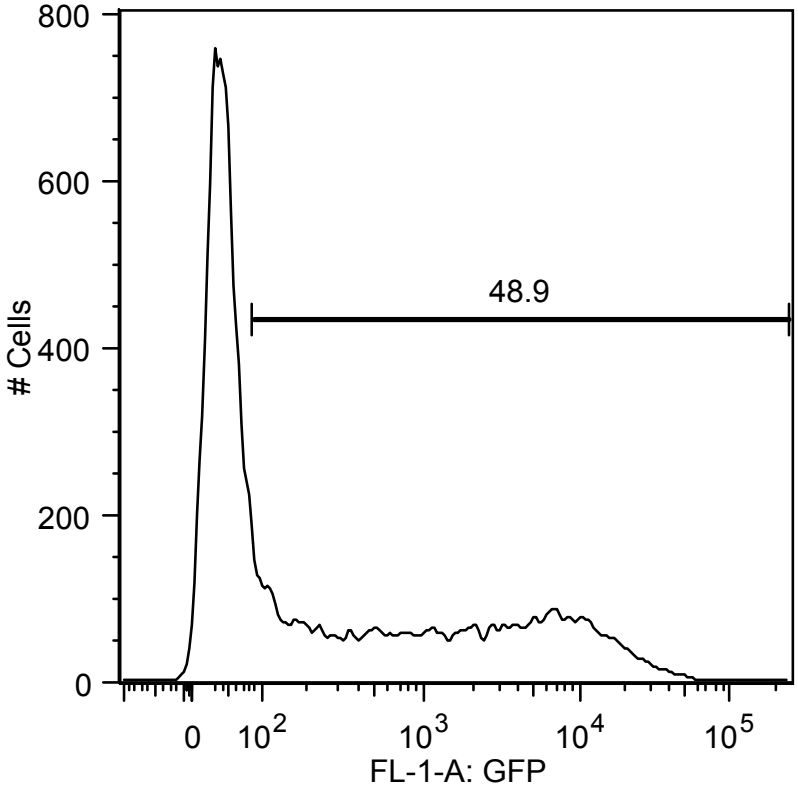
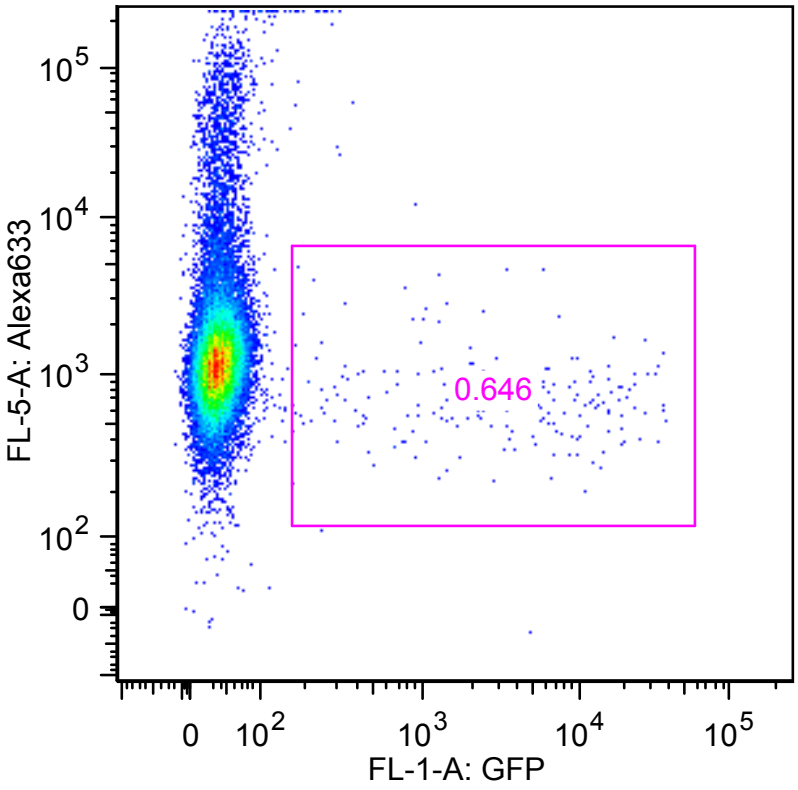


Figure 4.4. (cont'd)

4.4.3.b. GFP vs. Alexa-Fluor 633.

4.4.3.b.i. Anti-BF1*21:



4.4.3.b.ii. pTracer-CMV2:

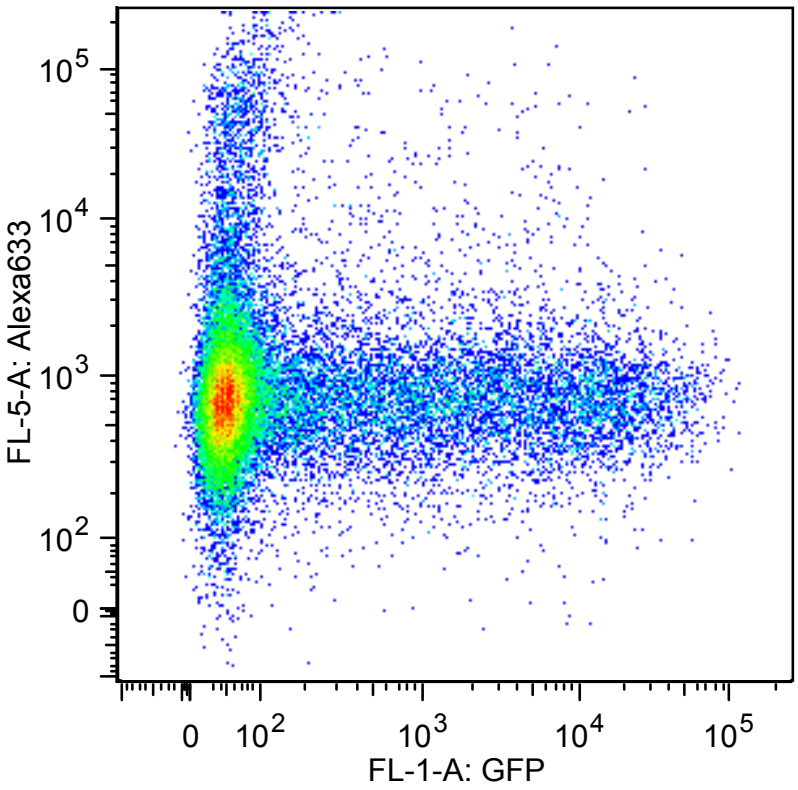
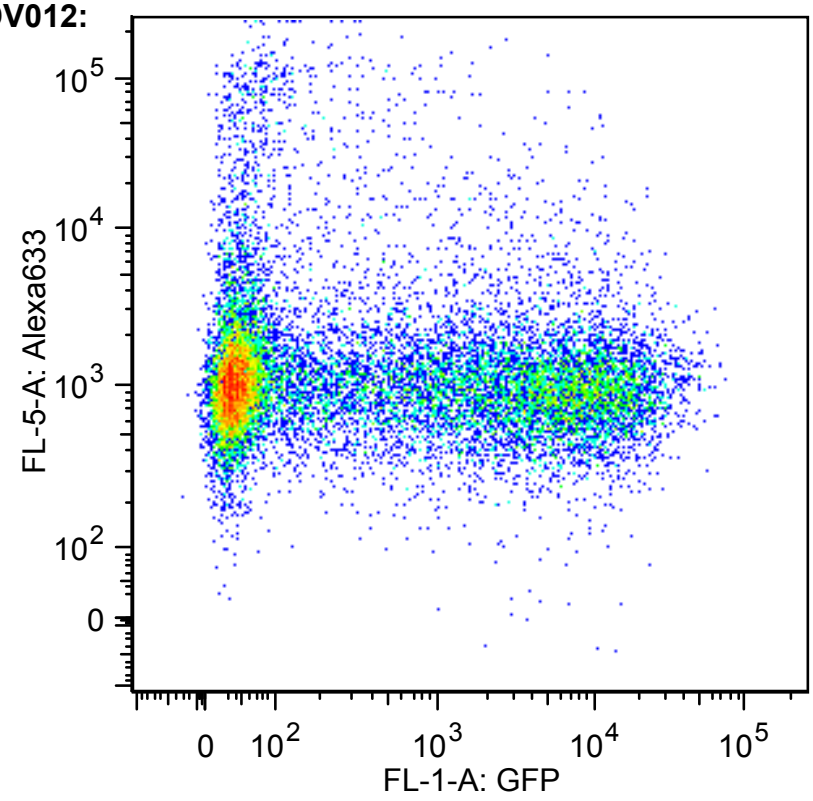


Figure 4.4. (cont'd)

4.4.3.b.iii. pTracer-CMV2-MDV012:



4.4.3.b.iv. pTracer-CMV2-MDV012ctg:

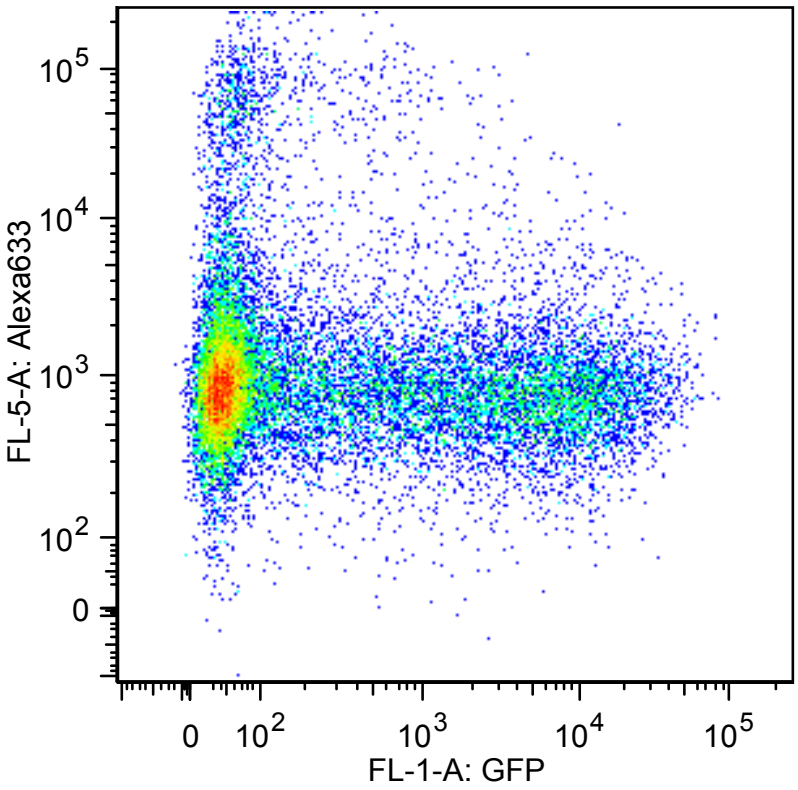
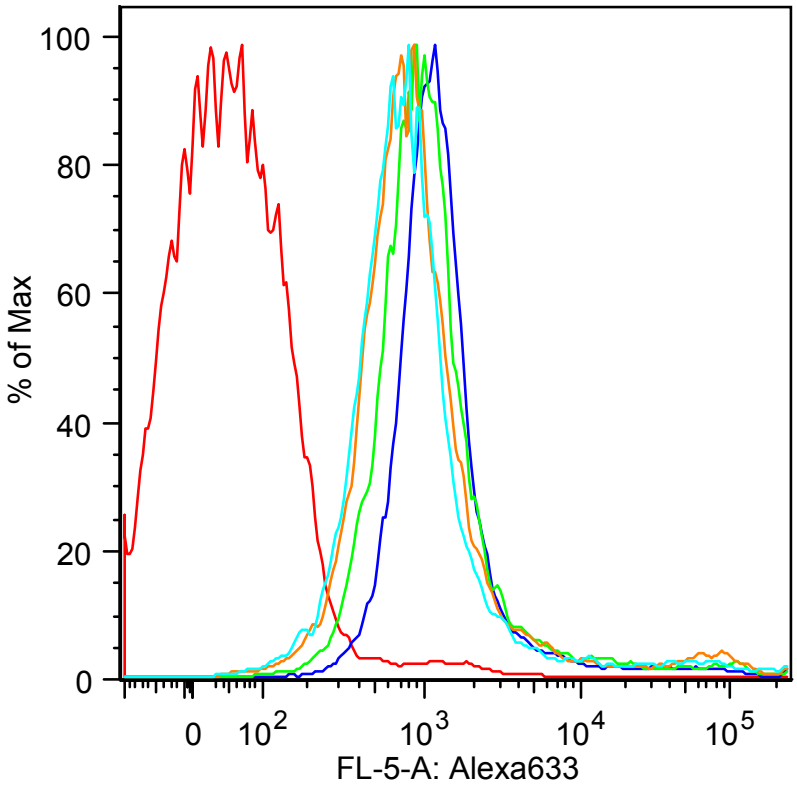


Figure 4.4. (cont'd)

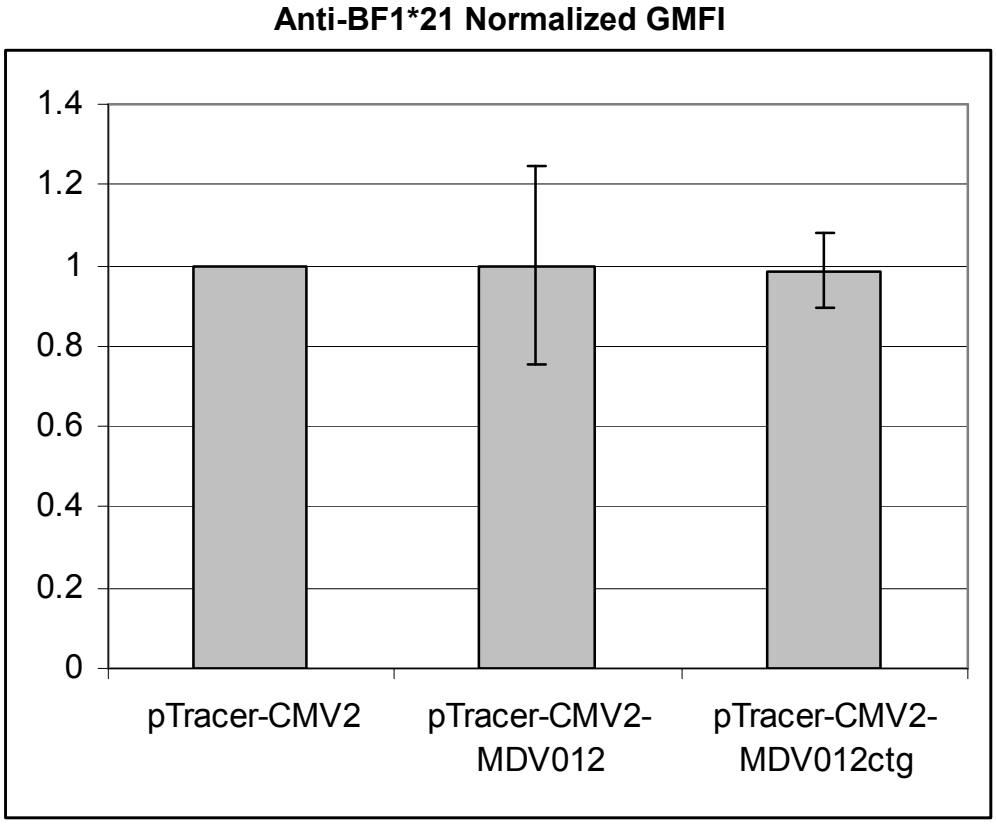
4.4.3.c. MHC class I minor (histogram). Anti-BF1*21/Alexa-Fluor 633 fluorescence intensity histogram of live, transfected cell populations (live cells for untransfected isotype- and anti-BF1*21-stained controls). Labels and GMFIs are noted in the box below.



Sample:	GMFI:
Isotype control	51
Anti-BF1*21	1325
CMV2	887
MDV012	1128
MDV012ctg	968

Figure 4.4. (cont'd)

4.4.4. Summary of the above 3 experiments (4.4.1-4.4.3). Staining with anti-BF1*21, as measured by geometric mean fluorescence intensity of Alexa-Fluor 633 secondary antibody on GFP+ cells. Average of Experiments 1-3, normalized to vector control (pTracer-CMV2). Error bars represent +/- standard deviation.



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