VIRAL MODULATION OF MHC CLASS II-MEDIATED ANTIGEN PRESENTATION

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ABSTRACT

Nan Wang

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Vaccinia virus (VV) has been used as a vaccine, yet safety concerns remain due to its viral immunoevasive properties. Among these, VV infection of antigen presentation cells (APC) perturbs MHC class II-mediated antigen (Ag) presentation. The goals of this project include: 1) to define mechanisms by which VV disrupts class II presentation; and 2) to examine whether disruption of the class II pathway by VV alters T cell responses *in vitro* and *in vivo*. A significant reduction in the expression of the class II chaperone, invariant chain (Ii), was observed during the late stage of VV infection. Yet surface expression of MHC class II molecules was maintained along with cell viability. To examine whether VV acts solely to disrupt host protein synthesis, B cells were treated with an inhibitor of translation-cycloheximide (CHX). Like VV, CHX negatively regulated Ii protein expression and class II presentation. Ii proteolysis also contributed in part to reduce Ii expression in VV infected and CHX treated APC. Yet only VV infection altered lysosomal protease expression, potentially influencing Ii degradation. Over-expression or ectopic-expression of Ii partially protected cells from VV-induced class II dysfunction. These studies suggest VV destabilizes class II molecules by disrupting Ii expression. To examine the presentation of viral Ags by class II, CD4 T cells from VV-primed mice were used. Viral proteins were presented by class II shortly after APC exposure to low concentrations of VV. The presentation of VV Ags correlated temporally with reductions in exogenous peptide presentation. At higher MOI ($\geq$ 1), class II presentation of VV Ags was reduced. To examine the in vivo effects of VV on Ag presentation, a mouse model of ovalbumin-induced airway hypersensitivity was used. Th2 cytokine production was reduced, while a novel inflammatory cytokine Interleukin-17 (IL-17) production was enhanced in asthmatic VV-infected mice. In health mice, repeated VV infections lead to enhanced CD8 T cell production of Interferon-γ (IFN-γ) and IL-17. Finally, antibodies to a viral protein H3 were generated and shown to preserve

class II presentation. Together these studies suggest VV disruption of the class II pathway may blunt T cell responses to VV.

Janice S. Blum, Ph.D., Chair

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ABBREVIATIONS

Ab	antibody
AEP	asparagine endopeptidase
Ag	antigen
APC	antigen presenting cell
AraC	arabinosylcytosine
BALT	bronchus-associated lymphoid tissue
BFA	brefeldin A
B-LCL	B lymphoblastoid cell line
BSA	bovine serum albumin
Cat	cathepsin
cDNA	complementary DNA
Chl	chloroquine
CHX	cycloheximide
CLIP	class II-associated invariant-chain peptide
CTL	cytotoxic T lymphocyte
DC	dendritic cell
EGFP	enhanced green fluorescence protein
EPO	epoxomicin
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorter
FcR	Fc receptors
GAD	glutamate decarboxylase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GILT	gamma-interferon-inducible lysosomal thiol reductase
H&E	hematoxylin and eosin
HEL	hen egg lysozyme
HI	heat inactivation
HLA	human leukocyte antigen
HSA	human serum albumin

HSC	heat shock cognate protein
HSP	heat shock protein
IFN-γ	gamma-interferon
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
i.p.	intraperitoneal
KD	kilodalton
KLH	keyhole limpet hemocyanin
LAC	lactacystin
Leu	leupeptin
LIP	leupeptin-induced peptide
LPS	lipopolysaccharide
m	minute(s)
MIIC	MHC class II containing compartment
MHC	major histocompatibility complex
MOI	multiplicity of infection
Mon	monensin
MVA	modified vaccinia virus Ankara
NKT	natural killer T cells
OAS	2'-5' oligoadenylate synthetase
OVA	ovalbumin
PAS	periodic acid-Schiff
PBMC	peripheral blood mononuclear cell
PMSF	phenylmethyl-sulphonylfluoride
rVV	recombinant VV
SLIP	small LIP
Th	T helper cell
TLR	Toll-like receptor
VV	vaccinia virus

INTRODUCTION

Vaccinia virus (VV), an enveloped virus, belongs to the poxviridae family of complex DNA viruses that replicate in the cytoplasm of host cells. VV has a linear, double-stranded DNA genome (about 200 kb) encoding more than 200 proteins. VV falls in the chordopoxvirinae subfamily and orthopoxvirus genera which include 3 genetically and antigenically related members: variola virus (smallpox), cowpox (the first vaccine for smallpox), and VV. Because of this homology, VV had been used as the vaccine to eradicate smallpox [1]. More recently, VV has been proposed as a vector to express foreign genes to generate potent vaccines for infectious agents and tumors. While VV is highly effective in inducing long-lasting protective immunity in a majority of healthy individuals, potentially serious complications following VV infection have limited enthusiasm for this virus as a universal vaccine reagent in immune deficient as well as hypersensitive individuals, the elderly and children [2-5]. Thus, investigation of the mechanisms by which VV perturbs host immunity remains important in terms of designing attenuated VV-based vaccines. Here, basic concepts of host immunity, the immune-evasion properties of VV, and VV vaccine development are reviewed.

1. Introduction to the host immune system

Host immunity is comprised of two parts - innate immunity and adaptive immunity. Innate immunity is quick acting and not specific for a pathogen or Ag. Adaptive immunity is induced later, is Ag specific and leads to the development of immunological memory. Detailed discussion of both innate and adaptive immunity is offered here.

1.1 Innate immunity

Innate immunity is the first line of defense promoting host protection against infection independent of the Ag or pathogen trigger. Many types of immune cells (i.e. phagocytes, neutrophils and natural killer (NK) cells etc.) and soluble factors (cytokines, chemokines, and complement) have evolved to protect the host from pathogen infection via pattern

recognition and other mechanisms. For example, after an epithelia barrier has been breached, the repeating patterns of molecular structures on the surface of a pathogen (termed pathogen-associated molecular patterns, PAMPs) such as bacterial lipopolysaccharide (LPS) are recognized by cell surface pattern recognition receptors (PRRs) such as Toll-like receptor-4 (TLR4) on surveying immune cells as well as epithelial cells. The ten TLRs identified in humans and mice detect PAMPs on most pathogens. Activated TLRs then induce pro-inflammatory cytokine and chemokine production to recruit and activate phagocytes and NK cells. TLR activation can also up-regulate co-stimulatory molecule expression on APCs to promote adaptive immunity. Other PRRs like the mannose-binding lectin (MBL) increase phagocytosis and activate complement to destroy pathogens [6, 7]. Soluble innate mediators including complement components, cytokines, and chemokines, also facilitate the activation of cellular or adaptive immunity.

1.2 Adaptive immunity

Adaptive or acquired immunity is characterized by clonal expansion of Ag-specific lymphocytes including T and B cells. The development of CD4 and CD8 T cells as well as Ag-specific antibody (Ab) producing B cells is critical for host elimination of pathogens and the induction of long-lasting immunological memory. The activation of T cells is dependent upon engagement with antigen presenting cells (APCs) such as dendritic cells (DCs), B cells, and macrophages. These professional APCs are characterized by their expression of detectable surface major histocompatibility complex (MHC) class I and class II as well as co-stimulatory molecules. APCs survey the host to internalize pathogens and antigens (Ags) such as proteins, lipids, nucleic acids and sugars. Protein Ags are processed within APCs by a variety of pathways, leading to the generation of antigenic peptides which bind MHC class I and class II proteins. The resulting peptide-class I or II complexes are recognized by T cell receptors (TCR) along with engagement of adhesion and co-stimulatory molecules to promote T cell activation. After being activated by peptide-class II complexes, CD4 T cells can be differentiated into 4 effector subsets, i.e. Th1, Th2, T regulatory (Tr)1 and Th17 cells, depending on

their profile of transcription factor controlled cytokine and chemokine expression in response to stimulation by APCs. Th1 cells predominantly produce IL-2 and IFN-γ to activate macrophages and induce localized inflammation. Th2 cells make IL-4, IL-13, and IL-5 and stimulate B cells to produce Ab. Th17 cells secrete IL-17 to help recruit granulocytes including neutrophils to sites of infection or inflammation. Tr1 produce TGF-β and IL-10 to suppress or down-regulate immune responses [8]. CD8 T cells are activated by MHC class I-peptide complexes on APCs. These cells frequently produce cytokines and express proteins promoting killing or cytolytic function, thus they are named cytotoxic T lymphocytes (CTL). CD8 CTL can induce apoptosis of infected or target cells. CD8 T cells producing IFN-γ and IL-4 are termed as Tc1 and Tc2 respectively [9], while CD8 T cells secreting IL-17 are known as Tc17 [10]. The differentiation of Th and CTL cells has been summarized in Fig. 1.

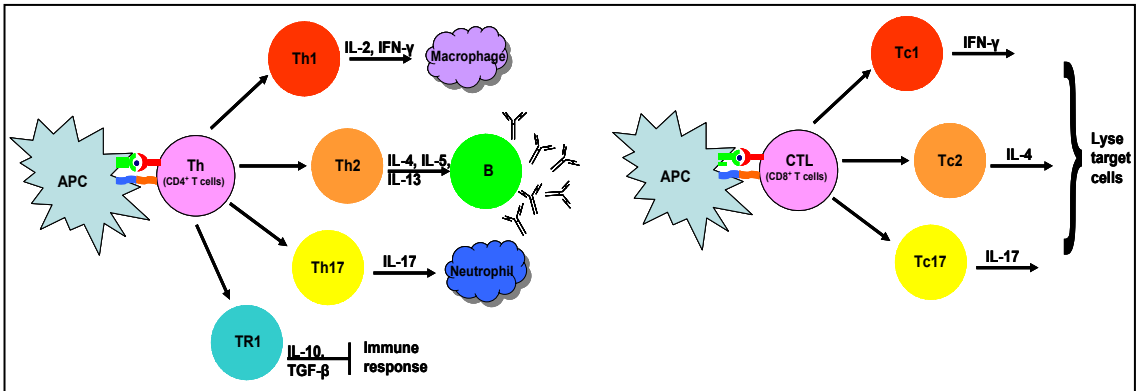


Fig. 1 Differentiation of Th and Tc cells. See details in the text.

B cells which display surface immunoglobulins (Igs), with or without Th2 cell help, are activated and mature to plasma cells which secrete Abs. Abs neutralize pathogens or tag infectious agents for clearance and destruction by phagocytes [6, 8, 11, 12]. As professional APCs, B cells also display MHC molecules and present Ag. B cells have specific Ag receptors or Igs on their surface. B cells uptake soluble proteins and solid Ag by pinocytosis and specific receptors respectively. Although B cells constitutively express MHC class II molecules, they need activation by Th cells to significantly increase co-stimulatory molecule expression.

1.3 Ag processing and presentation

Ag presentation leads to the generation and display of TCR ligands, and thus regulates adaptive immunity. There are two pathways, i.e. MHC class I and class II which activate CD8 and CD4 T cells respectively. These molecules bind and display peptides or epitopes from protein antigens for recognition by T cells. By contrast, CD1 and specifically CD1d proteins bind glycolipids and display these to NKT cells. NKT cells also express CD4 and CD8 surface markers. The basic concepts of these pathways are reviewed here.

1.3.1 MHC class I-mediated Ag presentation

MHC class I molecules typically present endogenous Ag including protein products of cytosolic replicating viruses and bacteria, as well as the incorrectly translated host proteins or peptides known as defective ribosomal products (DRiPs). Cytosolic Ags are processed by a multi-catalytic protease complex called the constitutive proteasome. During infection, the immunoproteasome can be induced upon cellular exposure to IFN-γ, α and β [13]. Proteasome processing of Ags leads to the formation of short peptides which are transferred to the ER by the transporter associated with antigen processing (TAP1/TAP2). Within the ER, newly synthesized MHC class I molecules are folded, chaperoned and retained in the ER by a series of proteins (calnexin, calreticulin, Erp 57 and tapasin). Peptides delivered into the ER by TAP are loaded onto class I molecules, and these MHC class I-peptide complexes are exported to the cell surface for display to CD8 T cells. In some APCs including macrophages and DCs, cross-presentation of exogenous Ags by class I molecules also occurs. For example, a virus infected cell is phagocytosed by a DC. The viral Ags are retrotransported into the cytosol of the DCs, processed and delivered to the ER for binding to class I molecules. Cross-presentation pathways appear to be essential for priming naïve CD8 T cells [14].

In addition to classical class I molecules encoded within the MHC gene complex, there are MHC class I-like molecules called CD1. CD1 molecules present lipid Ag to NKT

cells, which have both NK and T cell surface markers. Although CD1 molecules are structurally similar to MHC class I molecules, they behave like MHC class II molecules by transporting through the endosome-lysosomal pathway. The five human CD1 gene products are divided into 3 groups – group 1 including CD1a, CD1b and CD1c; group 2 containing CD1d and the intermediate group comprised of CD1e. Group 1 molecules present microbially derived lipids to diverse NKT cells while group 2 molecules present self lipids to invariant NKT cells with same TCR chain (Vα24-Jα18 in humans) [14]. Activated NKT cells secrete both Th1 and Th2 cytokines.

1.3.2 MHC class II-restricted Ag presentation

MHC class II molecules typically acquire exogenous Ags after endocytic or phagocytic uptake by professional APCs including B cells, DCs and macrophages. While Ag can be delivered into APCs by fluid phase uptake, receptors on APCs can enhance Ag internalization by 100-1000 fold to enhance Ag presentation. Ag processing can begin in endosomes and may increase upon delivery to lysosomes. Acidic proteases in these organelles such as cathepsins (Cat) S, L, B and D and asparagine endopeptidase (AEP) have been shown to differentially cleave Ags to yield smaller peptides. Endogenous Ags can also be transported to lysosomes by autophagy [14]. In addition, reductases such as the IFN-γ induced lysosomal thiol reductase (GILT) also facilitate Ag unfolding and proteolytic processing to peptides [15].

MHC class II molecules consist of αβ dimers which pair with the chaperone protein Ii in the ER. These αβIi complexes transit to the Golgi for glycosylation, and then they are targeted to the endosomal network where Ii is degraded by AEP, Cat S and Cat L [16-18]. Another MHC-encoded dimer complex, HLA-DM binds class II α and β to release residual Ii fragments from the class II ligand binding groove. This permits peptide fragments from processed Ags to bind to class II molecules. In some APCs including B cells and DCs, there is another class II like dimer termed DO which can physically bind to DM to partially inhibit DM's function [19]. The regulation of DM and DO binding may depend upon an acid pH environment. Newly formed peptide-class II complexes

rapidly transit to the surface of APCs for display to CD4 T cells. Thus, the intracellular maturation and trafficking of class II molecules are key to their acquisition of function.

1.3.3 Regulation of MHC class II function by Ii

Among the components of the class II pathway, Ii plays an essential role by stabilizing class II dimers, targeting class II to endosome-lysosomal vesicles, and preventing premature peptide binding to class II [14]. The importance of Ii in class II presentation has been demonstrated by studies using cell lines and animals with altered Ii expression. Ii loss induced either by antisense oligodeoxynucleotides or CHX significantly reduces MHC class II mediated Ag presentation, while enhancement of Ii expression by either transfection or knock-in improves class II function [20-22]. Ii reduction in APCs may have pathogenic implications. For example, in human diabetic cells impaired class II function is coupled with decrease Ii expression while correction of Ii expression by a vector rescues class II-mediated presentation [23].

The gene for Ii is located on chromosome 5 in humans and chromosome 18 in mice. Both human and mouse Ii genes are encoded outside the MHC gene loci on chromosome 6 and 17 respectively [14]. The Ii gene gives rise to a family of 4 protein isoforms, i.e. p33, p35, p41 and p43, dependent on the use of different translational initiation sites and alternative splicing [24, 25]. Newly synthesized Ii forms trimers, with each of the 4 isoforms potentially incorporated into homotrimers and heterotrimers. The predominant Ii isoforms found in APCs, are p33 homotrimers and p33/p35 heterotrimers. Each subunit of the Ii trimer binds noncovalently to MHC class II αβ heterodimer with help from the chaperone calnexin in the endoplasmic reticulum (ER). Following the formation of these nonamers, $(\alpha\beta Ii)_3$ complexes are directed to the Golgi and then endosomal network through targeting sequences locating in the cytoplasmic domain of Ii. Along the ER-Golgi-endosome pathway, post-translation modifications of Ii such as glycosylation, lipidation and phosphorylation also take place [26-28].

Throughout the endocytic pathway, Ii is degraded successively into 3 intermediates: 1) leupeptin-induced proteins (LIP); 2) small leupeptin-induced proteins (SLIP); and 3) class II-associated invariant chain peptide (CLIP) [29]. A leupeptin-insensitive protease(s) initiates the cleavage of Ii to produce LIP, studies suggest this maybe an aspartyl protease [30] or AEP [16, 31]. In B cells and DCs, the protease responsible for the later stages of Ii proteolysis steps is Cat S. By contrast, in cortical thymic epithelial cells Cat L plays this role in terminal Ii processing. In macrophages, however, both Cat S and Cat L as well as another cysteine protease- Cat F may be involved [32, 33]. As Ii processing progresses in the acidic late endosome-lysosomal organelles or MHC class II containing compartments (MIICs), CLIP, a terminal Ii peptide, is removed from the class II ligand binding groove by DM and replaced by processed antigenic peptides.

Ii assists with the folding, assembly, transport, and peptide occupancy of class II molecules. Without Ii, class II molecules may bind misfolded proteins or other chaperones and remain tethered in the ER. Indeed, for some class II alleles the absence of Ii hinders their folding and exiting from the ER. The absence of Ii could also alter the terminal glycosylation of class II in the Golgi, or result in mis-sorting of class II molecules and pre-mature degradation [34]. Some MHC class II molecules form high molecular mass aggregating in the absence of Ii [35]. Studies suggest that B cell development is negatively affected in Ii deficient mice. There are more mechanisms responsible for this besides the impaired Ag presentation [35-38]. Notably, CD4 T cell development and selection are defective in Ii knockout mice. Defects in T cell selection are likely due to the absence of Ii in thymic epithelial cells [39, 40]. This results in altered display of peptides by these class II molecules which escape the ER for surface presentation.

Ii also serves to chaperone DM to endosomal compartments where DM regulates peptide binding to class II molecules. DM functions to remove the Ii fragment CLIP from class II. DM also serves as a peptide editor. Thus, DM modifies the peptide repertoire for class II by increasing the formation and stability of high affinity class II-ligand complexes [35, 41]. Ii also protects DM from proteolysis and may help coordinate the coupling of DM

with class II by targeting DM to endosomal compartments such as MIIC [35, 42, 43]. By chaperoning both class II and DM, Ii plays an essential role in class II mediated Ag presentation.

1.3.4 Host protein synthesis and MHC class II pathway

It is well established that host protein synthesis is important for the function of the class II pathway for Ag presentation. Native protein Ag processing and presentation by class II molecules in B cells is disrupted by the protein synthesis inhibitor-cycloheximide (CHX) and a protein export inhibitor-brefeldin A (BFA) [44]. BFA may perturb intracellular Ag processing and/or peptide-MHC class II complex formation [45]. CHX was postulated to disrupt class II mediated-Ag presentation by reducing the pool of functional Ii in B cells [22]. Most of cell surface class II molecules are synthesized *de novo*, while a small number of class II may come from the recycled pool which transfers between the cell surface and the endosomal pathway [46]. CHX and BFA only inhibit production of newly synthesized but not the recycled class II molecules. Thus, the sensitivity of Ag presentation to CHX or BFA is dependent on the ratio of these two parts of class II molecules. Emetine, an irreversible inhibitor of protein synthesis, reduces intracellular class II protein levels but not cell surface class II [47]. This result may be consistent with a loss of Ii which stabilizes intracellular class II. Together, these studies suggest a step or steps in the class II pathway are sensitive to changes in host protein synthesis. Results from the current study support a loss of class II function when protein synthesis is disrupted and Ii levels decrease.

2. Pathogenesis of VV

The natural pathogenesis of smallpox is revealed by two associated incidences of viremia in infected individuals. The primary viremia occurs after variola virus moves from local respiratory lymphatics to spleen, liver and bone marrow. Following virus multiplication in these reticuloendothelial organs, secondary viremia is seen as virus traffics to distant sites like the skin [48]. By contrast, vaccinia inoculation in skin results only in local

lesion without systemic illness in immunocompetent individuals. Yet in immunocompromised patients, severe dissemination of VV occurs after vaccination. To mimic the physiological and pathological conditions induced after VV infection of humans, several murine models of VV infection utilizing intranasal (i.n.), intraperitoneal (i.p.), intracranial (i.c.), and intradermal (i.d.) virus delivery routes have been developed and applied [49]. While the i.n. model and the i.d. model are more physiologically relevant to natural infection and inoculation respectively, the i.p. model mimics the systemic viremia observed with the virus, and the i.c. model mirrors post-infection neurovirulence in some humans. Studies with these models provides evidence that viral evasion of both innate and adaptive immunity is important in VV pathogenesis.

2.1 Innate immune evasion properties of VV

Complications associated with VV inoculation such as: progressive vaccinia in patients with defects in cellular immunity; eczema vaccinatum in atopic patients; generalized vaccinia; postvaccination encephalomyelitis; and carditis, as well as research directed towards attenuating VV, have led to the discovery of multiple viral proteins which disrupt host immune responses [1, 48, 50]. Innate immunity is typically the first barrier to infectious agents and also an important target for VV. VV evades the host's innate immune system by several mechanisms. Specifically, VV produces factors which: 1) directly bind complement, cytokines or chemokines; 2) serve as decoy receptors for these innate pathways; 3) disrupt IFN (α, β, and γ) gene expression at the transcriptional and translational levels; 4) block infected cells from undergoing apoptosis; 5) perturb TLRs and their signaling pathways; and 6) promote the synthesis of steroids to suppress inflammation. Viral gene products linked to disrupting innate immunity and consequently, influencing adaptive immunity are listed in Table 1.

Table 1 Established mechanisms of VV evasion of innate immunity

VV protein	Mechanism of action	Reference
VV complement control protein	Binds and inactivates C4B and C3B	[51]
B8	Acts as a soluble receptor to bind and antagonize IFN-γ	[52]
B18	Acts as a soluble receptor to bind and antagonize IFN-α/β	[53]
B15	Acts as a soluble receptor to bind and antagonize IL-1β	[54]
C12	Acts as a soluble receptor to bind and antagonize IL-18	[55]
A53	Acts as a soluble receptor to bind and antagonize TNF	[56]
35Kd protein	Binds and antagonizes CC chemokines	[57]
A39	Acts as a soluble receptor to bind and antagonize semaphorin	[58]
E3	Acts as a ds-RNA binding protein to prevent the activation of dsRNA-dependent protein kinase (PKR) and 2'-5' oligoadenylate synthetase (OAS); inhibits ds-RNA induced apoptosis	[59-61]
K3	Acts as a pseudo-substrate of PKR; prevents the activation of eIF-2a by PKR	[62]
B13 or CrmA/Spi-2 protein	Inhibits the IL-1β-converting enzyme; blocks caspase 8 activity by closing the permeability transition pore of mitochondria	[63, 64]
F1	Inhibits apoptosis by stabilizing mitochondrial membranes and by binding and inactivating a proapoptotic molecule - Bak	[65, 66]
A46	Disrupts IL-1 induced nuclear factor (NF) κB activation; mimics the toll-interleukin 1 receptor (TIR); inhibits myeloid differentiation factor 88 (MyD88), TIR domain–containing adaptor inducing IFN-β (TRIF) and related adaptors to block mitogen-activated protein (MAP) kinases and NF κB as well as interferon regulatory factor 3 (IRF3) signaling	[67, 68]
A52	Inhibits IL-1 and TLR4 mediated NF-κB activation; serves as a dominant-negative version of MyD88 on IL-1, TLR4, and IL-18 signaling; inhibits TLR3 induced NF-κB activation by binding with interleukin 1 receptor–associated kinase 2 (IRAK2) and tumor necrosis factor receptor–associated factor 6 (TRAF6); activates TLR-induced p38 and c-Jun amino N-terminal kinase (JNK) to induce IL-10 production	[67, 69, 70]
N1	Inactivates NF-κB by inhibiting TIRs and TNF receptors, adapters such as TRAF, I-kappa B kinase-α, and I-kappa B kinase -β signaling; disrupts IRF3 signaling.	[71]
K1	Inhibits IκBα degradation to prevent NF-κB activation	[72]
A44	Induces steroid production to suppress inflammation	[73]

2.2 Viral Ag presentation by class I and class II molecules

VV vaccination induces both cellular and humoral immunity. Studies have shown that virus specific CD4 and CD8 T cells as well as Abs are detected in humans several months to years after immunization [74-77]. After vaccination, virus specific memory T and B cells persist in healthy individuals providing protection from variola infection for 50-70 years. The detection of both CD8 and CD4 T cells in humans and animals after exposure

to VV, suggest VV Ags are processed and presented by both MHC class I and class II molecules for T cell recognition.

VV enters host cells by at least two routes including direct fusion at the plasma membrane and by delivery into the acidic endosomal pathway with subsequent membrane fusion to the cytoplasm [78, 79]. Delivery of VV into the cytoplasm results in virus uncoating and the synthesis of new viral proteins in the cytoplasm. Studies suggest early viral Ags are processed likely by infected cells, yielding peptides which bind MHC class I molecules (Fig. 2A). These viral peptide-class I complexes transit to the cell surface for detection by CD8 T cells [80-82]. By contrast, studies to define viral peptides recognized by CD4 T cells in the context of class II molecules, suggest these peptides are derived from late viral structural Ags. This would be consistent with virus entry into cells by endocytosis or minimally, the delivery of late viral membrane proteins into cells via the endosomal network. Ags delivered into cells by endocytosis are denatured and proteolytically processed by endosomal and lysosomal proteases. The resulting antigenic peptides can bind class II molecules which reside and transit through endosomes to the cell surface (Fig. 2B). Thus, CD4 T cells may recognize peptides from VV late Ags bound to class II molecules after viral infection of professional APCs. Or alternatively, late viral Ags released during the lysis of infected cells may be endocytosed by APCs and processed for class II presentation to T cells. CD4 T cells are essential for the development of anti - viral CD8 T cells as well as the induction of virus - specific Ab. The observations of the long lasting VV specific CD4 T cells and Ab in vaccinees, as well as enhanced lethality of VV in mice with decreased CD4 or MHC class II expression highlights the importance of MHC class II presentation in VV-induced immune responses [75, 83]. Thus, studies to understand VV Ag presentation are important and may lead to new approaches to enhance immunity using VV-based vaccines.

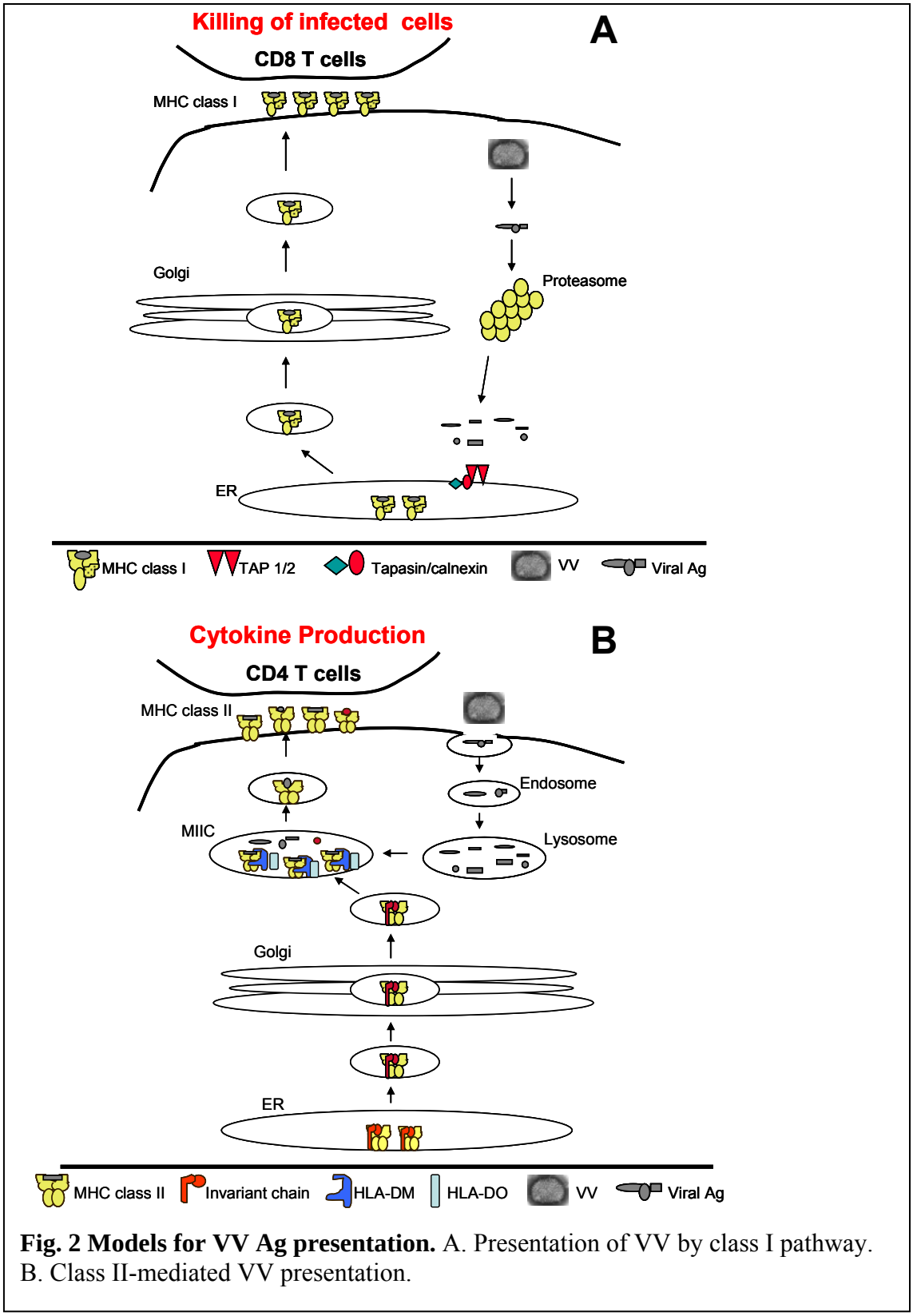


Fig. 2 Models for VV Ag presentation. A. Presentation of VV by class I pathway. B. Class II-mediated VV presentation.

2.3 Disruption of Ag presentation by VV

While VV infection and vaccination with live virus can elicit the activation of virus specific T cells, studies also suggest the virus has evolved mechanisms to thwart or dampen Ag presentation and T cell activation. Here, studies demonstrating VV-related defects in MHC class I presentation to CD8 T cells as well as alterations on CD1d and MHC class II presentation are discussed.

Selective inhibition of MHC class I presentation has been observed upon VV infection of fibroblasts. Using VV as a vector to express different cDNAs for different influenza Ags, it was observed that VV perturbed the presentation of some of influenza viral peptides by class I molecules. During late stage of VV infection, influenza haemagglutinin (HA) presentation to CTL was inhibited [84]. VV disruption of class I mediated-presentation of two influenza nuclear protein (NP) epitopes further supported selective disruption of the class I pathway. Here disruption occurred at both early and late stages of VV infection, although the severity of the inhibition was greater as viral infection progressed. VV induced class I dysfunction could be rescued by rapidly promoting the degradation of Ag by either Ag ubiquitination or deletion of NH_2-terminal signal sequence of the Ag, thus suggesting VV may disturb Ag processing [85]. VV infection also disturbed class I presentation of other influenza Ags including non-structural 1 (NS1) Ag [86]. Reduced surface class I expression has been observed during *in vivo* VV infection, perhaps partially explaining virus induced class I dysfunction [87].

CD1d molecules present lipid Ag to NKT cells. Studies by Brutkiewicz and colleagues showed that CD1d mediated-lipid Ag presentation is also perturbed by VV. The perturbation is mediated through the p38 pathway and is negatively regulated by ERK1/2. Intracellular CD1d trafficking appears to be altered by *in vitro* VV infection of fibroblasts. Murine splenic DC and macrophage expression of surface CD1 is reduced several days after *in vivo* VV infection [88, 89]. Two viral genes B1R (encoding a kinase) and H5R (encoding one substrate of the B1R gene product) appear to play important roles in

mediating VV induced CD1 dysfunction [90]. Yet, the precise mechanism(s) by which viral gene products alter CD1d function and Ag presentation remains unknown.

CD4 T cells play a critical role in the clearance of infectious VV [91]. The majority of CD4 T cells in humans and rodents, recognize peptides complexed to MHC class II molecules displayed on APCs. Thus, VV may target the class II pathway for Ag presentation as a means of immune evasion or modulation. A very early and transient reduction in CD4 T cell responses to several Ags after VV vaccination of humans, led to the suggestion of diminished APC function *in vivo* following exposure to VV [92]. Resting murine and human T cells are not readily infected by VV, although low levels of infection are observed with activated human T cells exposed to virus *in vitro* [93, 94]. By contrast, APCs are highly susceptible to VV infection *in vitro* and *in vivo* as shown by others and in this thesis [94-96]. *In vivo* and *in vitro* studies have shown that VV disrupts MHC class II mediated Ag presentation by professional (macrophages, DCs and B cells) and non-professional APCs (fibroblasts) [96, 97]. Thus, MHC class II molecules represent a likely target for viral evasion of host immunity, yet specific mechanism(s) by which VV disrupts APC function and the class II pathway remains to be elucidated.

Disruption of class II mediated Ag presentation by VV is a relatively general phenomenon. As shown in our previous study [97], VV perturbed the exogenous Ag (human serum albumin (HSA)), endogenous Ag (glutamate decarboxylase (GAD)) and peptide ($GAD_{273–285}$, hen egg lysozyme $(HEL)_{74–88}$, $HSA_{64–76}$, and IgG $I_{188–203}$) presentation by both newly synthesized and recycled class II molecules. VV inhibited class II function in both professional and non-professional APCs without changing total or surface class II expression levels up to 24 h of *in vitro* infection. This inhibition affected both cell lines (such as B cell and macrophage cell lines) and primary cells (such as DCs). Class II function in both human and murine cells could be disrupted. The inhibition was dependent on the duration and dose of VV while independent of virus replication level. Over-expression of endogenous Ag-GAD or provision of external co-stimulation could not rescue the class II function. However, VV infection disturbed the binding of peptide to class II molecules. In a latter study, we showed that *in vivo* VV

infection decreased class II function and expression in DCs. The mRNA of class II dropped much earlier than the protein did [96]. We saw the reduced class II protein expression level in the latter study instead of the earlier one may be due to the fact that we extended the observation time from 24 h (in the earlier paper) to 48 h (in the latter paper) post VV infection. The long half-life of class II protein, i.e. 36 h, may cause this difference.

2.4 VV modulation APC function

Whether VV disruption of MHC class I, class II and CD1d presentation is linked or via viral inactivation of common pathways in APCs remains unclear. In addition to alteration in Ag presentation, VV infection can perturb host protein synthesis, cell maturation or differentiation as well as secretion of soluble messengers such as cytokines. Here, a brief description of VV infection on each of these pathways is offered.

2.4.1 Inhibition of host protein synthesis and APC function

In mouse L cell fibroblasts, VV infection dramatically decreased host protein synthesis, presumably by hijacking cellular resources to assemble infectious virions [98]. Multiple viral components may regulate host protein expression including proteins present in infectious virions [99-101] as well as viral gene products transcribed after infection [102]. Studies with epithelial and fibroblast cells suggest VV may use multiple methods to block host DNA, RNA and protein synthesis. These include disruption of host nuclear DNA replication by viral encoded deoxyribonucleases [103]; interference with cellular RNA synthesis and processing by viral reduction of RNA polymerase II activity [104]; and enhancement of cellular mRNA degradation [105]. Despite loss of these key cellular functions, viral gene transcription remains active during the early and late stages of infection. Notably, not all host gene expression is blocked with VV infection, as the virus exploits host proteins to survive and replicate.

Few studies have examined the effects of VV disruption of host protein synthesis on APC function. Disruption of class II presentation was observed 1-2 h after VV infection [97]. In epithelia and some tumor cells, host protein synthesis was inhibited by VV within 1-2 h of infection [106]. Prior studies established that inhibition of host protein synthesis can diminish class II presentation using the well characterized protein synthesis inhibitor, cycloheximide (CHX) [22, 44]. CHX interrupts both the initiation and elongation of de novo protein synthesis by blocking: 1) binding of deacylated tRNA to reticulocyte ribosomes; 2) activation of transferase II; 3) release of tRNA from the donor site of ribosomes; and 4) aggregation of polysomes [107, 108]. However, few studies have specifically investigated whether VV perturbs the expression of host proteins critical for immune recognition. Yet, a recent analysis of changes in dendritic cell (DC) gene expression during attenuated VV infection did suggest alterations in the levels of some components of antigen presentation pathways [109]. While inhibition of host protein synthesis was postulated as one potential explanation for virus inhibition of class II presentation [110], we demonstrated sustained surface expression of class II proteins in APCs up to 24 h after *in vitro* VV exposure [97]. However, surface class II expression on DCs is reduced 48 h after *in vivo* infection [96]. Investigating how VV infection influences the expression of class II molecules and other factors required for APC function, is one of the important goals of this thesis.

2.4.2 Changes in APC maturation with virus infection

DCs serve as potent APCs bridging the innate and adaptive immune response. After capturing Ags, immature DCs migrate from peripheral tissues to secondary lymphoid organs and undergo a programmed maturation. These matured DCs express high levels of surface MHC class I, class II, and co-stimulatory molecules and secrete cytokines such as type I interferons, interleukins, and TNF-α which modulate host immune responses. VV infection can subvert DC function in part by blocking the function of some of these cytokines as summarized in Table 1. *In vitro* VV infection of DCs decreased the maturation of these cells in response to monocyte-conditioned medium, LPS or PGE_2/TNF-α [111]. By contrast, surface MHC class I, class II, CD40 and co-stimulatory

molecules such as CD86 expression on DCs is initially up-regulated upon *in vivo* VV infection [96]. One study has suggested *in vivo* VV acts to stimulate the maturation of DCs by triggering TLR2 activation [112]. Yet as shown by another study, while VV infection *in vivo* can promote increased surface marker expression consistent with DC maturation, these APCs display defects in class II Ag presentation [96]. Perturbation of normal DC maturation may contribute to VV disruption of Ag presentation pathways.

2.4.3 Alteration in APC cytokine production by VV

Using a murine epidermis-derived dendritic cell line, it was demonstrated that upon *in vitro* VV infection, normal production of various cytokines (TNF-α, IL-1β, IL-6, IL-10, IL-12, IFN-α and IFN-γ) was disrupted. Even after pre-activation with LPS or poly(I:C), TNF- α and IL-6 production is inhibited by VV *in vitro*. This inhibition is mediated by viral protein E3 and K1 through blocking the NF-κB pathway [113]. *In vivo* VV infection, on the other hand, increases primary splenic DC secretion of IL-10, IL-12, IL-6, IL-1β and TNF-α [96]. Comparison of these studies is complicated as DCs from different tissues as well as *in vitro vs. in vivo* infection were employed. Yet, changes in APC cytokine production with VV infection very likely influence host immunity and possibly APC function.

3. Development of VV-based vaccines

Cowpox was the 1st vaccine used to prevent smallpox transmission. VV, is a less virulent but closely related virus, which has been used to successfully eradicate smallpox worldwide. Currently, due to concerns of virus weaponization and cross-species infection by other more virulent poxviruses such as monkeypox virus, the effectiveness and safety of vaccinia virus have been re-evaluated. VV is a relatively safe vaccine with overall complication rate at 4 per 100,000 vaccinees [114]. It can protect vaccinees from variola infection for 50-70 years. However, the severity of complications lead to the generation of an attenuated strain-Modified Vaccinia Ankara (MVA) which has defects in

replication and virulence [115]. Both VV and MVA are effective in inducing immunity against a few poxviruses including monkeypox [116].

Besides being a poxvirus vaccine, VV continues to be proposed as a vector to selectively express Ags from infectious agents or tumors. By replacing VV nonessential genes such as thymidine kinase (TK) via homologous DNA recombination, foreign genes derived from infectious pathogens or tumors could be expressed in host cells by VV. VV displays many properties which make it desirable as a vaccine vector. These properties include: the easy generation and purification of recombinant VV; the virus's large insertion capacity; the virus's wide host range; high levels and precision-controlled expression driven by the virus; and the virus's lack of host genomic integration [1, 117]. However, the serious complications (heart, brain, and skin inflammation) after VV inoculation in immune-deficient patients and healthy individuals can not be ignored. Contraindications of VV vaccination include eczema or atopic dermatitis, human immunodeficiency virus (HIV) positive patients, and transplant or chemotherapy receiving patients. In other conditions such as asthma, the effects of VV vaccination are unknown. These complications have led to the design of attenuated VV vaccines for use in humans. Here, the development of VV and attenuated VV derived vaccines is reviewed.

3.1 Usage of VV as a vector to express genes derived from pathogens

VV has been widely used as a vector to express pathogen-derived genes for study and to induce host immunity against various infections. Recombinant VV expressing heterologous viral genes such as human immunodeficiency virus (HIV) gp160 induces both humoral and cellular immunity against HIV [118]. VV expressing hepatitis B virus surface Ag (HBVsAg) [119]; rabies glycoprotein [120]; influenza virus hemagglutinin [121]; respiratory syncytial virus (RSV) G or F glycoprotein [122, 123]; and RSV nucleocapsid (N) protein [124] provoke neutralizing primary and memory Abs against these corresponding viruses. VV driven expression of nonstructural immediate-early protein pp89 of murine cytomegalovirus (MCMV) [125]; lymphocytic choriomeningitis virus (LCMV) nucleoprotein or glycoprotein [126]; friend murine leukemia helper virus

(F-MuLV) envelope protein [127]; and RSV M2 protein [128] stimulate CTL responses in mice. F-MuLV gag protein encoded by VV, on the other hand, induces CD4 T cell expansion *in vivo* [129]. Mucosal immunity or IgA secretion which is important for fighting respiratory tract infection could be achieved by either i.n. delivery of a recombinant VV containing the H1 gene of influenza [130] or enteric inoculation of recombinant VV expressing RSV F glycoprotein or the influenza virus hemagglutinin [131, 132]. A VV recombinant vaccine can also exert antiviral function by encoding pro-inflammatory cytokine such as IL-5 and IL-6, which helped to increase IgA production [133, 134].

VV-based vaccines for infection are not limited to viral pathogens, as recombinant VV encoding bacterial and parasitic vaccines have also been developed. For example a nonamer CTL epitope from the listeriolysin O protein expressed in a VV vector stimulates the expansion of protective CTL responses against bacterium *Listeria monocytogenes* [135]. A recombinant VV encoding the surface protein of *Neospora caninum tachyzoite*, NcSAG1 or NcSRS2, induces both humoral and cellular immunity against this parasite [136].

3.2 Tumor Immunization using VV vectors

A cause of cancer malignancies is that the host is tolerant of the tumor and host immunity is not activated. Thus, two immunological tools being tested against cancer are: 1) enhancement of tumor immunogenicity; and 2) disruption of inhibitory factors that lead to immune tolerance. High expression of tumor-associated Ags (TAA) is a major method being used to increase immunogenicity. Recombinant TAAs such as rat neu oncogene-encoded transmembrane glycoprotein p185 [137], carcinoembryonic antigen (CEA) [138] and human melanoma-associated glycoprotein p97 [139] induce humoral and/or cellular immunity against tumors. Expression of genes from tumorigenic viruses such as polyoma virus [140] or F-MuLV [127] using VV based vectors, can help promote host immune responses to lyse these tumors. Encoding other adjuvant pro-immunity molecules within the VV genome also helps increase anti-tumor immune response upon

in vivo infection. Co-expression of TAA together with co-stimulatory molecules (B7-1 and B7-2) using VV vectors was found to enhance tumor-fighting effects [141]. Insertion of pro-inflammatory cytokines such as IL-2 and IL-12 into VV vectors further strengthens the anti-tumor effects of these cytokines [142]. VV encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) also effectively increases immunogenicity of an originally poorly immunogenic murine colon adenocarcinoma cell line, MC-38 [143]. As a pathogen itself, VV infection alone stimulates oncolysis of melanoma and metastatic breast, kidney, and colon carcinoma [144]. Besides TAA and immuno-modulators, VV can be used to express tumor suppressing genes to enhance tumor deterioration. For example, VV expression of wild-type p53 inhibits human (U-373MG, U-87MG, LN-Z308) and rat glioma cell (9L, C6) growth and mediates tumor apoptosis [145].

3.3 Development of attenuated VV-based vaccines for poxviruses, other infectious agents and cancer

Various strains of VV including Dryvax and Lister were used as vaccines to annihilate smallpox transmission. However, severe complications after VV vaccination in some individuals led to the design of less virulent VV-based vaccines. Those attenuated vaccine strains have lost virulent genes following multiple passages in cell culture, or via selective deletions of virulence genes, and/or insertions of pro-inflammatory cytokines by genetic engineering. Examples of live attenuated strains include LC16m8 with a B5R mutation which was derived from Lister [146], CVI-78 or the New York City Board of Health (NYCBOH) strain which has proven effective in eczema patients [147], and replication defective Modified Vaccinia Ankara (MVA) which lacks many immunoregulatory genes existing in the parent Ankara strain and does not replicate in mammalian cells [148]. NYVAC is derived from the Copenhagen strain by deleting 18 open reading frames (ORFs) of virulent genes. These include many immune evasive genes such as B13R, K1L and N1L [149]. Encoding pro-inflammatory cytokines in VV can in some cases help to decrease VV virulence. For instance, both IL-2 and IL-15 expressing recombinant VV reduce viral evasion of host immunity [150, 151]. These

attenuated vaccines are effective and safe to be administrated in immune-deficient subjects [149-153]. Yet VV encoding IL-4 or IL-17 have proven to have deleterious effects as vaccines [154, 155]. Thus engineering modified VV-based vaccines is an on-going effort.

Besides being used as vaccines for poxviruses, modified VV strains may also be used as vectors to express foreign genes from other infectious agents or cancer. Virus replication in MVA is blocked at a late stage of morphogenesis in mammalian cells, yet viral protein synthesis remains intact. Thus, MVA may potentially be used as an expression vector with a higher degree of safety. Indeed, recombinant MVA (rMVA) is highly immunogenic and effective in protecting animals from influenza, parainfluenza, measles virus, flaviviruses, CMV, severe acute respiratory syndrome (SARS) coronavirus, simian immunodeficiency virus (SIV), and HIV infections [117, 156-159]. Vaccines based on rMVA have been tested for bacteria such as *Mycobacterium tuberculosis* and for parasites such as plasmodium [160-162].

rMVA vectors encoding cancer related viral Ags such as Epstein-Barr virus-associated nasopharyngeal carcinoma Ag and human papillomavirus (HPV) E2 Ag have been tested as vaccines [163-165]. Recombinant MVA encoding TAAs- CEA; oncofetal antigen 5T4; and mucin (MUC)-1 together with host immunity enhancement treatments appear effective in treating various cancers [166-168]. To improve vaccine immunogenicity, both the enhancement of immune-stimulatory factors and the reduction of immune inhibitory factors have been tested. For example, co-expression of multiple co-stimulatory molecules and/or pro-inflammatory cytokines such as IL-2 has been employed to boost vaccine immune stimulation, or alternatively blockade of cytotoxic T-lymphocyte antigen 4 (CTLA-4) [169]. Activation of APCs is another way to break tumor tolerance. Increased anti-melanoma responses by transfer autologous $CD34^+$ cell-derived DCs transduced *ex vivo* with a rMVA encoding the human tyrosinase gene has been studied [170]. Notably, intralesional infection with MVA alone induces anti-melanoma immunity [171].

Besides being used individually, MVA could also be used together with a DNA vaccine to increase antigenicity. Primary immunization with the DNA plasmid followed with a booster immunization using recombinant MVA (rMVA) encoding the same or a related pathogenic proteins induces immune responses against several infections. For instance, studies have tested a DNA vaccine expressing SIV Gag, Pol, Vif, Vpx, Vpr, and HIV-1 Env, Tat, and Rev genes followed with the rMVA booster encoding SIV Gag, Pol, and HIV-1 Env genes [172]; combinations of DNA and rMVA both encoding *plasmodium falciparum* multiple epitope (ME) - thrombospondin-related adhesion protein (TRAP) [173], and combination of the classical bacille *Calmette-Guérin* DNA vaccine with rMVA expressing *Mycobacterium tuberculosis* antigen 85A [174] greatly enhances host immunity against SHIV (an SIV and HIV chimera), malaria and tuberculosis respectively.

Other attenuated VV strains are also useful as vectors to express foreign genes. For example, NYVAC engineered to express the Japanese encephalitis virus (JEV) prM, E, and NS1 genes, protects pigs from JEV viremia [175].

4. Summary and Objectives

APCs appear to be highly susceptible to VV infection *in vitro* and potentially *in vivo* leading to alterations in the function of these cells. In this thesis, the efficiency of VV infection *in vitro* and *in vivo* was quantified using a recombinant virus encoding EGFP. To extend our previous observation that VV perturbs MHC class II mediated Ag presentation, the effect of this virus on class II-restricted Ag presentation was tested using immortalized and primary human or murine APCs. The ability of VV to destabilize class II ligand binding was investigated along with the role of cellular transformation on viral disruption of the class II pathway. The effects of heat inactivation and genetic manipulation of VV on Ag presentation were also studied to elucidate VV-derived factors that could affect class II function.

Previous studies had suggested that in some cases, disruption of class II protein function was linked to alterations in host protein synthesis. Because VV infection can perturb the

expression of some host proteins, studies were carried out to test for change in the expression of class II molecules and their cofactors during VV infection of APCs. While no change in class II protein levels were detected with virus infection at 24 h, a marked reduction in Ii cellular abundance was noted. Studies were carried out to determine mechanistically whether VV infection of APCs altered Ii synthesis and/or degradation. Parallel studies were carried out using a chemical inhibitor of protein expression.

To establish the relative contribution of Ii loss in class II dysfunction, studies were carried out using cell lines lacking or expressing Ii. Biochemical and functional assays were used to compare the effect of variable Ii expression on VV disruption of the class II presentation.

Ii protein levels dropped only during the late stages of VV infection. Yet, studies suggested an earlier loss of class II function due to VV infection. The competition between VV and other Ags for class II presentation could at early time be responsible for class II disruption. Studies were carried out to look at virus Ag presentation using wild type or modified virus. CD4 T cell responses to virus infected cells were monitored by T cell proliferation assay to detect IL-2 and ELISA to check other cytokines. Recall CD4 and CD8 responses after rechallenge *in vivo* with virus were also studied. To study the effect of VV on *in vivo* Ag presentation, ovalbumin (OVA)-induced asthmatic mice were infected by VV. Histological analysis and cytokine production was assessed. Finally, the ability of an H3 (a viral envelope protein) epitope specific Ab in blocking VV infection and protecting class II function was investigated.

MATERIALS AND METHODS

Viruses

VV, Western Reserve (WR) strain was grown, cultured, gradient purified, and titered as previous described in CV-1 (African green monkey kidney) cells [97]. To culture VV, CV-1 cells were infected with VV at MOI 0.1 for 30 m followed by culturing for additional 2-3 d until all cells were round. To harvest and purify VV, infected CV-1 cells were centrifuged, resuspended in 10 mM Tris HCl (pH 9.0), homogenized on ice, and centrifuged again to remove nuclei. The supernatants were sonicated, underlaid with an equal volume of 36% sucrose in 10 mM Tris HCl (pH 9.0), and centrifuged at 33000 g for 80 m at 4˚C. Pellets resuspended in 1 mM Tris HCl (pH 9.0), were aliquoted, and stored at -80˚C. To titer VV, CV-1 cells were infected with serially diluted VV for 30 m followed by incubation for additional 3 d, then stained with 0.1% crystal violet in 20% ethanol. The numbers of the plaques formed in cells infected with VV at the highest dilution (i.e. the most diluted VV) were averaged to determine the titer (pfu/ml). To heat inactivate VV, undiluted, purified VV was incubated at 60˚C for 1 h to yield HI-VV [176]. To generate replication deficient virus, purified VV was exposed to UV light (254 nm) for 20 m to generate UV-VV [97]. The B1R and H5R temperature-sensitive viruses, were obtained originally from Drs. R. Condit (University of Florida, Gainesville, FL) and P. Traktman (Medical College of Wisconsin, Milwaukee, WI) respectively [90]. BSSH (VV expressing soluble truncated D8L gene product) was obtained from Dr. E. Niles (State University of New York, NY) [177]. CD4-VV (recombinant VV (rVV) encoding the human CD4 molecule); mIi-VV, rVV encoding murine Ii; EGFP-VV, rVV encoding Enhanced Green Fluorescent Proteins (EGFP) [178] were obtained from Dr. R. Brutkiewicz (Indiana University School of Medicine (IUSM), Indianapolis, IN) or Dr. J. Yewdell (National Institutes of Health, Bethesda, MD). VSC, rVV encoding *E. coli* β-galactosidase (β-Gal) behind an engineered high expression early late promoter [179], was obtained from Dr. G. Alkhatib (IUSM, Indianapolis, IN). All viruses were propagated and purified by the Poxvirus Core Facility at IUSM.

Cell lines

CV-1 (African green monkey kidney cells); TK$^-$143B (human osteosarcoma cells); M1DR4, a human fibroblast cell line transformed with SV-40 [180, 181] and retrovirally transduced with human MHC class II molecules HLA-DR4; M1DR4Ii, M1DR4 cells co-transfected with a plasmid encoding human Ii; L1DR4, a murine fibroblast cell line [182] retrovirally transduced with DR4; L1DR4Ii, L1DR4 cells co-transfected with a plamid encoding human Ii were cultured in DMEM (Invitrogen Life Technologies) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Priess, a human B lymphoblastoid cell line (B-LCL) expressing DR4; PriessGAD, Priess cells retrovirally transduced for constitutive expression of the 65-kD form of human glutamic acid decarboxylase (GAD); Frev, another B-LCL; Frev/SMA, Frev cells transfected with a mutated form of the human immunoglobulin (Ig) κ chain or SMA [183]; Raji, a B-LCL; 7C3DR4, the human B-LCL Swei transfected with murine class II molecules I-A^b [184] and retrovirally transduced for constitutive expression of DR4; T2DR4DM, a mutant human B-LCL retrovirally transduced with DR4 and transfected with plasmids encoding DM α and β [185-187]; and the murine B cell tumor TA3 (expressing two alleles of murine class II molecules or I-A $^{k\times d}$) [188] were cultured in IMDM (Invitrogen Life Technologies) with 10% heat-inactivated calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Murine T cell hybridomas recognizing DR4 including: 33.1 specific for $GAD_{273–285}$, 17.9 specific for human serum albumin $(HSA)_{64–72}$ and 1.21 specific for Ig $\kappa_{145\text{-}159}$; actin 15.10 specific for actin and I-A^b [189]; B04 specific for $HEL_{74–88}$ and I-A^b; TS2.1 recognizing influenza hemagglutinin $(HA)_{126\text{-}138}$ and I-A^d [190]; as well as the mouse B cell tumor line 1153 (expressing I-A^b) were cultured in RPMI 1640 (Invitrogen Life Technologies) with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 50 μM beta-mercaptoethanol (2-ME). HT-2, an IL-2 dependent T cell line was grown in RPMI 1640 media with 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-ME and 20% T stim (BD Biosciences). For quantification of IL-2 using HT-2 cells, T stim was omitted from the culture media.

Isolation of primary APCs from human peripheral blood mononuclear cells (PBMCs) and murine primary splenocytes

Human PBMCs were isolated from buffy coats (Indiana Blood Center) using Ficoll-Paque PLUS (GE Healthcare) and centrifugation. T cells were depleted from these PBMCs with anti-CD3 Ab coated microbeads (Miltenyi Biotec). To isolate murine APCs, spleens of C57BL/6 mice were harvested, homogenized and red blood cells (RBC) were lysed with RBC lysis buffer (0.15M NH_4CL, 10mM $KHCO_3$, 0.1mM Na_2EDTA in H_2O, pH 7.4). T cells were removed with anti-CD4 and anti-CD8 Ab coated microbeads (Miltenyi Biotec) to obtain monocytes, B cells, DCs along with lesser numbers of granulocytes. When required, splenic cells were fractionated using HISTOPAQUE-1083 (Sigma) to remove dead cells. In some studies APCs were pre-activated with 1μg/ml LPS (Sigma) for 24 h. To separate individual APC populations, microbeads coated with Abs to CD11c, CD11b or B220 (Miltenyi Biotec) were used sequentially for positive selection of murine DCs, macrophages or B cells. All studies using mice were reviewed and approved by the Laboratory Animal Resource Center (LARC) at IUSM prior to initiation.

Peptides and Ags

$GAD_{273–285}$ (IAFTSEHSHFSLK), $HEL_{74–88}$ (NLCNIPCSALLSSDI), $HSA_{64–76}$ (VKLVNEVTEFAKTK), $HA_{126-138}$ wild type (HNTNGVTAACSHE), $HA_{126-138}$ mutation T128V (HNVNGVTAASSHE) [190] and AENK (an inhibitor for AEP) [16] were produced using F-moc technology and HPLC to a purity of >90%. VV viral peptides which bind class II molecules were provided by Dr. Alessandro Sette (La Jolla Institute for Allergy and Immunology, San Diego, CA). HSA protein (protease free preparation) was obtained from Sigma.

Antigen presentation or T cell assay

APCs were cultured in the absence or presence of VV for 2-14 h to permit virus infection. APCs were then washed, and fixed with 0.5% paraformaldehyde for 10 m at

4˚C, followed by co-culture with T cell hybridomas for 24 h. In some assays, Ag or peptide was added to cells during virus infection, prior to washing, fixation and co-culture to detect T cell activation as described below. VV infection was observed in >90% of the treated B cell as detected by viral Ag expression [97]. To monitor the effect of virus replication on GAD presentation, PriessGAD cells were treated with VV +/- the viral DNA polymerase inhibitor – arabinosylcytosine (AraC, 10 μM, from Sigma) for 14 h before GAD specific T cell addition. To investigate the effect of protein synthesis inhibition on endogenous Ag presentation, PriessGAD or FrevSMA cells were treated with 10 μg/ml CHX (Sigma) for 0-6 h. To study the effect of CHX on exogenous Ag or peptide presentation, Priess cells were incubated with 0-10 μM HSA protein; HSA peptide; Ig κ II peptide; or GAD peptide +/- CHX (10 μg/ml) for 6 h. After CHX treatment, APCs were washed, fixed and co-cultured with Ag-specific T cells. To compare the effects of CHX and VV on exogenous Ag presentation, APCs were treated with 1-10 μM HSA protein with or without (+/-) 10 μg/ml CHX for 6 h or +/- VV (MOI=10) for 14 h, paraformaldehyde fixed and co-cultured with HSA-specific T cells. To determine the effects of CHX or VV on peptide presentation, Priess cells were co-incubated with 1-10 μM $HSA_{64–76}$ or $GAD_{273–285}$ peptide +/- CHX or VV, washed, fixed and co-cultured with peptide-specific T cells. APC viability was assessed by trypan blue exclusion during exposure to virus or CHX, and conditions optimized to ensure little to no decrease in cell viability during these treatments.

To test the effect of increased intracellular Ii expression on class II presentation, fibroblast cells lacking Ii (M1DR4 and L1DR4) and M1DR4 and L1DR4 engineered to express Ii (M1DR4Ii and L1DR4Ii) were incubated with GAD peptide (up to 10 μM) +/- VV (MOI up to 10) for 6 h prior to fixation and addition of GAD specific T cells. Similar comparison analysis was carried out with human B cells expressing normal levels of Ii (7C3DR4) and T2DR4DM cells which contain 4 copies of genomic Ii. To investigate the effects of recombinant VV which encode Ii, PriessGAD cells were infected with mIi-VV (rVV encoding murine Ii); CD4-VV (rVV encoding human CD4) or wild type VV at MOI=10 for up to 24 h, then fixed and co-cultured with GAD specific T cells.

T cell activation was quantified by IL-2 production using the IL-2-dependent proliferation of HT-2 cells [191] in hybridoma assays. All assays were performed in triplicate, and the relative mean proliferation and standard deviations expressed as cpm.

Immunoblot or Western Blot analysis

Cells infected with VV (MOI = 10) +/- AraC or treated with CHX (10 μg/ml) for different times, were harvested and lysed in buffer (10mM Trizma Base, 150mM NaCl and 1% Triton-X 100 with 1% protease inhibitors: N^{α}-tosyl-lysine-chloromethylketone and phenylmethylsulphonyl fluoride). Cell lysates were centrifuged to remove nuclei prior to the addition of SDS sample buffer. Samples of 40 μg or 400 μg (the latter for cathepsin analysis only) of total cell proteins were fractionated by SDS-PAGE followed by transfer to nitrocellulose membranes. Membranes were probed for: DRα monomer expression using the monoclonal antibody (mAb) DA6.147; DRαβ dimer using mAb L243; MHC I heavy chain using mAb 3B10.7; GAD using a polyclonal Ab (Sigma-Aldrich); Ii using mAb Pin 1.1; Cat S using Cat S Ab (Biovision); Cat B using Cat B mAb (Calbiochem); Cat D using Cat D mAb (Calbiochem); Cat L using the mAb CPLH 36.1 [192]; DMβ using a rabbit Ab to DMβ peptide (TPLPGSNYSEGWIS); and GILT using a rabbit Ab to GILT. Cellular actin was detected using the mAb-Pan Actin Ab-5 (Lab Vision). VV Ags were detected with a rabbit polyclonal VV specific Ab (Cortex Biochem) to demonstrate cellular VV infection. Viral proteins D8 and H3 were detected with corresponding specific rabbit Abs-C8 (immunogen: soluble D8 protein) and anti-H3 Ab (immunogen: full length H3 protein) respectively [193, 194]. Densitometric analysis of Western blots was carried out with software ImageJ (NIH website) using cellular actin or GAPDH expression as a relative internal standard.

To monitor the effects of VV on Ii maturation and processing, PriessGAD cells were cultured with VV (MOI=10) for 2 h to permit infection, followed by incubation with agents to disrupt Ii transport or proteolysis. Thus, cells were incubated with Brefeldin A (BFA, 50 ng/ml, Calbiochem) to block protein transit from ER to Golgi; Leupeptin (Leu, 500 μM, Sigma) to block lysosomal cysteine proteases; monensin (Mon, 1 μM,

Calbiochem) to block protein exit from the Golgi; chloroquine (Chl, 0.3 mM, Sigma) to block endosome and lysosome acidification; Phenylmethyl-sulphonylfluoride (PMSF, 50 μM, Sigma) to block cellular serine proteases; lactacystin (LAC, 250 nM, Sigma) to block the proteasome; epoxomicin (EPO, 200 nM, Sigma), a proteasome inhibitor; E64 (0.5mM, Sigma), an irreversible cysteine protease inhibitor; and AENK (1 mg/ml) to block AEP for an additional 12 h before cell harvesting and cell protein analysis by Western Blotting. In the 2×Leu group, Leu was added again at 6 h before cell harvesting to ensure protease inhibition during the course of infection. To study the effects of CHX on Ii processing, PriessGAD cells were cultured with proteolysis inhibitors for 1h, then CHX (10 μg/ml) was added to the media for an additional 4 h followed by immunoblotting of cell lysates and protein quantification by densitometry. The working concentrations of drugs used in these studies were derived from literature [195, 196]. The incubation periods used were selected after pilot studies to optimize cell viability, inhibitor action and detection of target proteins in uninfected cells. Cell viability of >80% after each treatment was confirmed by trypan blue staining.

Immunoprecipitation

PriessGAD cells treated +/-VV or CHX were harvested, lysed and pre-cleared by incubation with 50 μl of protein G coupled beads/sample for 30 m. Protein G coupled beads were spun down and the cell supernatant was collected. The anti DRα mAb DA6.147 was added to the pre-cleared cell lysates and incubated at 4˚C overnight. 100 μL of Protein G coupled beads were added to each sample and incubated for an additional 1 h in the cold. DR molecules bound to protein G coupled beads were eluted by addition of reducing SDS buffer and boiling for 5 m. Eluates were resolved on SDS-PAGE and Western analysis was performed using specific Abs (e.g. Pin 1.1 for Ii).

Quantification of protein synthesis

To monitor total new protein synthesis, APCs were culture with l-lysine [4,5-^{3}H] and l-leucine [3,4,5-^{3}H] (ICN Biomedicals Inc) in lysine- and leucine-deficient minimum

essential media (Sigma) with increasing concentrations of CHX (up to 50 μg/ml) for 6 h. Cellular proteins were filter captured to quantify radiolabeled- amino acid incorporation. At the concentration of 10 μg/ml, CHX efficiently decreased cellular protein synthesis (about 85%) with minimal loss of cell viability (>90%). During analysis of Ii processing and expression, PriessGAD cells were incubated with BFA (50 ng/ml) or Leu (500 μM) +/- CHX (10 μg/ml) in the [^{3}H]-amino acid containing media for 6 h before harvesting and quantification of radiolabel incorporation to ensure BFA or Leu treatments did not alter inhibition of host protein synthesis by CHX.

[^{35}-S] methionine incorporation and pulse-chase assay

To assess the effects of VV on host protein synthesis, PriessGAD cells (1×10^{7}/sample) were infected with VV (MOI=10) for 0, 2, 6, and 14 h before addition of 0.5 mCi of [^{35}S]-methionine (specific activity: 1175 Ci/mmol, MP Biomedicals) in 0.5ml cold methionine free RPMI media /sample for 1 h. Cells were harvested, lysed and immunoprecipitated with Pin 1.1 for Ii and DA6.147 for DR. The precipitated samples were resolved on the SDS-PAGE followed by Coomassie Blue gel staining and autoradiography. Coomassie Blue staining was performed to ensure equal sample loading. For pulse-chase assays, PriessGAD cells were starved in methionine free RPMI media for 1 h and labeled with 0.5 mCi of [^{35}S]-methionine (specific activity: 1175 Ci/mmol) for another hour. Samples were chased in media containing excess methionine +/- VV (MOI=10) and/or E64 (500 μM) for 0, 2, 6, and 14 h. Samples were immunoprecipitated, subjected to SDS-PAGE and analyzed by autoradiography of SDS-PAGE.

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative real time PCR

PriessGAD cells were treated +/- VV (MOI = 10) for up to 14 h. Total RNA was extracted from these cells with an RNEasy Mini kit (Qiagen) following the manufacturer's instructions. cDNA was generated from RNA using an Advantage RT for PCR kit from BD Biosciences. Primers for PCR amplification were designed using the

Custom Primers - OligoPerfect™ Designer software (Invitrogen). The primers used for human Ii (CD74) were 5'-GCT GTC GGG AAG ATC AGA AG-3' (sense) and 5'-GCC ATA CTT GGT GGC ATT CT-3' (antisense); for DRα (HLA-DRA) 5'-CAA AGA AGG AGA CGG TCT GG-3' (sense) and 5'-AGC ATC AAA CTC CCA GTG CT-3' (antisense); for human Cat S (CTSS) 5'-GGA TCA CCA CTG GCA TCT CT-3' (sense) and 5'-CCA GCT TTC CTG TTT TCA GC-3' (antisense); for human Cat B (CTSB) 5'-GCT ATC CTG CTG AAG CTT GG-3' (sense) and 5'-CAT TGT CAC CCC AGT CAG TG-3' (antisense); for human Cat D (CTSD) 5'-AGC TGG TGG ACC AGA ACA TC-3' (sense) and 5'-CTC TGG GGA CAG CTT GTA GC-3' (antisense); for human Cat L (CTSL) 5'-TGT GGT TCT TGT TGG GCT TT-3' (sense) and 5'-CAG GCC TCC ATT ATC CTG AA-3' (antisense); for human actin (ACTA1) 5'-AGA AAA TCT GGC ACC ACA CC-3' (sense) and 5'-CCA TCT CTT GCT CGA AGT CC-3' (antisense); for human Early Endosome Antigen1 (EEA1) 5'-GAA GAA AAG GAG CAG CAT GG-3' (sense) and 5'-GGT CCT TAT CGC CAA TTT GA (antisense); for human AEP (LGMN) 5'-GAA ACG CAA AGC CAG TTC TC-3' (sense) and 5'-GCA AGG AGA CGA TCT TAC GC-3' (antisense); and for human HSC70 (HSPA10) 5'- AGC TGT AAG ACG CCT CCG TA -3' (sense) and 5'- GTG ACA TCC AAG AGC AGC AA -3' (antisense); for viral E3L 5'-CGC AGA GAT TGT GTG TGA GG-3' (sense) and 5'-AAC GGT GAC AGG GTT AGC AC-3' (antisense); for viral D8L 5'-CAA ATC GGA CAA CCA TCT CA-3' (sense) and 5'-CCA TTA GAT CCG CCA ATA CG-3' (antisense); for viral H3L 5'-GCG GCC GCC ATG GCG GCG GCG AAA ACT CC-3' (sense) and 5'-GCG GCC GCC TTA GAT AAA TGC GGT AAC G-3' (antisense). GAPDH primers were obtained from the Advantage RT for PCR kit. Amplification reactions were performed using 1.1×ReddyMix™ PCR Master Mix (ABgene) with different cycle times in a MJ Research thermal cycler. The number of amplification cycles for semiquantitative analysis was 28 cycles except for HSC70, Cat S, Cat B and Cat D (32 cycles); and Cat L (40 cycles). The cycling parameters used were: 95˚C, 15 sec; 50˚C, 30 sec; and 68˚C, 1 m. PCR products (10 µl) were electrophoresed on 1% agarose gels, stained with SYBR® safe DNA gel stain (Invitrogen), and detected with UV transillumination using ChemiDoc™ XRS (Bio-Rad). mRNAs for early viral gene product E3L and late gene product D8L were monitored as evidence of VV infection. Host cell HSC70 (HSPA10)

mRNA was used as a control for sample loading. Similar procedures were used to detect relative mRNA abundance after CHX treatment. Ii (CD74) mRNA expression levels in CHX treated samples were evaluated and averaged from three independent experiments with the standard deviation indicated.

To detect human Ii (CD74), DRα (HLA-DRA) and HSC70 (HSPA10) mRNA levels by quantitative real time PCR, RNA isolation and cDNA synthesis were performed as above. TaqMan® Gene Expression Assays with specific primers and probes (Hs00269961_m1 for CD74; Hs00219575_m1 for HLA-DRA and Hs00852842_gH for HSPA10) were incubated with TaqMan® Fast Universal PCR Master Mix and cDNA templates. Samples were amplified 40 cycles using a 7500 Real-Time PCR System (Applied Biosystems) with the following parameters: 95˚C, 15 sec; and 60˚C, 1 m. HSPA10 was used as the endogenous reference while 0 h relative quantification of CD74 or HLA-DRA mRNA was used as the calibrator.

To detect murine mRNAs by semi-quantitative and realtime PCR, the following specific primers were used: for I-A^b (H2-Ab1) were 5'-CTG TCT GGA TGC TTC CTG AGT TT-3' (sense) and 5'- CAG CTA TGT TTT CAG TCC ACC-3' (antisense); for murine Ii (Cd74) 5'- AGA GAG CCA GAA AGG TGC AG -3' (sense) and 5'- ATC TTC CAG TTC ACG CCA TC -3' (antisense); for murine Cat L (Ctsl) 5'- CTG CTT GGG AAC AGC CTT AG -3' (sense) and 5'- TGA GCG TGA GAA CAG TCC AC -3' (antisense); for murine Cat S (Ctss) 5'- CCA TTG GGA TCT CTG GAA GA -3' (sense) and 5'- AGC CCT TCT CTC TCC AGT CC -3' (antisense); and for GAPDH (Gapdh) 5'- CCA GGT TGT CTC CTG CGA CGA CT -3' (sense) and 5'- ATA CCA GGA AAT GAG CTT GAC AAA GT -3' (antisense). Semi-quantitative PCR was performed as described above. For real time PCR, SYBRgreen master mix (Applied Biosystems), primers for specific genes and cDNA templates were mixed before running with 7500 Real-Time PCR System for 40 cycles.

DNA sequencing

To confirm the identity of the PCR product amplified using primers specific for human Cat L (CTSL) , PriessGAD cells were cultured +/- VV (MOI = 10) for 14 h followed by mRNA isolation and cDNA synthesis. After amplification of cellular mRNA using the CTSL primers, the reaction products from each sample were separated on a 1% agarose gel, and the amplified cDNA bands were excised and extracted using a QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. The cDNAs were sequenced by the DNA Sequencing Core Facility at IUSM using a Perkin Elmer / Applied Biosystems 3100 Genetic Analyzer and Big Dye Terminator chemistry v3.1. CTSL cDNA amplified from uninfected PriessGAD cells was sequenced, and this cDNA sequence was compared with the sequence from VV infected PriessGAD cells using ClustalW software from the European Bioinformatics Institute. To prove the identity of these amplified cDNA sequences as CTSL, nucleotide-nucleotide BLAST software from the National Center for Biotechnology Information was used. Proteins translated from these cDNA sequences were predicted using Expasy software from the Swiss Institute of Bioinformatics.

Flow cytometry and cell sorting

Cells (5×10^5) were incubated with a primary Ab or appropriate isotype Ab for 30 m on ice followed by washing and treatment with a fluorescence labeled secondary Ab (e.g. phycoerythrin (PE)-conjugated rabbit anti-mouse IgG or cyanine 2 (Cy2) conjugated donkey anti-rat IgG (Jackson ImmunoResearch) at 1:1000 dilution for additional 30 m followed by extensive washing. In some cases, direct fluorescein isothiocyanate (FITC) or PE conjugated Abs such as FITC Mouse Anti-Human CLIP Ab (BD bioscience) or FITC conjugated anti-human CD74 (Ii) (eBioscience) were used directly without the addition of a labeled secondary Ab. To detect intracellular molecules (such as HLA-DM), cells were permeabilized with 0.05% saponin in PBS (200µl/sample) for 15 m at 37˚C. Subsequent steps for treating cells were all using 0.05% saponin added to the wash-staining buffer (1% BSA in PBS). When using the EGFP expression virus, cells were co-

stained with PE-labeled Abs to avoid fluorescence overlap that would be expected using FITC. EGFP occupies fluorescent channel 1 as FITC does. Thus, to study the infection of different APC populations, murine splenic APCs treated +/- the EGFP-VV were co-stained with PE labeled Abs to distinguish cell types. Cell surface expression of CD11c was used to detect DCs, CD11b for macrophages, and B220 as a marker for B cells (Miltenyi Biotec). Samples were analyzed by flow cytometry using Cell Quest software. For sorting EGFP positive cells, cells (2×10^7/sample) infected with EGFP-VV (MOI=10) for 14 h were harvested, fixed in 1% paraformaldehyde for 20 m on ice. The cells were washed and resuspended in 2 ml media /sample. Sorting was done by Flow Cytometry Resource Facility at IUSM.

Generation of recombinant VV

The procedure to generate recombinant virus was modified from the method described previously [97, 197]. Briefly, full length cDNA of human Ii genes (CD74, named here as hIi) isoform b cloned from PriessGAD cells was inserted into a transfer vector-zero pCR®-Blunt vector (Invitrogen) to engineer flanking restriction sites of EcoRI (New England Biolabs). This hIi cDNA was then ligated into the EcoRI site of the recombinant plasmid pSC59 to yield pSC59-hIi. pSC59 is a plasmid containing engineered overlapping early and late VV promoters designed for higher expression of inserted genes compared with the natural promoter containing plasmid [198]. The hIi insertion in pSC59-hIi was flanked by VV TK sequences, which provide sites for homologous recombination with VV. The uncut and HindIII (New England Biolabs) cut pSC59-hIi were electrophoresed on 1% agarose gels, stained with SYBR® safe DNA gel stain, and detected with UV transillumination to confirm the appropriate insertion fragment. Plasmid orientation of the hIi insert and the sequence of hIi was confirmed by DNA sequencing. To recombine the pSC59-hIi with VV, VV (MOI=0.05) infected TK^-143B cells (confluence: 85%) were transfected with pSC59-hIi (20 μg) by lipofectamine (Invitrogen). Virus particles were harvested after 2 days with 3 freeze-thaw cycles and used to infect 143 TK^- cells in presence of 5-bromodeoxyuridine (BUdR, 25 ug/ml). The basis of this selection is that the insertion in pSC 59 disrupts the TK gene of VV by

homologous recombination. Only viruses without an active TK gene can survive in the presence of BUdR and form plaques. Three rounds of this selection and amplification were performed before the final harvest of the recombinant VV containing hIi (hIi-VV). hIi-VV was titered in CV1 cells. hIi expression was also tested in CV1 cells infected by hIi-VV (MOI=10) with Western analysis using VSC or β-Gal-VV (recombinant VV encoding β-Gal also derived from pSC59) as the insertion vector control . The mIi-VV used in the Western analysis and following functional assays was provided by Poxvirus Core Facility using pSC11 as the recombinant vector. pSC11 contains both early and late VV promoters [199, 200].

CD4 T cell proliferation/cytokine production assay following in vivo exposure to virus or viral Ags

C57BL/6 mice were infected with WR VV (i.p. 10^6 pfu/mouse) at day 0 and housed for 1, 2, 7 d prior to termination during primary infection assays. Similarly, mice were i.p. infected for 7 days with same dose of modified or mutant VV [e.g. UV irradiated VV, heat inactivated VV, or BSSH (VV with truncated D8L)] prior to sacrifice and analysis. To examine T cell memory or recall responses to virus, in some studies animals were i.p. infected with 10^6 pfu/animal WR VV at day 0, housed for 21 days which time the animal was re-infected by i.p.. After 4 days these mice were terminated and analyzed. Control studies using uninfected mice were carried out in parallel. To harvest immune cells from these animals for analysis, superficial and mesenteric lymph nodes as well as spleens from infected mice were harvested, homogenized and positively selected with anti-CD4 Ab coated microbeads (Miltenyi Biotec) to capture $CD4^+$ T cells. The CD4 T cells were cultured with *in vitro* VV infected or viral peptide added APCs (1153 or primary splenic APCs). The APCs were inactivated by either fixation with 0.5% paraformaldehyde (for 1153) or irradiation (1000 rad, for splenic cells). The cell number and ratio of CD4 T cells to APCs were optimized according to pilot assays. For example, 10^5 cells of each cell type (or a ratio 1:1 for CD4 T cells to APCs) were placed in each well of a 96-well plate for T cell proliferation assay. 10^6 cells of each cell type (1:1 for CD4 T cells to APCs) were placed in a well on a 24-well plate for cytokine analysis. In all cases, CD4 T

cells were co-cultured with APCs for 72 h. CD4 T cell proliferation was monitored by [^{3}H]-thymidine incorporation. Cytokines (IL-2, IFN-γ, IL-4, TNF-α and IL-17 etc) produced by CD4 T cells were assayed with a conventional ELISA and multi-analyte ELISArrary analysis (see following).

Enzyme linked immunosorbent assay (ELISA)

To detect cytokines produced by T cells after re-stimulation, capturing Abs (for IL-2, IFN-γ, IL-17, IL-4 and TNF-α; BD Bioscience) were coated on 96-well EIA/RIA plates (Corning Incorporated) at 4˚C overnight. Cell media from APC-T cell co-culture assays were incubated at 4˚C overnight with plates coated with Abs to cytokines. To detect Ab captured cytokines, Biotin labeled Abs specific for cytokines (BD Bioscience) followed by Streptavidin-HRP (Thermo Scientific) were used. To detect Abs binding to viral protein, purified viral recombinant proteins such as H3 were used to coat the plates. Then, rabbit Abs eH3 or eH3c were added to H3 protein coated plate at 4˚C overnight followed by addition of goat anti-rabbit IgG Fc-HRP (Jackson ImmunoResearch Laboratories). Finally, HRP activity was measured using ABTS (BioFX).

Multi-analyte ELISArray kits for mouse Th1 / Th2 / Th17 cytokines (SA Biosciences) were used according to the manufacturers' instruction. Briefly, conditioned media from T-APC cultures (50 μl/well) as well as negative (no addition) and positive controls (recombinant cytokines) were added to microtiter plates coated with Abs to specific cytokines for 2 h at room temperature. Next, Biotin labeled Abs specific for cytokines were added, followed by Avidin-HRP and HRP-substrate. A total of 12 cytokines were analyzed, i.e. IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IL-23, IFNγ, TNFα and TGFβ-1 during this approach.

Cytokine production by memory CD8 and CD4 T cells upon restimulation with a mitogen PMA and ionosphere iononmycin

CD8/CD4 T cells were isolated from C57BL/6 mice after two injections with WR VV (see CD4 T cell proliferation/cytokine production assay) using anti-CD8/CD4 Ab coated microbeads (Miltenyi Biotec). These cells were restimulated with 50 ng/ml phorbol myristate acetate (PMA, Sigma) and 500 ng/ml ionomycin (Sigma) in the presence of GolgiPlug (BD Biosciences) for 4 h to induce cytokine production and accumulation in the Golgi. Surface CD4 and CD8 molecules were detected by incubating cells with the corresponding fluorescently conjugated Abs (eBioscience) for 30 m at 4˚C. After fixation with 1% formaldehyde, cells were washed twice with the wash-staining buffer (1% BSA in PBS) containing 0.05% saponin followed by intracellular staining for IL-17, IFN-γ, and granzyme B using fluorescently conjugated specific Abs (eBioscience) for 30 m at 4˚C. Flow cytometric analysis was performed as mentioned above. *In vitro* restimulation and cell staining for these assays were done by Norman Yeh from Dr. Mark Kaplan's laboratory at IUSM.

In vitro IL-17 polarization assay

Isolated splenic CD4 or CD8 T cells from untreated C57BL/6 mice were activated with soluble anti-CD3 Ab (2 μg/ml; BD Pharmingen) and anti-CD28 Ab (1 μg/ml; BD Pharmingen) in the presence of various splenic APC populations (total APCs, DCs, B cells and macrophages) or lymph node derived APCs under conditions favoring the development of IL-17 producing T cells. In these studies, T cell IL-17 polarization was induced by adding anti-IFN-γ (10 μg/ml; BD Pharmingen), anti-IL-4 (10 μg/ml; BD Pharmingen), TGF-β1 (1 ng/ml; R&D Systems), IL-6 (100 ng/ml; Peprotech), and IL-1β (10 ng/ml; eBioscience) in culture media at 37˚C [201]. On day three, fresh media containing IL-2 (20 U/ml; Roche) was added to these cells. Cells were cultured for 2 additional days prior to intracellular IL-17 staining and analyzed by flow cytometry. The *in vitro* polarization, cell staining and flow cytometric analysis were done by Norman Yeh.

Ovalbumin (OVA)-induced asthma model

To study the effects of virus infection on Ag driven inflammation in the mouse, an OVA induced asthma model was used. At day 0, Balb/c mice (five per group) were randomly divided into 4 groups including: 1) no asthma and no infection or control; 2) asthma only; 3) VV infection only; and 4) asthma + VV infection. The mice in the asthma alone (group 2) or asthma + VV (group 4) groups were i.p. injected with 20 μg OVA (Sigma-Aldrich) with 2mg Imject Alum (Pierce) twice at days 0 and 7 respectively. From day 14 to 19 each OVA primed mouse was i.n. sensitized with 50 μg OVA/day for six consecutive days. The sensitization of mice using OVA to induce asthma was carried out by Dr. Sarita Sehra at Dr. Mark Kaplan's laboratory. Three days after the i.n. sensitization (day 22), the mice in VV alone (group 3) or asthma + VV (group 4) groups were intra-tracheal (i.t.) infected with VV (10^3 pfu/mouse) by Dr. Robert Presson, Department of Medicine, IUSM. Four days after VV infection (day 26), the lungs and splenocytes from these mice were harvested for histology and cytokine production assays respectively by Dr. Gourapura Renukaradhya, Jeremy Eltz and Beau Champ of the Poxvirus Core Facility under the direction of Dr. Randy Brutkiewicz, IUSM.

For histology, lungs were perfused with PBS and fixed in 10% formalin overnight. The next morning the tissues were transferred to 70% ethanol prior to paraffin embedding by Dr. Keith Condon, IUSM. Tissue samples were sectioned and haematoxylin and eosin (HE) and periodic acid-Schiff (PAS) stained to detect inflammatory cell migration into the lungs as well as mucous production. Tissue sample histology and mucous production quantification were assessed by Dr. David Wilkes, Department of Medicine, IUSM.

For cytokine production assays, 1.5 x 10^6 splenocytes from each mouse were cultured in 24 well plates +/- 100 μg/ml OVA for 72 h before analysis by ELISA using IL-5, IL-13, IL-17, and IFN-γ specific Abs. Samples were assayed in triplicate and the mean and standard deviation determined. These assays were carried out by Dr. Gourapura Renukaradhya.

Synthesis of viral D8 and H3 recombinant proteins using an E coli protein expression system

Truncated VV proteins D8 and H3 lacking their transmembrane domains were expressed in DE3 *E. coli* cells using plasmids provided by Dr. W. Chang (Academia Sinica, Taiwan). The recombinant viral proteins were tagged with tandem histidine residues and purified with the QIAexpressionist kit (Qiagen) according to the instructions from the manufacturer. To optimize the production of the histidine conjugated viral D8 and H3 proteins, cells were cultured +/- isopropyl β-D-1-thiogalactopyranoside or IPTG (Qiagen, 0.4 mM for H3 and 0.8 mM for D8) to monitor recombinant protein expression. Lysates from these cells were fractionated by SDS-PAGE, transferred to nitrocellulose membranes and probed with either D8 and H3 specific Abs or rabbit anti-6-His-HRP Ab (His-Ab, Bethyl). The purified H3 protein was used to test the binding specificity and affinity of the eH3 or eH3c Abs by ELISA.

Protective H3 Ab analysis

Individual rabbits were immunized with either the keyhole limpet hemocyanin (KHL) conjugated peptides $H3_{44\text{-}61}$ (CPEKRNVVVVKDDPDHYKD) or the peptide $H3_{244\text{-}259}$ (CVAEHRFENMKPNFWSR) to induce the eH3 Ab (to $H3_{44\text{-}61}$) and the eH3c Ab (to $H3_{244\text{-}259}$) respectively. Both pre-immune and immunized sera were harvested from each rabbit. Ab titers and the specificity of these Abs were verified by Western analysis and ELISA using purified VV and H3 protein respectively. Infection efficiency of PriessGAD cells by EGFP-VV (MOI=10, 6 h) in the absence or presence of the eH3/eH3c Abs was tested using flow cytometry with human vaccinia immune globulin (VIG) as a positive control which blocks VV infection. Pre-immune sera were used as the negative control. Presentation of endogenous GAD by PriessGAD cells upon VV (MOI=10, 6 h) infection in the presence or absence of the eH3/ eH3c Abs was monitored using an Ag presentation assay. For both flow cytometry and Ag presentation assays, 1 volume of VV (MOI=10) was pre-incubated with 3 volumes of various Abs for 40 m at 37˚C. To rule out non-specific Fc receptor binding to Abs, cells were first treated with FcR blocker ($2\mu l/1\times10^6$

cells, Miltenyi Biotec) prior to the addition of virus +/- virus-specific Abs. When FcR blocker was present, cells were pre-incubated with this reagent at 37˚C for 30 m before the addition of virus.

RESULTS

Chapter 1 VV infection of APCs and disruption of class II mediated-Ag presentation

Although T cells are resistant to VV entry, APCs (such as DCs and macrophages) are susceptible to VV infection *in vitro* and *in vivo* [94-96]. APC dysfunction after *in vivo* and *in vitro* exposure to VV has been established [92, 96, 97]. Specifically, VV perturbs MHC class II mediated Ag presentation by both professional and non-professional APCs [96, 97]. Here, to confirm the susceptibility of B cells, macrophages and DCs to VV infection, the infection efficiency of immortalized B cells and primary APCs was compared both *in vitro* and *in vivo* after VV inoculation. To validate VV-induced disruption of class II function, Ag presentation was tested in several human and murine B cell lines as well as murine primary APCs upon VV infection. VV disruption of class II presentation was rapid, occurring in <1 h of virus infection. Class II Ag presentation was disrupted by live VV as well as replication deficient virus [97]. Although inhibition of host protein synthesis had been postulated, specific mechanism(s) for viral disruption of the class II pathway remained to be elucidated [110]. Thus, the effect of virus on the expression of key components of the class II pathway was investigated using human B lymphoblasts. To explore the viral factors contribute to disruption of class II function, T cell assays were carried out using VV +/- the virus specific DNA polymerase inhibitor arabinofuranosylcytosine (AraC). This inhibitor also blocks the synthesis of virus late gene products [202]. Heat inactivated VV and viruses lacking specific genes reported to regulate other pathways for Ag presentation were also tested for their effects on the class II pathway. Thus, these initial studies began to probe how virus infection influences class II presentation.

1.1 Efficiency of APC infection by VV

Previous studies have shown that APCs are susceptible to VV infection while T cells are more resistant to virus entry [94-96]. To investigate the *in vitro* infection efficiency of APCs, a human B cell line-PriessGAD, murine splenic APCs (depleted of T cells by microbeads coated with Abs to CD4 and CD8) and T cells (cells positively selected using microbeads coated with Abs to CD4 and CD8) were infected with EGFP-VV (MOI=10) for 24 h. The EGFP-VV is a recombinant VV encoding enhanced green fluorescent protein (EGFP) under the control of virus early-late gene promoter [178]. Cells expressing EGFP were detected by flow cytometry. To further analyze the efficiency of infection for primary APCs, these cells were further identified using PE labeled Abs specific for phenotypic surface markers, i.e. B220 for B cells, CD11c for DCs and CD11b for macrophages. Using murine splenic cells, pilot experiments showed that the relative abundance of each APC population within the spleen remained constant after 24 h of *in vitro* VV infection. Thus, we did not observe the loss of discrete cell types during this *in vitro* infection analysis. As listed in Table 2, PriessGAD cells were the most susceptible cell type with more than 80% cells infected after 24 h of incubation with VV. By contrast, the infection efficacy for mixed population of murine primary APCs was much lower with only 20% of cells containing EGFP-VV. However, higher infection was observed for specific populations of splenic APCs. Potent APCs such as DCs, the efficiency of virus infection was 72%. Macrophages also had a high efficiency of infection at 51%. Since the majority of the primary APCs in the spleen are B cells with an infection efficiency of about 20%, the overall efficiency for the mixed splenic population of APCs was close to this number. DCs and macrophages are also known to be more endocytic and they can take up more material by phagocytosis [203], thus these higher efficiencies of infection might be expected compared to murine B cells. T cells, on the contrary, displayed only limited infection, i.e. 8% $EGFP^+$ 24 h post VV infection. The *in vivo* infection efficiency for different APCs was also studied. C57BL/6 mice were i.p. infected with 2.5×10^8 pfu of EGFP-VV/mouse for 24 h. Animal spleens were then harvested, homogenized and depleted of T cells by microbeads coated with Abs to CD4/CD8. The remaining splenic cells were then stained with different PE-conjugated

Abs to detect different APC populations (B cells, DCs and macrophages) and analyzed by flow cytometry. Although less than observed with *in vitro* virus exposure, *in vivo* inoculation of mice also resulted in virus EGFP detected in individual APC populations at about 10-30% (Table 2). The data on infection efficiencies for individual APC populations was normalized relative to the abundance of each cell type in the spleen. Thus, as previous studies have demonstrated [94-96], APCs but not T cells could be effectively infected by VV.

Table 2 Infection efficiencies after 24 h VV inoculation

Cell type	Infection route	Virus EGFP $^{+}$ cells (%)
PriessGAD (human B-LCL)	*In vitro*	81
Primary murine DCs	*In vitro*	72
	In vivo	30
Primary murine macrophages	*In vitro*	51
	In vivo	27
Primary murine B cells	*In vitro*	24
	In vivo	10
Primary murine APCs (mixed population of splenic APCs)	*In vitro*	25
	In vivo	17
Primary murine T cells	*In vitro*	8

For *in vitro* infection, human B-LCL or isolated primary cell populations were infected with EGFP-VV (MOI=10) for 24 h. For *in vivo* infection, cells were harvested from EGFP-VV (2.5×10^{8} pfu) infected C57BL/6 mice. APC populations were distinguished with PE-conjugated Abs, and the relative abundance of each cell type in the spleen, i.e. 75% B cells, 18% macrophages and 7% DCs was used to calculate the percent infected cells.

1.2 Disruption of class II-mediated Ag presentation by VV

Our studies showed that VV perturbs MHC class II-mediated Ag presentation by both professional and non-professional APCs [97]. To extend these results, endogenous GAD Ag presentation by PriessGAD cells upon VV infection was tested using T cell assays. As shown in Fig. 3A and B, VV infection of PriessGAD reduced MHC class II presentation of GAD in a manner dependent on viral MOI and duration of infection. PriessGAD cells were infected with VV and then treated with paraformaldehyde to inactivate the virus prior to co-culture of these APCs with T cells. Exposure of APCs to VV at an MOI of 10 resulted in a dramatic inhibition of class II Ag presentation with little or no change in cell viability. VV induced class II dysfunction not only in human

but also in a murine cell line. Presentation of a naturally processed Ag, actin by the murine B cell tumor 1153 diminished in a dose dependent manner when 1153 was exposed to VV (Fig. 3C). The effects of virus on class II presentation by primary murine spleen cells were also investigated. Murine splenic cells depleted of T cells, were infected with EGFP-VV (MOI=10) or under mock conditions for 14 h. The virus infected cells were fixed and sorted by FACS to ensure analysis of only cells expressing viral EGFP. HEL peptide presentation by murine spleen cells mock infected or isolated based on EGFP expression was analyzed. The EGFP-VV positive cells failed to present HEL peptide while the non-infected spleen cells efficiently presented HEL (Fig. 3D). Thus, 100% VV infected APCs lost their Ag presentation ability completely. Therefore, cell infection correlated with the loss of Ag presentation *in vitro*. Similar to human B-LCL, little to no change in cell viability was observed during virus infection of these murine cell lines and primary APCs.

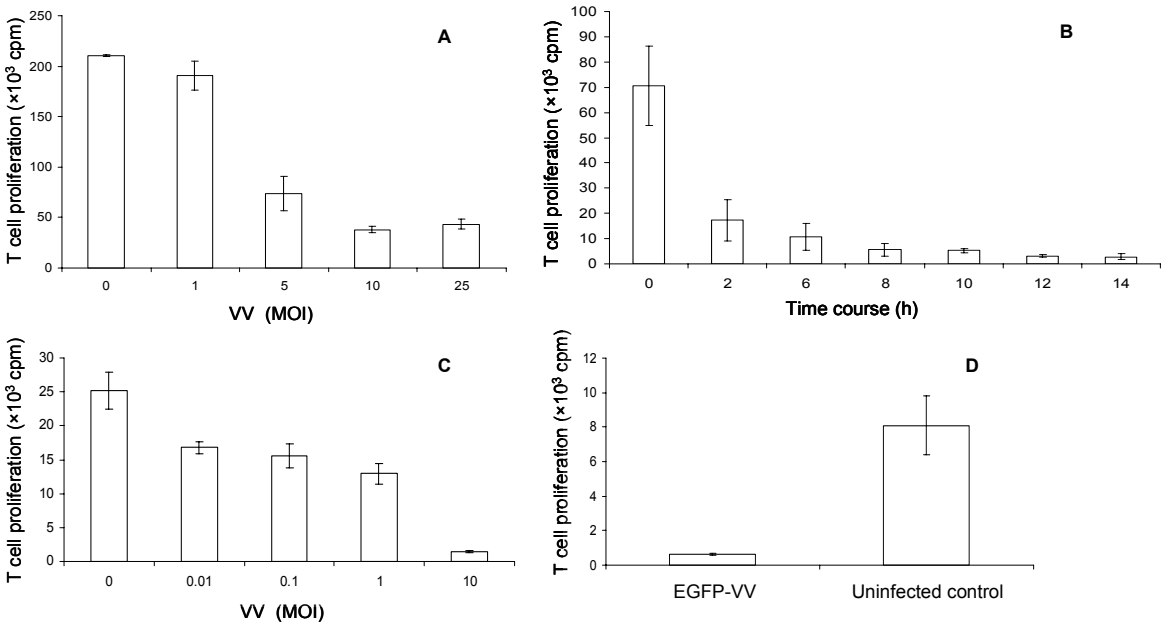


Fig. 3 Disruption of class II function by VV infection of established cell lines and primary cells. A. Inhibition of class II presentation by VV was dependent on viral MOI. PriessGAD cells were infected with VV (MOI 0 to 25) for 6 h, prior to fixation and analysis of GAD Ag presentation. B. Temporal progression of VV inhibition of class II function. PriessGAD cells were incubated with VV (MOI=10) for 0 to 14 h, then assayed for GAD Ag presentation. C. Endogenous actin presentation by murine B cell tumor upon VV infection was dependent on MOI. 1153 B cells were infected with VV (MOI up to 10) for 6 h and fixed prior to actin-specific T cell addition. D. Loss of class II function correlated with VV infection of primary APC. Splenic cells were harvested from C57BL/6 mice. Semi-purified APC were obtained by depleting T cells with microbeads coated with Abs to CD4 and CD8 molecules. The CD4/CD8$^-$ spleen cells were infected with EGFP-VV (MOI=10) for 14 h followed by fluorescence activated cell sorting (FACS). A parallel sample of these cells was mock infected. EGFP positive cells after sorting and non-infected cells were incubated with 10 μM HEL peptide for 6 h prior to co-culture with HEL-specific T cells and analysis for T cell activation (see materials and methods).

1.3 Viral early-late gene products in disruption of class II presentation

The efficacy of VV inhibition of class II presentation may be influenced by both virus associated factors and/or a cellular component from the host. To further investigate the role of virus-derived products, the following functional assays were undertaken. Our previous study showed that replication of VV is dispensable for the inhibitory effect on endogenous GAD presentation using UV and psoralin treated VV [97]. This treatment also blocks virus production of early gene transcripts, and thus the expression of virus

early gene protein products. Thus, these results suggest that a viral late gene or host product may be involved in disrupting class II presentation. Using arabinofuranosylcytosine (AraC) which specifically inhibits viral DNA polymerase, similar inhibitions were observed regardless of AraC presence at 6 h. AraC can also block the synthesis of virus late gene products [202]. Indeed at longer times of cell culture with AraC or untreated VV, we noted less inhibition with the AraC treated cultures suggesting a virus late gene may be involved (Fig. 4A). Viral particles contain several abundant late proteins which are carried into cells and often promote infection [204]. To examine if these virion proteins must be in their native conformation, VV was heat treated (60˚C for 1 h). Heat-inactivated VV has been used as a control for studies with live VV [205]. VV that has been heat-inactivated can not form plaques and is generally thought to be non-antigenic [206]. As shown in Fig. 4B, heat-inactivated VV was less effective in blocking GAD Ag presentation compared to live VV. Thus, the native structure and virion infection appear to be important. In subsequent studies, we found even heat treated virus enters cells, thus the most likely requirement is virion protein conformation or function.

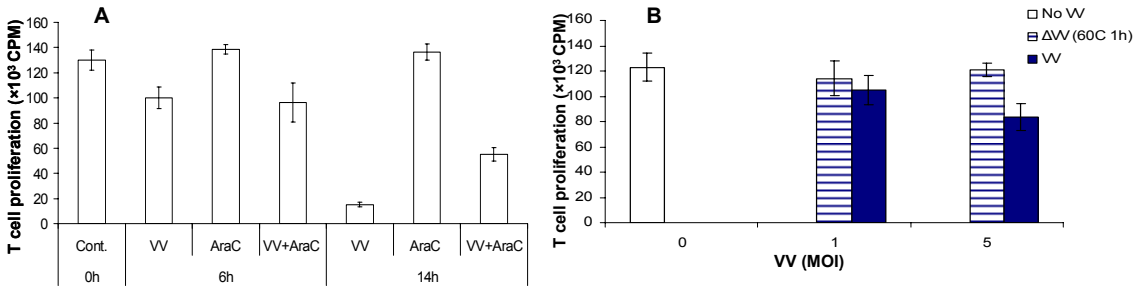


Fig. 4 Viral factors contributing to the inhibitory property of VV. A. Inhibition of DNA replication and late gene expression partially preserved class II function. PriessGAD cells were treated with VV +/- the viral DNA polymerase inhibitor-arabinosylcytosine (AraC, 10 μM) for 0, 6 and 14h. T cell assay was performed to monitor GAD presentation. B. Heat inactivation of VV ablates the ability of the virus to disrupt the class II pathway. VV, untreated or heat inactivated, was incubated with PriessGAD cells for 6 h prior to T cell addition and assays for T cell activation.

CD1-mediated lipid presentation pathway is also disrupted by VV [90]. CD1 molecules are structurally similar to class I while behaving like class II molecules by using the endosome-lysosomal pathway. The CD1 family is divided into three groups. The group 2 member CD1d molecules are expressed on hematopoietic cells, hepatocytes and

gastrointestinal epithelium [89]. CD1d molecules present lipids to invariant Vα24-Jα18 TCR containing NKT cells [14], like class II molecules present peptide to CD4 T cells (Fig. 5A). Activated NKT cells fulfill their function by secreting Th1 and Th2 cytokines. Like the disruption of class II presentation, VV also perturbs CD1d-mediated lipid presentation. It has been demonstrated that the products two viral genes, B1R and H5R, may be involved in VV-induced CD1d dysfunction [90]. B1R is an early viral gene encoding a kinase required for viral replication [207, 208]. The H5R gene product, a substrate of B1 kinase, is a transcription factor expressed at both early and late stages and is involved in the transcription of VV late genes [209, 210]. We tested if viral B1R and H5R genes were necessary for class II perturbation using temperature sensitive (TS) mutants. Neither B1 nor H5 proteins were necessary for class II inhibition since at both permissive (31˚C) and non-permissive (38˚C) temperatures, GAD Ag presentation was equally disrupted using the TS mutant viruses and VV (Fig. 5B). Specifically, about 20% GAD presentation remained when PriessGAD cells were infected with viruses (MOI=1) and less than 10% remained when cells were infected with higher MOIs independent of B1 and H5 protein function. These results were also consistent with our previous study showed that VV disrupted class II presentation is independent of viral replication [97].

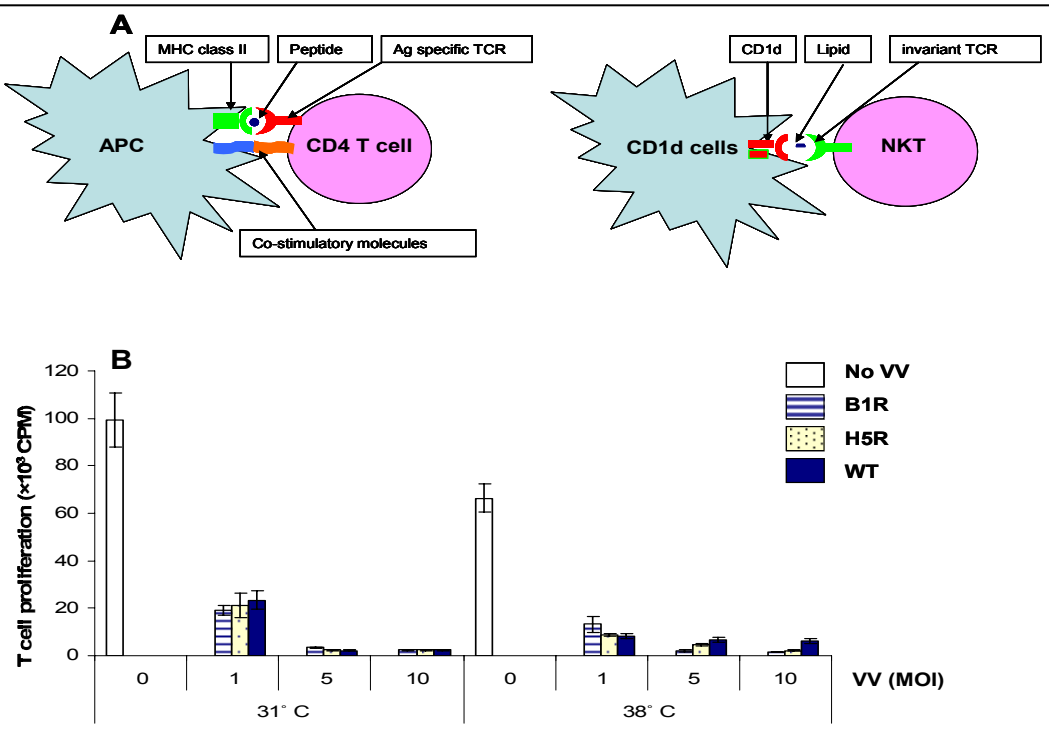


Fig. 5 The effect of virus B1 and H5 proteins on class II presentation. A. Ag Presentation by class II and CD1d pathway. Processed peptide is presented by class II molecules on the surface of APC to CD4 T cells, which express Ag specific TCR. Interaction between co-stimulatory molecules is required for CD4 T cell activation (Left panel). Lipid Ag is presented by CD1d containing cells to NKT cells expressing invariant TCR (Right panel). B. Inhibition of endogenous GAD presentation was independent of virus B1R and H5R gene product functional expression. PriessGAD cells were infected with VV or temperature sensitive mutant viruses (MOI 0 to 10) for 6 h at permissive (31˚C) or non-permissive (38˚C) temperature prior to T cell assay. Viral mutants with temperature sensitive B1 and H5 proteins were obtained from Dr. R. Brutkiewicz, IUSM.

1.4 Inhibition of class II function by VV is not due to EBV-associated genes in B-LCL

Our studies demonstrated similar losses in class II function upon VV infection of human or murine cells. Yet there remained some concerns that the human B-LCL examined in our studies expressed Epstein Barr virus (EBV) gene products as result of their transformation using this virus. Thus, studies were carried out to examine the effects of VV infection using a human B cell tumor line lacking EBV genes. BJAB is a human B cell tumor line which does not express hallmark EBV Ags such as EBV nuclear antigen (EBNA) associated with EBV transformation [211]. BJAB cells were retrovirally

transduced with class II DR4 for these studies and surface expression of class II molecules was confirmed by flow cytometry. GAD peptide presentation was dramatically inhibited by VV infection (MOI=10) of BJAB-DR4 cells (Fig. 6A). Similar to our results with EBV transformed B cells, virus inhibition of class II presentation could be partly reversed by adding higher concentration of peptide to BJAB-DR4 cells. Inhibition of GAD peptide presentation by BJAB-DR4 cells was similar to that observed with EBV^+ Priess cells (Fig. 6B). Thus, we concluded that EBV transformation did not influence the ability of VV to disrupt class II presentation. Notably, as expected, the parent cell line BJAB, which lacks the appropriate class II DR4 molecules, failed to present GAD peptide even at the highest dose. This result confirms the specificity of our T cell assays using these B cell lines.

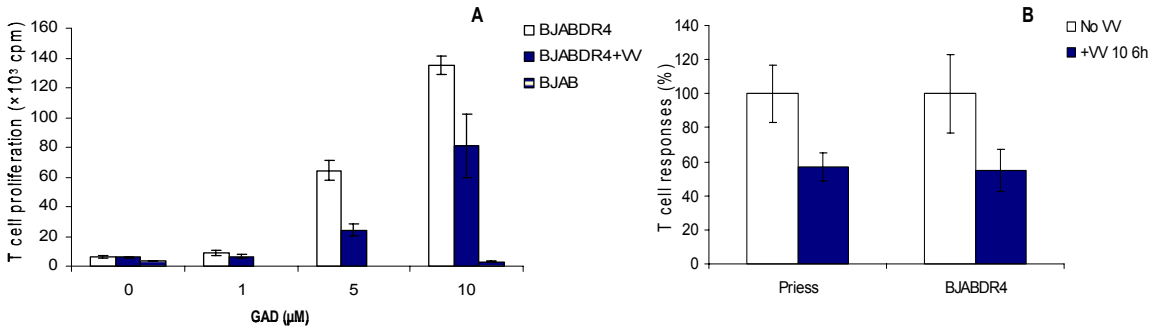


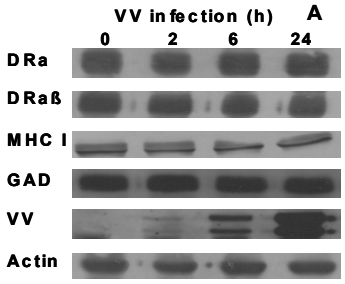
Fig. 6 VV inhibition of class II function was not influenced by EBV transformation of B cells. A. GAD peptide presentation was not affected by EBV transformation. BJAB or BJAB-DR4 cells were incubated with GAD peptide (up to 10 μM) +/- VV infection (MOI=10) for 6h before T cell addition and analysis of T cell activation. B. VV induced comparable inhibition of GAD presentation by Priess cells and by BJAB-DR4. Priess and BJAB-DR4 cells were incubated with 0.5 μM or 10 μM GAD peptide respectively in the presence or absence of VV (MOI=10) for 6 h prior to T cell addition. Higher concentration of GAD peptide was used with BJAB-DR4 cells due to their lower expression of class II DR4 allele after retroviral transduction. Samples without VV infection were set to 100% for each cell line and relative peptide presentation upon VV treatment was measured.

1.5 Expression of key components of the class II pathway with VV infection

Inhibition of host protein synthesis and recruitment of host resources to build virions are important cytopathic effects of VV. Inhibition of host protein synthesis is typically associated with virus early gene expression, and thus might correlate temporally with the

loss of class II function in infected APCs. Reduction in components required for the class II pathway could be one mechanism by which the virus disrupts this pathway. Studies to evaluate the effects of VV on host protein synthesis were historically carried out in Hela cells or epithelial cell lines such as BSC [212, 213]. More recent microarrays of host gene expression examined Hela cells and DCs upon VV infection [109, 214].

To examine the expression of components of class II pathway upon VV infection, Western analysis was applied using specific Abs. Protein levels for both MHC class I and class II molecules were assessed during 24 h of VV infection. VV infection did not significantly diminish steady state expression of MHC class II DRα monomers or DRαβ dimer levels up to 24 h (Fig. 7A and B). Surface class II levels as detected by flow cytometry were not altered by VV infection [97]. The constant surface expression of class II molecules even after 24 h VV infection was also demonstrated for murine 1153 and TA3 B cell lines using flow cytometry (data not shown). The relatively high stability or long half-life of assembled class II, for example, class II complexes in human B cells display half-life of approximately 36 h [215], may account for the preservation of these proteins upon VV infection. Quantification of the data in Fig. 7B confirmed that human class II DRα monomer, DRαβ dimer, MHC class I heavy chain and endogenous Ag GAD expression remained constant up to 24 hours after VV infection. VV protein expression, on the other hand, increased progressively.



B

Relative expression (%)		
Protein	VV infection (h)	
	0	24
DRa	100	114
DRaß	100	91
MHC I	100	95
GAD	100	110

Fig. 7 Sustained expression of MHC molecules and GAD protein during VV infection. A. MHC class I, class II and GAD steady state expression was maintained during VV infection. PriessGAD cells were infected with VV (MOI=10). Samples were immunoblotted to detect MHC class II DRα; DRαβ complexes; MHC class I; GAD; VV late proteins (30-35 KD) and actin. B. Densitometric analysis of protein expression. For samples in panel A, each protein's abundance at 0 and 24 h was calculated relative to actin as a control for loading. Protein abundance at time 0 h was normalized to 100% for comparison with samples exposed to VV for 24 h. Standard error in densitometry for replicate studies was ≤ 15%.

In contrast to host protein abundance, most cellular mRNAs decreased significantly after VV infection. For example, as shown in Fig. 8A, HLA-DRA (encoding DRα) mRNA abundance was reduced gradually over time following VV infection. mRNA for house keeping genes like ACTA1 (encoding actin) and GAPDH (encoding glyceraldehyde-3-phosphate dehydrogenase or GAPDH) also dropped progressively upon VV infection. As expected, mRNA for viral E3L was detected early during infection. Messages for some host genes including HSPA10 (encoding heat shock cognate protein 70 or HSC70) did not change up to 14 h after infection, therefore the message for this gene was used as a loading control in RNA analysis. To more accurately estimate the alteration in HLA-DRA mRNA, real time PCR was applied. Again, HLA-DRA mRNA levels diminished progressively post VV infection using HSPA10 as the endogenous control (Fig. 8B). A reduction in mRNA for MHC class II was also confirmed in an *in vivo* VV infected mouse model. C57BL/6 mice were infected with VV (i.p.10^6 pfu/mouse) up to 2 d. Spleen cells from these animals were harvested and used to isolate different APC populations. Murine class II gene H2-Ab1 (encoding I-A^b) mRNA decreased in a time dependent manner for DCs and B cells. The H2-Ab1 mRNA reduction was not significant in macrophages (Fig. 8C). To examine H2-Ab1 mRNA alteration in more detail, a shorter time course of *in vivo* infection was used to assess class II mRNA in DCs (Fig. 8D). Again, H2-Ab1 mRNA abundance was reduced progressively after infection.

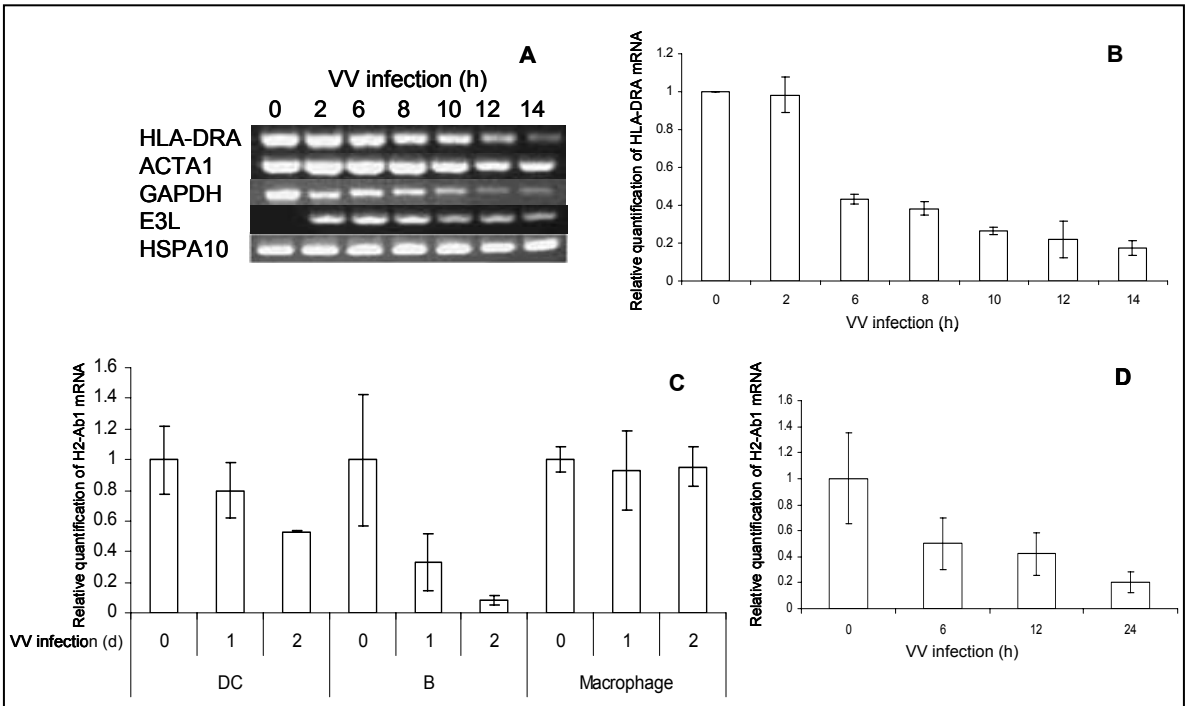


Fig. 8 Reduction in class II mRNA upon *in vitro* and *in vivo* VV infection. A. Alteration in cellular and viral mRNAs after VV infection. PriessGAD cells were infected with VV (MOI=10) for up to 14 h and mRNA was isolated for semi-quantitative RT-PCR using primers for HLA-DRA, ACTA1, GAPDH, HSPA10, and viral early gene E3L. Analysis was carried out with 32 amplified cycles for HSPA10 and 28 cycles for all other genes. Viral E3L gene transcripts were assessed to confirm virus gene expression. Host HSPA10 expression was maintained during infection. B. Quantitative real time PCR analysis of HLA-DRA mRNA during VV infection. mRNA was isolated from PriessGAD cells infected with VV up to 14 h and converted to cDNA. cDNA was amplified with HLA-DRA and HSPA10 specific primers for up to 40 cycles. Quantification of HLA-DRA mRNA level was calculated relative to HSPA10 message level. 0 h or the non-infected sample was used as the calibrator. The results of three individual experiments were averaged. C. Decrease in H2-Ab1 mRNA in APCs after *in vivo* VV infection. Mice infected with VV (1×10^6 pfu/mouse) for up to 2 d were followed by harvesting of spleen cells. DCs, B cells and macrophages were isolated with corresponding Ab coated microbeads. Extraction of RNA and synthesis of cDNA was preformed prior to realtime PCR analysis. D. Progressive reduction in H2-Ab1 mRNA of *in vivo* infected DC detected by realtime PCR. Mice were infected with 1×10^6 pfu VV up to 24 h before isolation of DCs. For C & D, relative quantification of H2-Ab1 mRNA levels was normalized with Gapdh as the endogenous control. H2-Ab1 mRNA from non-infected or 0 d/h samples was used as the calibrator for comparison. At each time point, values were averaged from three mice in the same group.

1.6 Destabilization of peptide-class II complexes by VV

Our previous study showed that VV destabilizes class II at an early stage of infection by disrupting peptide binding [97]. Since GAD peptide can directly bind cell surface class II without intracellular processing, we first examined the effect of VV on GAD peptide presentation. Functional assay showed a reduction in GAD peptide presentation upon VV infection (Fig. 9A). These results confirmed that VV may disturb the binding of peptide to class II. To further prove the destabilization of peptide-class II complexes by VV, the presentation of different influenza hemagglutinin (HA) peptides were tested in the absence or presence of VV (Fig. 9B). These HA peptides bind class II with different stabilities [190]. VV inhibition of wild type (WT) HA (half-life of peptide:I-A^d complexes is 26 h) presentation was more significant (about 50%) than that of the more stable HA mutation-T128V (TV) (half life of peptide:I-A^d complexes is 85 h) presentation (about 28%). Thus, stability of peptide association with class II molecule was another factor affecting VV disruption of class II function. The peptides that can form more stable complexes with class II molecules were more resistant to VV inhibition. These results proved indirectly that VV may disturb class II function by destabilizing class II-peptide complexes.

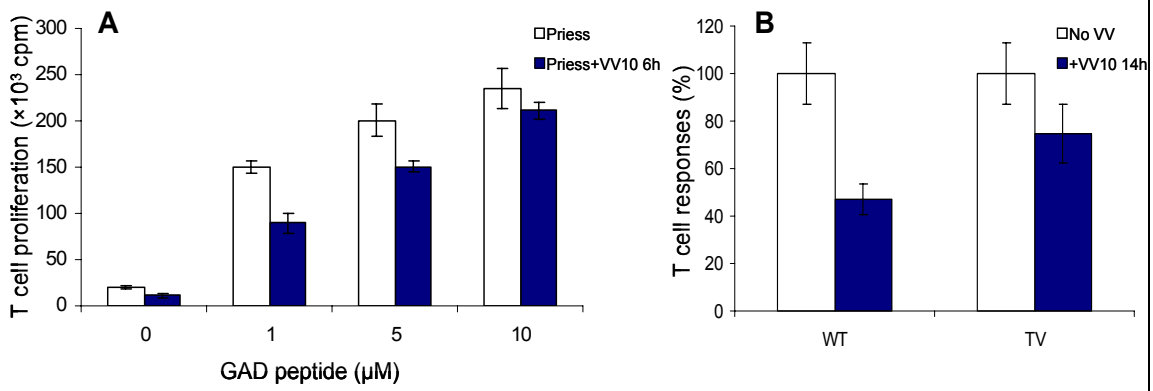


Fig. 9 VV infection of APC disturbed class II-peptide presentation. A. The effect of VV on GAD peptide presentation. Priess cells were incubated with GAD peptide (up to 10 μM) and VV (MOI=10) for 6 h. Cells were washed, fixed, co-cultured with GAD specific T cells and assayed for T cell activation. B. Stable peptide-class II complex was relatively resistant to VV infection. TA3 cells were co-incubated with 5 μM of wild type HA peptide (WT) or T128V mutated HA peptide (TV) and VV (MOI=10) for 14 h prior to the addition of HA specific T cells and analysis of the T cell activation. Relative T cell responses for each peptide were normalized to no VV samples as 100%.

Together the data presented in this chapter confirm that APCs but not T cells were vulnerable to VV infection. VV disrupted class II mediated peptide and Ag presentation by immortalized cell lines and primary APCs, heat treatment destroyed the ability of VV to perform this activity. The efficacy of inhibition positively correlated with the level of cellular infection. Virus replication and early gene products were not required for disrupting class II function. Yet, native virions and possibly viral late gene products appear to be important in disrupting class II presentation. EBV transformation did not affect the ability of VV to disrupt class II function. VV reduced class II expression at mRNA levels but not at protein levels. Destabilization of peptide-class II binding is one of the mechanisms VV used to disturb class II -mediated Ag presentation.

Chapter 2 Loss of invariant chain (Ii) upon VV infection of APCs

Ii is essential for the class II pathway by chaperoning both class II and DM molecules. Ii expression level correlates positively with the class II presentation ability [20-22]. Stabilization of class II dimers is one of the most important functions of Ii. Previously, we showed that VV destabilizes peptide-class II complexes. Thus, we explored whether VV induces class II destabilization by affecting Ii. Indeed, consistent with the VV induced destabilization results, a significant reduction in Ii at the protein and mRNA levels was found. To further demonstrate the adverse effects of disrupting protein synthesis on Ii expression and class II function, a classical protein synthesis inhibitor-cycloheximide (CHX) was used. CHX mimicked the inhibitory effects of VV in term of Ii protein level and class II function. Yet, there were significant differences in how VV perturbs the class II pathway, suggesting virus infection alters more than host protein synthesis. Apparent differences in Ii proteolysis were observed with VV and CHX treatment of APCs. Only VV but not CHX altered Ii processing. Virus infection led to losses in the expression of specific cathepsins required for Ii and Ag processing, while Cathepsin L is induced. Likely, the reduction in Ii expression during VV infection is caused by changes in both synthesis and processing. Over-expression of Ii partially reversed VV-induced class II dysfunction, further confirming the contribution of Ii loss to the disruption of class II function.

2.1 Diminished Ii levels in VV-infected APCs

Upon screening the expression of several components of class II presentation pathway by Western analysis, we observed a significant reduction in Ii protein expression. As shown in Fig. 10A, the steady state level of cellular Ii decreased in a time dependent manner upon VV infection of PriessGAD cells. Ii protein levels were slightly decreased at 2 h of infection with an even more significant drop between 8-12 h. Densitometric quantification revealed a 15% reduction in cellular Ii expression at 2 h and an 80% decrease in Ii levels 12-14 h following VV infection. Since Ii is crucial for class II Ag presentation, the loss of Ii may contribute in part to VV disruption of MHC class II-

mediated Ag presentation, particularly at later stages of viral infection. To further examine the correlation between class II dysfunction and Ii reduction upon VV infection, the expression of Ii and other components along the Ag presentation pathway was compared using VV or UV-irradiated VV. Our previous study had shown that UV-treated VV diminished class II function to a level slightly less than untreated VV [97]. As shown in Fig. 10B, the reduction in cellular Ii for PriessGAD cells treated with UV-inactivated VV was less severe compared with cells infected by VV. Again class I and II protein levels remained constant. The partial preservation of Ii may possibly explain the decreased perturbation of class II mediated Ag presentation by UV-treated VV. Another method to block VV replication is to use a viral specific polymerase inhibitor, arabinosylcytosine (AraC). AraC treatment of VV also blocks synthesis of viral late genes. Similar to the effect of UV treated VV, AraC partially preserved Ii expression (Fig. 10C). The effects of AraC were observed post 6 h infection, when virus replication and late protein expression occur. Thus, we conclude that loss of cellular Ii at later stages of infection appears to be linked to class II dysfunction.

To monitor the synthesis of Ii upon VV infection, a [^{35}S]-methionine incorporation assay was performed. Here, PriessGAD cells were infected with VV for up to 14 h with radiolabeled methionine added at different times prior to immunoprecipitation of Ii and class II proteins. At 6 h, VV infection decreased Ii synthesis significantly yet DR synthesis was preserved (Fig. 10D). Even at 14 h, synthesis of new class II is detected but little if any Ii could be isolated from infected cells. To determine if the reduction in Ii expression was due to a global inhibition of host gene expression by VV, the relative abundance of CD74 (encoding Ii) mRNA was measured using semi-quantitative RT-PCR analysis. CD74 mRNA levels progressively decreased upon VV infection temporally preceding the drop in Ii protein expression (Fig. 10E). By contrast, host HSPA10 (encoding HSC70) mRNA levels did not change up to 14 h after VV infection. To more accurately detect alterations in CD74 mRNA levels after VV infection, real time PCR was performed using HSPA10 as the endogenous control. Quantification of CD74 mRNA revealed a 65% decrease after 6 h and an 80% drop after 10 h post VV infection (Fig.

10F). These results indicate that the diminished expression of host CD74 mRNA contributed to the reduced steady state levels of Ii protein during VV infection.

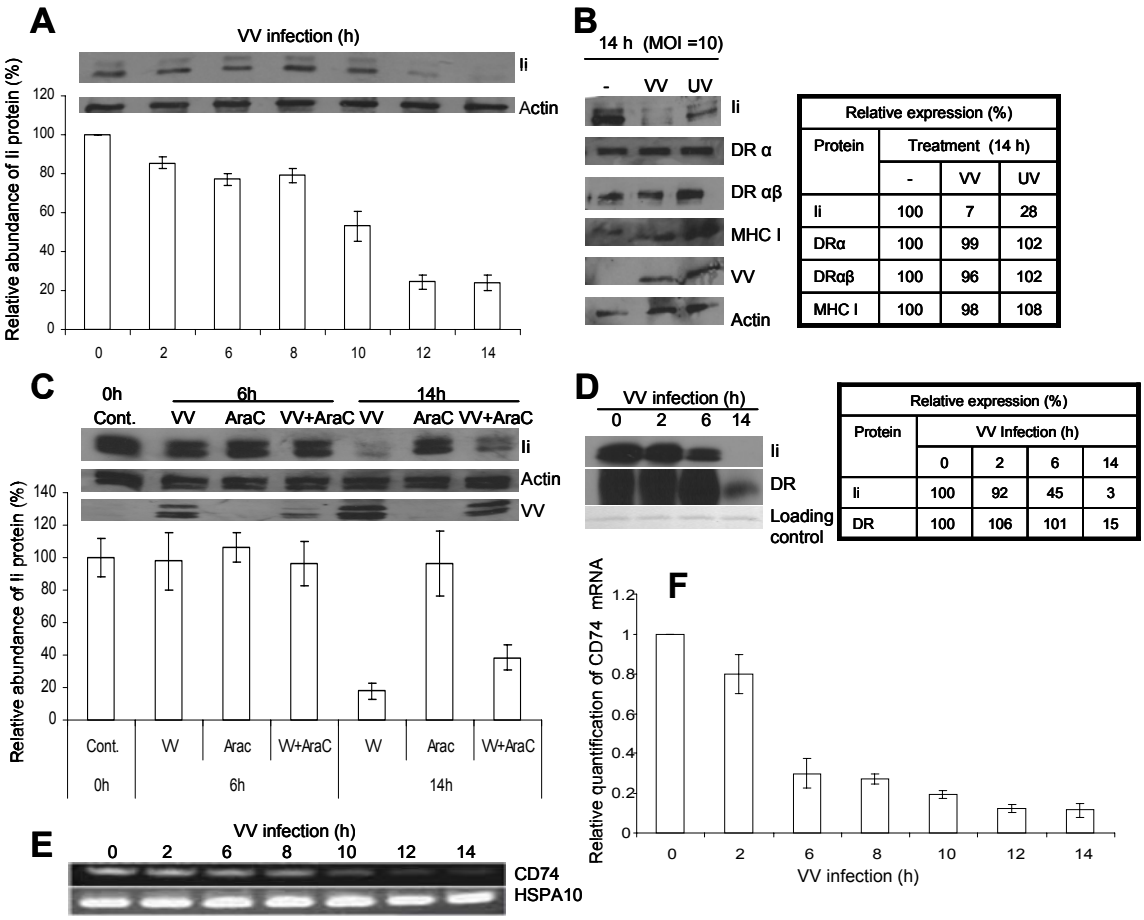


Fig. 10 VV infection reduced Ii expression at both the protein and mRNA levels. A. VV reduced Ii steady state protein levels during infection of APC. Samples of PriessGAD cells infected with VV for up to 14 h were Western blotted for Ii expression. Actin was used as the loading control. Densitometric quantification of Ii protein expression was calculated relative to actin abundance at each time point after VV infection of B cells (averaged from three separate experiments). B. Relative less loss of Ii upon cell exposure to UV-irradiated VV. PriessGAD cells were incubated with VV or UV treated VV at MOI 10 for 14 h prior to Western Blot analysis using specific Abs to Ii, MHC class II DRα, class II DRαβ dimers, MHC class I, VV and actin. Densitometric analysis of protein expression is listed besides the blots. Each protein's abundance was calculated relative to actin. Treated samples were normalized to control, untreated samples which had been set as 100%. C. Inhibition of DNA replication and late gene expression partially preserved Ii expression. PriessGAD cells were treated with VV +/- the viral DNA polymerase inhibitor –AraC (10 μM) for 0, 6 and 14h. Western blot analysis was performed to monitor Ii expression. Quantification of Ii protein expression was calculated relative to actin abundance at each treatment (averaged from three experiments).

Fig. 10 legend (Cont'). D. Exposure of B cells to VV decreased the synthesis of Ii protein between 2-6 h. PriessGAD cells were infected with VV for 0 - 14 h, and at specific times (0, 2, 6, 14 h) aliquots of cells were labeled with 0.5 mCi of [^{35}S]-methionine for 1 h. Following this brief radio-labeling, cells were washed and detergent lysates prepared. Cell lysates were immunoprecipitated with Abs specific for Ii or class II proteins and these immunoprecipitated samples were resolved by SDS-PAGE. Electorphoretic gels were fixed, stained with Coomassie Blue and dried prior to autoradiography. Proteins loaded in each sample lane were monitored by Coomassie Blue staining and as shown here, support equal sample loading. Quantification data of relative abundance of Ii or DR normalized to loading controls is listed besides. 0 h samples were set equal to 100%. E. VV infection reduced B cell CD74 mRNA levels in a time dependent manner. PriessGAD cells were infected with VV (MOI=10), mRNA levels for CD74 and HSPA10 were analyzed by RT-PCR. Data shown after mRNA amplification for 28 cycles for CD74 and 32 cycles for HSPA10. The relative abundance of HSPA10 mRNA was tracked as the loading control. F. Quantitative real time PCR analysis of CD74 mRNA during VV infection. mRNA was isolated from PriessGAD cells infected with VV for 0-14 h and converted to cDNA for amplification with CD74 and HSPA10 specific primers for 40 cycles. Quantification of CD74 mRNA levels was determined relative to HSPA10 message levels. Data were averaged from three separate experiments.

The observed reduction in cellular Ii upon VV infection was not limited to PriessGAD cells. When other human B-LCL such as Raji and Frev were tested, similar decreases in Ii expression were observed (Fig. 11A). To further investigate whether Ii reduction is a general phenomenon observed in primary cells upon *in vitro* VV infection, Ii protein and mRNA levels were monitored in human PBMCs and murine splenic cells following VV infection. To purify these APCs from peripheral blood or tissues, T cells were depleted from the total cell population with either microbeads coated with Abs to CD3 (for human PBMCs) or CD4 plus CD8 (for murine splenic cells). Similar to what was observed in B-LCL, Ii expression in human primary APCs dropped remarkably at both the protein and mRNA level upon VV infection (Fig. 11B). However, in total murine splenic APCs, Ii protein levels remained unchanged upon VV infection (Fig. 11C to E). Several methods were used to isolate and treat the murine APCs including: isolating live cells using Ficoll or isolation of B cells using microbeads coated with B220 Abs (Fig. 11C) or pre-activating APCs with LPS, a Toll like receptor ligand, before infection with VV (Fig. 11E). Notably, Ii protein in murine APCs remained unchanged even following VV infection at the highest dose (i.e. MOI=10) and the longest time (i.e. 24 h) as shown in

Fig. 11D. UV irradiated VV was tested in parallel with VV in each of the studies with murine APCs and no alteration in Ii protein was observed (data not shown). The constant expression of surface and intracellular Ii in murine splenic APCs after incubation with VV or UV irradiated VV was also demonstrated by flow cytometric analysis (data not shown). While the half-life of human Ii is about 1-2 h [216, 217], the half-life of murine Ii is longer than 2 h [218]. The increased stability of murine Ii protein compared to human Ii may account for this discrepancy. Murine Cd74 (encoding Ii) mRNA, however, increased after LPS stimulation and was reduced upon VV infection as expected (Fig. 11E). Thus, it is likely over longer times, Ii protein expression would drop for these murine APCs.

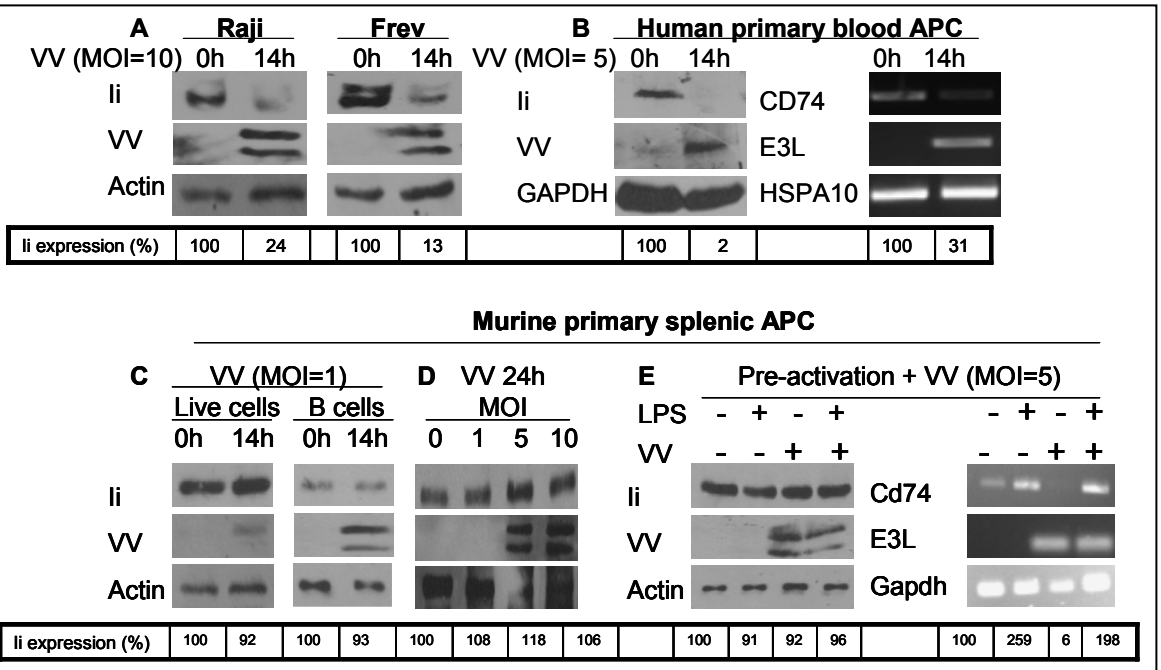


Fig. 11 Loss of Ii in B-LCL, human and murine primary APC with VV infection. A. Human B-LCL Raji or Frev cells were infected with VV (MOI=10) for 14 h prior to Western Blot analysis with Ii, VV and actin specific Abs. B. Reduction of Ii protein and mRNA in human primary APC post VV infection. PBMCs were depleted of T cells prior to infected with VV at MOI=5 for 14 h followed with Western Blot analysis (left column) or RT-PCR (right column). GAPDH and HSPA10 were used here as the loading controls for proteins and mRNAs respectively. C. Ii protein remained constant in VV infected total murine primary splenic APC with confirmed viability and B cells. Murine splenic cells were depleted of T cells then selected with ficoll reagent (left column) or microbeads coated with B220 Abs (right column) to isolate B cells followed with VV (MOI=1) infection and Western Blot analysis. D. The abundance of murine Ii protein did not change up to 24 h infection with VV. Splenic cells depleted of T cells were infected with VV up to MOI 10 for 24h before Western Blotting analysis. E. Ii protein remained constant while Cd74 mRNA altered in LPS pre-activated and VV infected splenic cells. Splenic cells without T cells were pre-incubated with LPS (1 μg/ml) for 24 h prior to addition of VV (MOI=5) for 14 h. Western blot analysis (left column) and RT-PCR analysis (right column) were performed for detecting Ii expression. Quantification data is listed for Ii expression (protein/mRNA) under corresponding panels. Abundance of Ii was normalized to loading controls, i.e. actin/GAPDH for protein or HSPA10/Gapdh for mRNA. Abundance of Ii in samples without treatment was set equal to 100%. Experiments were repeated for at least three times.

To examine whether Ii expression was regulated during *in vivo* VV infection, we assessed Ii expression in primary murine APCs isolated from animals that received i.p. VV. Following *in vivo* VV infection (2 d for all APC types or 24 h for DCs only), animal

spleens were harvested and different APC populations were isolated using Ab coated microbeads. Cd74 (encoding Ii) mRNA levels following infection were assessed using real time PCR. As shown in Fig. 12A, Cd74 mRNAs decreased in a time dependent manner in each of the APC populations. The most significant reduction in Cd74 mRNA occurred in B cells and DCs. Cd74 mRNA also diminished progressively when DCs were infected for a shorter time period (Fig. 12B). These studies are consistent with *in vitro* infection experiments suggesting viral infection decreases Ii expression at mRNA levels.

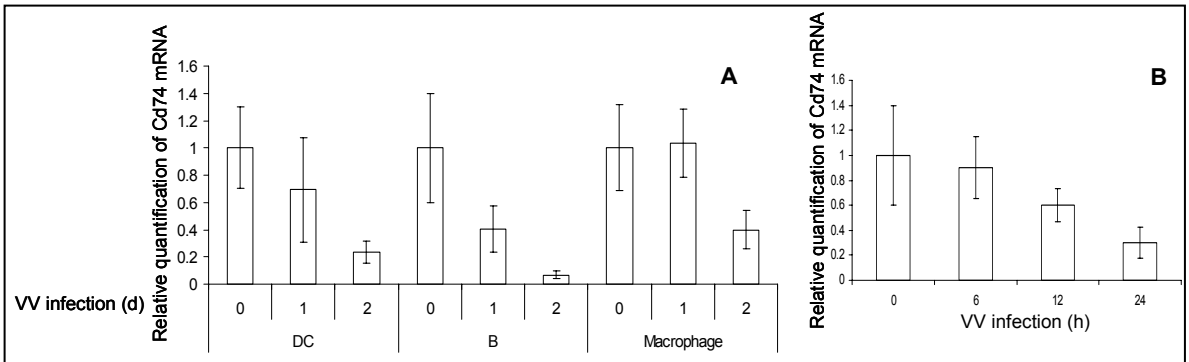
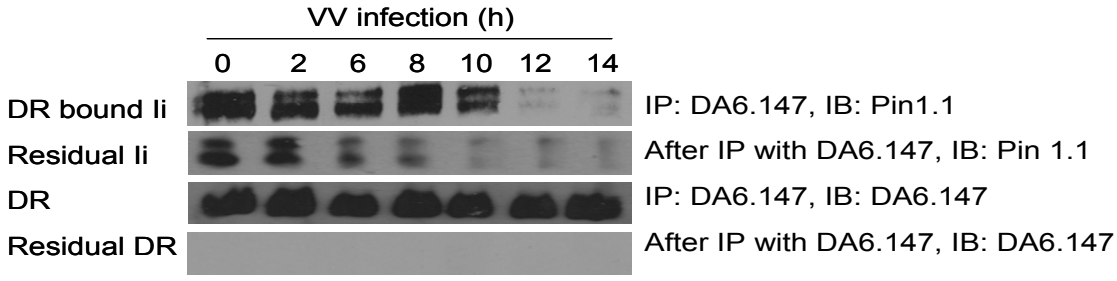


Fig. 12 Loss of Cd74 **mRNA upon *in vivo* infection of APC.** A. Progressive reduction of Cd74 mRNAs in different APC after *in vivo* VV infection. The infection of mice, the isolation of APC, the extraction of RNA, and the synthesis of cDNA were same as described in Fig 8C. Real time PCR was performed using murine Cd74 and Gapdh specific primers. B. Loss of Cd74 mRNA in DC after *in vivo* VV infection. Mouse infection, DC isolation and cDNA preparation were performed as in Fig. 8D. For A & B, relative quantification of Cd74 mRNA levels was normalized with Gapdh as the endogenous control. Cd74 in non-infected or 0 d/h sample of each APC population was used as the calibrator and set up as 1. All other time points were calculated compared with the calibrators. In each time point for each population, three mice were included. Data shown here are representatives from at least three separate experiments. Cell and mRNA samples for this study were prepared by Drs. Yongxue Yao and Ping Li.

The main function of Ii is to chaperone class II molecules along the antigen presentation pathway. Since total Ii protein levels decreased significantly upon VV infection of human APCs, the amount of functional Ii or class II DR associated Ii, was assessed using an immunoprecipitation assay. DR bound Ii, similar to total Ii, was remarkably reduced upon VV infection in a time dependent manner. Total class II DR protein, however, remained constant (Fig. 11). Reduced DR associated Ii protein following VV infection further suggested that diminished Ii might contribute to the disruption of class II function.

The residual Ii that existed in cell lysates after DR Ab pulldown also decreased in a time dependent manner post VV infection. This may represent Ii associated with class II DP or DQ molecules or free Ii. The decrease in this pool of Ii appeared to occur even more quickly than the loss of DR-Ii molecules. There was no residual DR protein detected after DR Ab pulldown confirming that the precipitation using the DR specific Ab was efficient (Fig. 13).



DR-Ii/DR	1	0.74	0.74	0.7	0.59	0.08	0.06
Residual Ii/DR	1	0.91	0.54	0.41	0.14	0.15	0.07

Fig. 13 Reduction of functional Ii levels upon VV infection. PriessGAD cells were infected with VV (MOI=10) for up to 14 h followed by immunoprecipitation (IP) with DR specific Ab-DA6.147. Ii associated with these Ab captured class II were detected by Western analysis. Residual Ii represents Ii proteins left in cell lysates after class II Ab pulldown. Cell lysates were resolved on SDS-PAGE and immunoblotted (IB) with Ii specific Ab Pin1.1 or DR specific Ab DA6.147. The quantification of DR bound Ii and residual Ii normalized to DR is list below the graph. 0 h samples were set equal to 1. Data shown here is representative of five individual experiments.

A previous study showed that some class II alleles aggregate in the endoplasmic reticulum of spleen cells from Ii deficient mice [35]. To investigate whether the reduced expression of Ii by VV could result in DR aggregation, PriessGAD cells were infected with VV (MOI=10) for up to 14 h. Following infection, DRα and DRαβ protein expression was assessed by Western analysis. If aggregation occurs, class II positive bands with larger molecule weights will aggregate near the top of SDS-PAGE gel. Under these conditions, we failed to detect aggregation of class II molecules. As shown in Fig. 14A, no aggregation was observed up to 14 h post VV infection. Other studies have shown that DM, another important factor along the class II pathway, is also chaperoned by Ii [35, 42, 43]. Since VV decreased Ii expression significantly, we tested if VV infection could also affect DM expression. To determine if DM protein levels change upon VV infection, PriessGAD cells were infected with VV (MOI=10) for 14 h followed

by Western analysis and flow cytometry. Both methods failed to detect any alteration of DM expression upon VV infection (Fig. 14B and C).

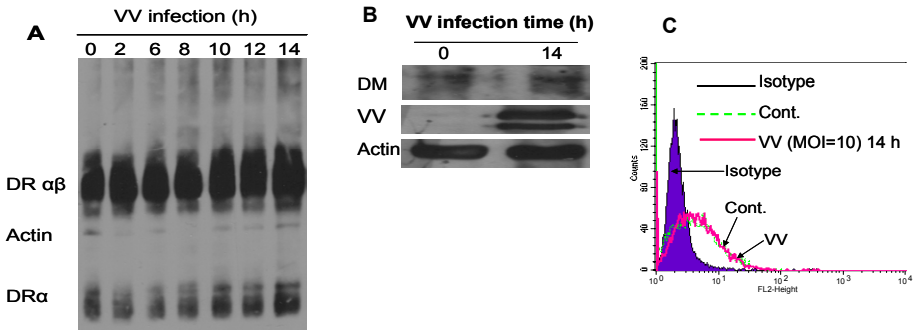


Fig. 14 No aggregation of class II DR or changes in the expression of DM upon VV infection. A. The expression of DR upon VV infection. PriessGAD cells were incubated with VV (MOI=10) for 0 to 14 h. Cells were harvested, lysed, samples mixed with non-reducing SDS buffer without boiling, and resolved on SDS-PAGE. After transfer to nitrocellulose membranes, the blots were probed with DRαβ Ab-L243, actin Ab and DRα Ab-DA6.147. DRαβ dimers of approximately 55-60 KD as well as DRα monomers (35 KD) were found without protein aggregation. B. DM protein expression upon VV infection. PriessGAD cells were cultured +/- VV (MOI=10) for 14 h, cells harvested, lysed and protein samples then immunoblotted using DM specific Ab. VV proteins were detected as evidence of infection. Actin was used as the loading control. C. Intracellular DM protein levels upon VV infection. PriessGAD cells were infected as in panel B. Cells were permeabilized with 0.05% saponin containing PBS for 15min. Permeabilized cells were then stained with Ab to DM or isotype control Abs for 30 m followed by the 2nd PE-conjugated rabbit anti-mouse Ig G at 1:1000 dilution in buffer containing 0.05% saponin. Finally flow cytometric analysis were performed. Dash line represented the non-infected cells while dark line represented infected cells. The shaded histogram represented isotype-matched irrelevant Ab staining of control or infected cells. Intracellular DM expression was unchanged after VV infection. At least five separate experiments were repeated for each panel.

Thus, *in vitro* VV infection reduced Ii mRNA and protein expression levels in human B-LCLs and PBMCs without inducing DR aggregation or reduction in DM protein levels. DR associated Ii also diminished upon VV infection. Both *in vitro* and *in vivo* VV infection led to Cd74 mRNA reduction in primary murine cells. Little loss of murine Ii protein was observed using primary murine cells. However there may be a decrease in Ii protein expression in a longer period. Other class II pathway components only decreased at the mRNA level during VV infection.

2.2 Inhibition of class II function and reduction of Ii protein expression by CHX treatment of APCs

VV inhibition of class II-mediated Ag presentation as well as Ii expression, led us to propose that this observed reduction in Ii synthesis may contribute in part to class II dysfunction. The protein synthesis inhibitor - CHX was used to further explore the relationship between Ii expression and class II function. CHX interrupts both the initiation and extension of *de novo* protein synthesis at the translational stage [107, 108]. Earlier studies with murine cells showed that CHX perturbs class II presentation of native Ags but not peptides [22, 44]. Thus, here using human cell lines, the effects of CHX on class II presentation of exogenous and endogenous Ags as well as peptides were tested. In addition, the effect of CHX on the expression of Ii and other components of the class II Ag presentation pathway was assessed. First, a [^{3}H]-labeled leucine and lysine incorporation assay was used to select the optimal concentration of CHX for treating cells. Priess or PriessGAD cells were labeled with [^{3}H] leucine and lysine in media with different concentrations of CHX. These studies suggested that as early as 6 h after CHX exposure, human B cell protein translation is markedly reduced. At longer times of treatment, CHX induced cytopathic effects hence our studies were limited to cells treated with CHX for 6 h. Cells were harvested after a 6 h incubation +/- CHX, and protein synthesis was quantified as the amount of [^{3}H] labeled amino acid in the cells. CHX decreased both Priess and PriessGAD cellular protein synthesis in a dose dependent manner (Fig. 15A). At a concentration of 10μg/ml, CHX efficiently decreased cellular protein synthesis with minimal effects on cell viability (>90% of cells were viable as detected by trypan blue). Thus 10 μg/ml CHX was used as a working concentration in the following biochemical and functional experiments. CHX treatment inhibited endogenous GAD Ag presentation in a time dependent manner (Fig. 15B). CHX treatment for 30 m reduced the presentation of endogenous GAD by about 45% compared to the 0 h group. To investigate whether CHX inhibition of the class II pathway is a general phenomenon, HSA Ag presentation by class II was investigated. Notably, presentation of the HSA peptide was more efficient than that of the full length protein, the latter requires increased processing before presentation. Yet, CHX disrupted both HSA peptide and protein

presentation significantly, with a higher dose of HSA peptide partially able to compensate for this inhibitory effect. The efficacy of CHX inhibition was greater for the HSA protein than for the HSA peptide possibly suggesting that protein translation is required for Ag processing as well as peptide association with class II (Fig. 15C). The presentation of another peptide-GAD was more resistant to CHX inhibition. As shown in Fig. 15D, at 1 μM of GAD peptide, only a 30% loss in class II function was observed in comparing Priess cells treated with CHX and a control group of cells. Again, CHX inhibition was diminished when the dose of GAD peptide was increased. These studies are similar to work with murine cells, except for the observed CHX sensitivity of peptide presentation by human B cells [22, 44]. The susceptibility of HSA peptide presentation to CHX inhibition may be due to a requirement for the HSA peptide to be internalized and processed by APCs in early endosomes before presentation by class II molecules [219]. Yet other peptides including GAD can directly bind to surface class II molecules independent of processing [220]. Studies using protein Ag suggest CHX inhibition of class II presentation was somewhat dependent on the source of Ag. Presentation of an abundant endogenous SMA, a mutated form of Ig κ, was not effected by treatment of cells with CHX (Fig. 15E). Similarly, class II presentation of a high affinity peptide, κ II, was also resistant to CHX exposure (Fig. 15F). These studies suggest a selectivity to CHX inhibition of class II function. Using peptides at high concentrations or using a high affinity ligand (such as κ II), the inhibitory effects of CHX can be overcome.

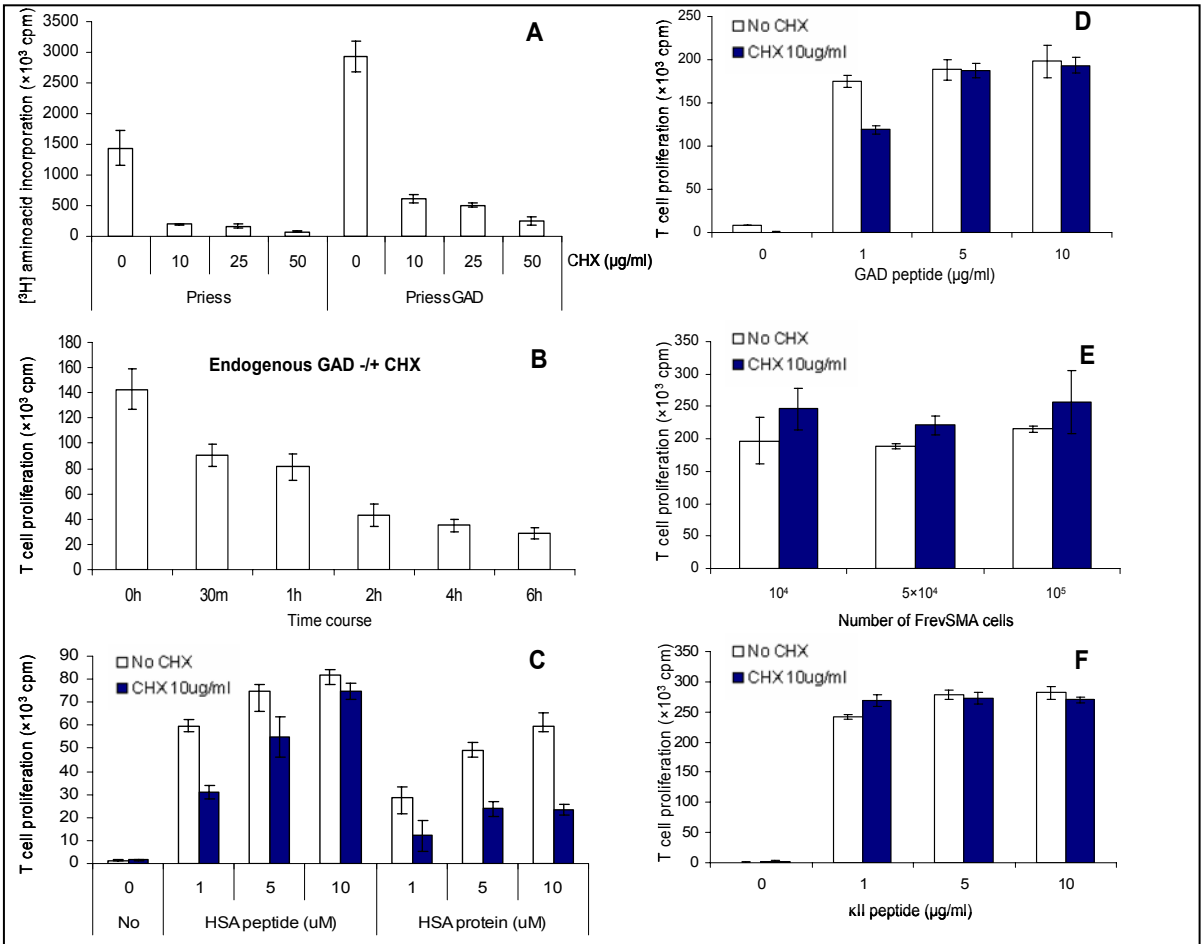
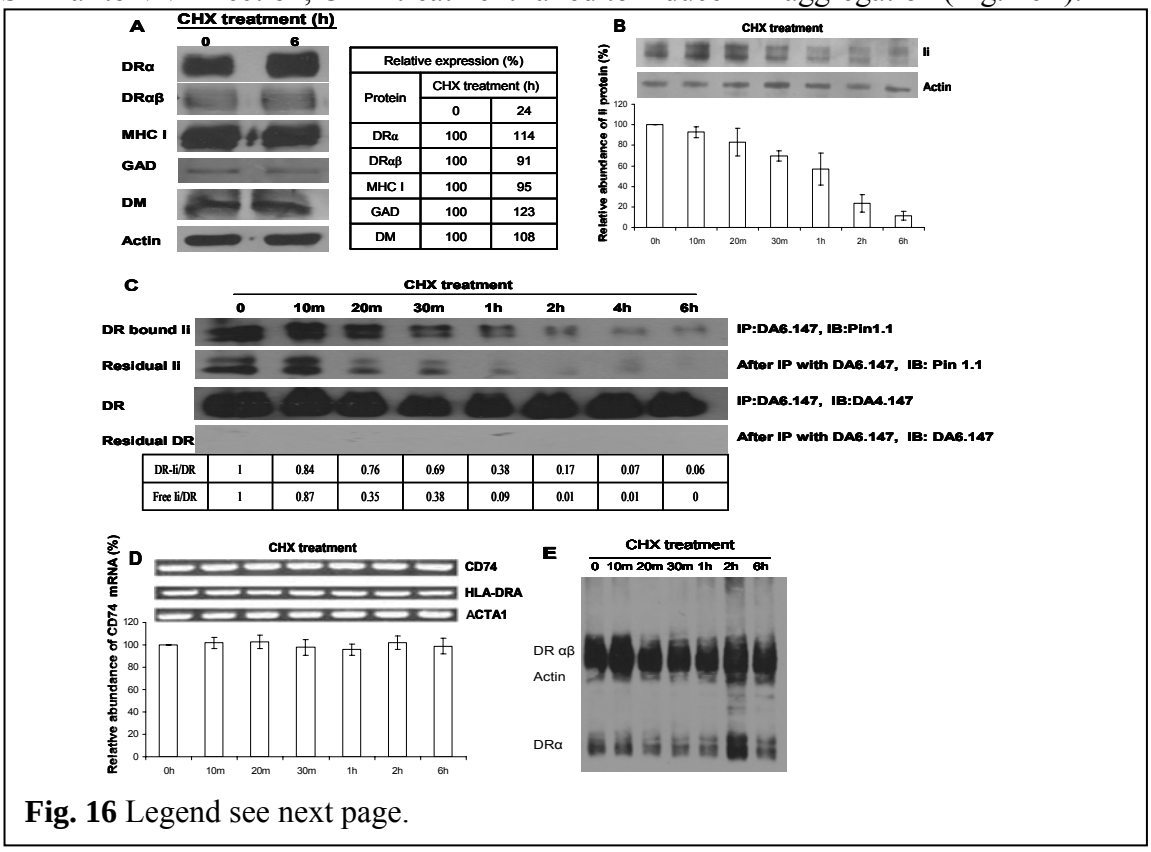


Fig. 15 Inhibition of class II-mediated Ag presentation by CHX. A. CHX decreased cellular protein synthesis in a dose dependent manner. Priess and PriessGAD cells were grown in L-lysine [4,5-^{3}H] and L-leucine [3,4,5-^{3}H] containing media with increasing concentrations of CHX for 6h. Cells were harvested, transferred to 96-well plates, these samples were frozen overnight prior to capture of the lysed cell material on filters for quantitative analysis of [^{3}H]-incorporation. B. The inhibitory effect of CHX on endogenous GAD Ag presentation. PriessGAD cells were incubated with 10 µg/ml CHX for 6 h, fixed with paraformaldehyde, then co-cultured with GAD specific T cells. T cell activation was measured as described in Materials and Methods. C. CHX treatment disrupted HSA epitope presentation. Priess cells were incubated with 0-10 µM HSA peptide or protein +/- CHX for 6 h prior to fixation, washing and the addition of HSA specific T cells. D. Inhibition of GAD peptide presentation by CHX. Priess cells were cultured with 0-10 µM GAD peptide +/- CHX for 6 h prior to fixation, washing and the addition of GAD specific T cells. E. The effect of CHX on endogenous SMA Ag presentation. Variable numbers of Frev/SMA cells which express SMA, a mutant form of Ig κ, were cultured +/- CHX for 6 h before fixation, washing and the Ig κ II specific T cell addition. F. The effect of CHX on Ig κ II peptide presentation. Frev cells were incubated with 0-10 µM Ig κ II peptide +/- CHX for 6 h before fixed, washed and co-cultured with Ig κ II specific T cells. Experiments were repeated at least three times.

After confirming functional inhibition of class II upon CHX treatment of APCs, the effects of CHX on the expression of key components from the class II pathway were addressed. As shown in Fig. 16A, expression of many cellular proteins remained constant up to 6 h following CHX treatment. Steady-state protein expression was unchanged by CHX treatment for molecules such as class II, class I, endogenous GAD Ag and DM. Maintenance of DM expression was also confirmed by flow cytometry (data not shown). Similar to VV infection, CHX treatment decreased Ii protein levels in a time dependent manner (Fig. 16B). After treatment with 10 μg/ml CHX, 70% of Ii protein remained at 30 m, 22% remained at 2 h and 10% remained at 6 h. This time course correlated with the decreased function of class II molecules (Fig. 15B). Not only total Ii, but also functional Ii (DR bound Ii) and residual Ii expression was reduced upon CHX treatment (Fig. 16C). Residual Ii may represent a free pool of Ii or Ii associated with class II DP and DQ molecules. Consistent with CHX inhibiting at the level of cellular translation, cellular mRNA for Ii did not change up to 6 h post CHX treatment. As shown in Fig. 16D, mRNAs for CD74, HLA-DRA and ACTA1 all remained constant in CHX treated cells. Similar to VV infection, CHX treatment failed to induce DR aggregation (Fig. 16E).



Relative expression (%)		
Protein	CHX treatment (h)	
	0	24
DRα	100	114
DRαβ	100	91
MHC I	100	95
GAD	100	123
DM	100	108

DR-Ii/DR	1	0.84	0.76	0.69	0.38	0.17	0.07	0.06
Free Ii/DR	1	0.87	0.35	0.38	0.09	0.01	0.01	0

Fig. 16 Legend see next page.

Fig. 16 CHX rapidly reduced cellular Ii protein levels without changing MHC class II expression. A. Class II as well as class I protein expression remained constant upon CHX treatment of B cell lines. PriessGAD cells were treated with CHX (10 μg/ml) for 6 h and lysed for Western blot analysis. Actin protein expression was used as a control for sample loading. Densitometric analysis of protein expression is listed beside the blots. Protein abundance at 6 h plus CHX was compared with 0 h samples using actin as the endogenous loading control. Data is representative of six experiments. B. CHX treatment of APC rapidly reduced Ii protein levels. PriessGAD cells were treated with CHX (10 μg/ml), followed by Western blot analysis using an Ii-specific Ab. The average abundance of Ii steady state protein relative to cellular actin after CHX treatment of B cells from three independent studies is shown. C. Reduction of functional Ii and residual Ii upon CHX treatment. PriessGAD cells were treated with CHX (10 μg/ml) for up to 6 h followed by immunoprecipitation (IP) with DR specific Ab-DA6.147. Residual Ii represents Ii protein left in the cell lysates after DA6.147 pulldown of class II. Cell lysates were resolved on SDS-PAGE, transferred to membranes and probed with Ii and DR Abs respectively. The quantification of DR bound Ii and residual Ii normalized with DR is listed below the graph. Samples from 0 h were set as 1. Data shown here are representative from five individual experiments. D. CHX treatment of B cells did not change Ii mRNA levels. PriessGAD cells were treated with CHX (10 μg/ml) for up to 6 h and analyzed by RT-PCR using CD74, HLA-DRA and ACTA1 specific primers. ACTA1 was used as the loading control. Data showed were from 28 cycles of amplification. Quantification of CD74 mRNA levels after CHX treatment (average from three RT-PCR experiments) is shown. E. DR protein expression after CHX treatment. PriessGAD cells were incubated with CHX (10 μg/ml) for up to 6 h. Cells were harvested, lysed, mixed with non-reducing SDS buffer without boiling, resolved on SDS-PAGE and transferred to membranes, and probed with DRαβ Ab-L243, actin Ab and DRα Ab-DA6.147. Representative of six experiments are shown.

Thus, CHX decreased class II function and Ii protein expression as had been observed following VV infection of human B cells. CHX appears to act quickly to halt host protein synthesis, possibly explaining why CHX more rapidly disrupts class II function and Ii expression. Like VV, CHX blocked Ag and peptide presentation, although some selectivity was observed. Thus, at high concentration of some peptides, the effects of CHX were overcome. High doses of peptide also overcome VV disruption of class II function. Several proteins required for the class II pathway remained constant upon CHX treatment, yet a loss of Ii protein expression after CHX treatment of cells, mirrored a loss in class II function. In addition, no aggregation of class II or reduction in DM expression was observed. Consistent with its activity at the translational level, CHX did not change host mRNA expression.

2.3 Comparing the effects of VV infection and CHX treatment on Ag presentation and Ii expression

Both VV and CHX negatively influenced class II function and Ii protein expression. Kinetically, the inhibition of endogenous GAD presentation was similar upon VV or CHX exposure of APCs (data from Fig. 3B *vs.* Fig. 15B were replotted in Fig. 17A to facilitate comparison). However, differences existed between the two agents. For example, CHX inhibited Ii expression more rapidly than VV did (Fig. 10A *vs*. Fig. 16B replotted in Fig. 17B). Also, only VV but not CHX reduced CD74 mRNA levels (Fig. 10E *vs*. Fig. 16D replotted in Fig. 17C).

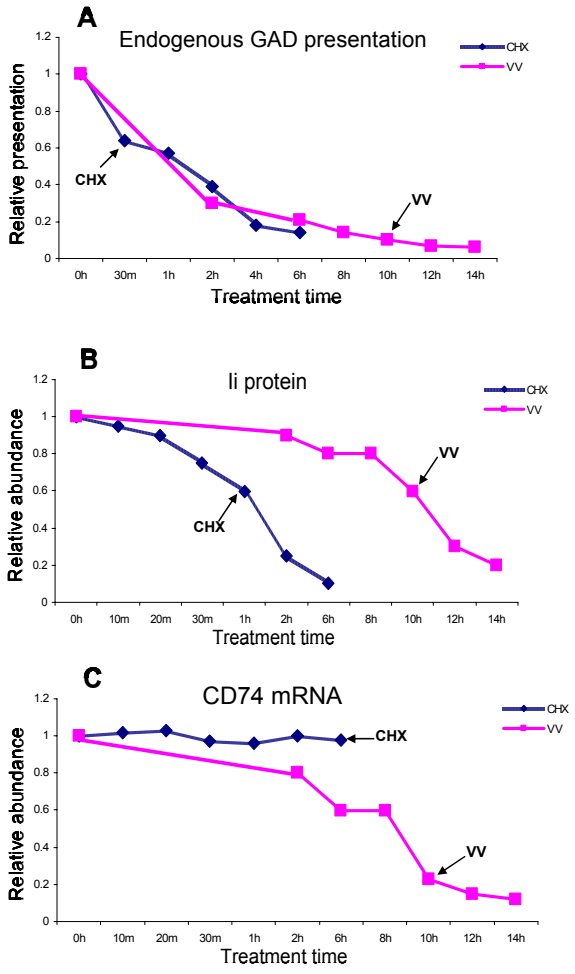


Fig. 17 Comparison of VV infection (MOI=10) and CHX (10 µg/ml) treatment of APC in functional assays of endogenous GAD presentation and Ii expression. A. Inhibition of class II presentation of endogenous GAD Ag. Data was replotted from Fig. 3B and Fig. 15B. B. Reduction in Ii protein expression. Data was replotted from immunoblotting studies from Fig. 10A and Fig. 16B using actin as an endogenous control. C. The effects of VV and CHX treatment on B cell CD74 mRNA. Data was replotted from Fig. 10E and Fig. 16D using endogenous controls ACTA1 (for CHX treated samples) or HSPA10 (for VV infected samples). For all figure panels, 0h samples were set equal 1. Subsequent samples were plotted relative to the 0 h samples.

To further compare the effects of VV and CHX on the presentation of Ags other than endogenous GAD, additional T cell assays were performed. Using HSA protein as shown

in Fig. 18A, exposure of Priess cells to either VV or CHX similarly reduced exogenous Ag presentation. Pulsing APCs with higher concentrations of HSA protein partially overcame the inhibitory effects of CHX and VV (data not shown). In contrast to VV infection, published studies have shown a reduction in class II mediated Ag presentation but not peptide presentation upon murine B cell exposure to CHX [22, 44]. However, in the current studies with human cells, CHX treatments diminished HSA peptide presentation to a similar extent to VV (Fig. 18B). GAD peptide presentation on the other hand, was more vulnerable to down modulation by VV infection compared with CHX treatment (Fig. 18C). Both the HSA protein and peptide must be processed prior to presentation by class II, this may account for their enhanced sensitivity to CHX treatment. By contrast as indicated above, GAD peptide can directly bind class II. This may explain the relative resistance of GAD peptide presentation to CHX treatment. VV consistently inhibited the presentation of both HSA and GAD peptides. Thus, while both CHX and VV disrupt MHC class II-mediated Ag presentation by B cell lines, differential effects of these agents on the presentation of some peptides were observed.

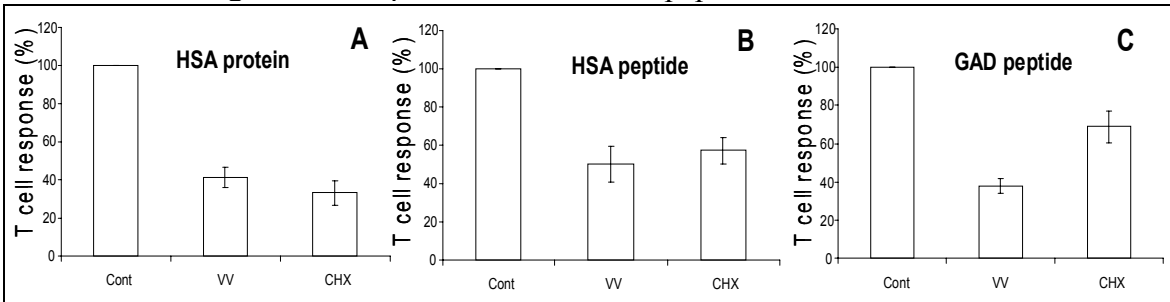


Fig. 18 Both VV infection and CHX treatment of human B-LCL decreased MHC class II presentation. A. VV or CHX treatment of B cells inhibited exogenous HSA Ag presentation. Priess cells incubated with HSA Ag (1μM) were treated with VV (MOI=10) for 14 h or CHX for 6 h prior to cell fixation with paraformaldehyde, then co-cultured with HSA-specific T cells. T cell activation was measured as described in Materials and Methods. B. VV or CHX treatment of B cells inhibited HSA peptide presentation. Priess cells were incubated with HSA peptide (1μM) and VV for 14 h or CHX for 6 h prior to fixation and analysis of T cell activation. C. VV or CHX treatment of B cells inhibited GAD peptide presentation. Priess cells were incubated with GAD peptide (1μM) and VV for 14 h or CHX for 6 h before cell fixation, and the addition of GAD-specific T cells. T cell activation was evaluated. For panel A-C, relative responses of T cell are shown for APC +/- treatment. For these studies, T cell activation for untreated or control samples were set to 100%. Data is representative of at least three separate experiments.

2.4 Alteration of Ii processing upon VV infection

The observed differences in the steady state abundance of Ii after VV or CHX treatment, suggested that host synthesis inhibition may not be the only factor influencing Ii levels in VV treated cells. The proteolytic cleavage of Ii upon transit to endosomal compartments also influence Ii steady state abundance in B cells. It has been established that some viral proteins, such as human immunodeficiency virus type 2 (HIV-2) and simian immunodeficiency virus virion-associated protein Vpx, can interact with Ii and reduce Ii expression levels by elevating Ii degradation [221]. Therefore, Ii degradation may also contribute to the loss of this class II chaperone in VV or CHX treated cells. To address this possibility, B-LCL were exposed to VV or CHX in the presences of various agents known to block Ii proteolysis. Drugs such as brefeldin A (BFA), monensin (Mon), leupeptin (Leu) and chloroquine (Chl) block Ii maturation and processing at different stages, therefore the accumulation of full length and Ii fragments can be assessed in cells treated with these agents by Western analysis. Specifically, BFA blocks protein transport from the ER to the Golgi complex by activating the GTPase Arf1p [222], and therefore inhibits Ii degradation completely [223]. Monensin is a carboxylic ionophore that traps proteins in the Golgi by disturbing Na^+ and K^+ exchange, thus Ii protein can not transit to endosomes like MIIC for degradation [26]. Leupeptin inhibits cysteine and serine proteases such as Cat B, Cat L, and Cat S competitively and reversibly in MIIC, consequently interrupting the terminal stages of Ii degradation and promoting the accumulation of leupeptin-induced peptide or LIP fragment [195, 224]. Chloroquine, a lysosomotropic drug, works as a weak base which is retained in acidic compartments, increasing the pH of endosomes and lysosomes [225, 226]. Thus chloroquine obstructs the early and late stages of Ii degradation by disrupting MIIC acidification [227].

PriessGAD cells were pretreated with VV (MOI=10) for 2 h followed by the addition of BFA (50 ng/ml), Leu (500 μM), Mon (1 μM) or Chl (0.3 mM), and further incubation for an additional 12h. Following these treatments, Western analysis was performed with the Ii N terminal specific mAb - Pin1.1. For these studies, cells were inoculated with VV prior to drug treatments to minimize any effect of these agents on virus infection. For CHX treatment, PriessGAD cells were incubated with the above drugs for 1 h followed

by CHX (10 μg/ml) treatment for an additional 4 h. The effects of VV and CHX on the accumulation of full length Ii and the fragment of LIP are shown in Fig. 19A and B respectively. As shown in Fig. 19A, VV treatment of cells decreased full length Ii levels to 46% of that observed with control cells. BFA, Leu, Mon and Chl cell treatments resulted in relative accumulation of full length Ii to 158%, 120%, 131% and 123% of control treated cells. Leu treatment of APCs as previously reported [195] resulted in Ii and LIP accumulation due to inhibition of endosomal-lysosomal proteases including Cat S. VV infection decreased the accumulation of full length Ii in BFA and Leu treated samples. For VV + BFA group, Ii levels decreased to 50% of BFA treatment alone; while for the VV + Leu group: Ii decreased to 60% of Leu treatment alone sample. These results suggest that VV did not radically affect the early steps (i.e. before LIP cleavage) of Ii processing. LIP degradation, however, increased dramatically at this later stage of VV infection, i.e. only about 10% LIP was detectable after PriessGAD cells were treated with Leu and VV compared to those treated with Leu alone. Thus, VV infection may influence the late steps (i.e. after LIP cleavage) of Ii processing. Contrary to the significant reduction in full length Ii accumulation after BFA and Leu treatment, virus infection had significantly less effects on Mon or Chl induced Ii accumulation, i.e. cellular Ii levels remained at 90% after Mon or Leu treatment of VV infected cells. A recent study showed in addition to fusion at the plasma membrane at neutral pH, VV also enters host cells through a low-pH endosomal pathway. Monensin (10 μM), working as an endosomal acidification inhibitor, decreases VV entry by more than 70% [78]. Since Chl also elevates pH in the lysosomal-endosomal pathway, we inferred Chl may also disrupt VV access. When PriessGAD cells were co-incubated with Mon or Chl, we observed lower infection efficacies (i.e. about 75%) compared with BFA or Leu treated cells (set equal to 100%). The ability of Mon to decrease VV infection correlated with a published report using the same working concentration (i.e. 1 μM) of Mon [78]. By contrast, when cells were treated with CHX plus these drugs, a less complicated situation was observed. CHX treatment of cells reduced Ii and the LIP fragment equivalently independent of cell exposure to BFA, Leu, Mon or Chl (Fig. 19B). The comparable reductions in full length Ii by CHX also suggest this drug was similarly able to block Ii synthesis even in the presence of BFA, Leu, Mon or Chl treatment.

To further study the role of proteolytic degradation in the loss of Ii and Ii LIP fragment in cells exposure to VV, studies were carried out focusing on the use of protease inhibitors. The reduced levels of LIP in Leu treated cells following VV exposure, raised questions as to whether complete inhibition of cellular cysteine proteases was achieved. Thus, an additional dose of Leu (i.e. 2×Leu) was added to cells during the incubation as well as the irreversible cysteine protease inhibitor-E64. As shown in Fig. 19C, VV infection decreased the accumulation of full length Ii by Leu (1×) to about 50% and cellular LIP levels to 10% in cells similar to Fig. 19A. For VV infected cells, addition of two doses of Leu at 2 and 8 h resulted in higher levels of LIP accumulation confirming that cysteine proteases catalyze the late stages of Ii processing in VV infected cells. The use of E64 also led to the accumulation of Ii LIP fragments in VV infected APCs. Together, these studies suggest cysteine proteases function in Ii turnover in control or VV infected cells. Yet, blocking of these enzymes required more potent inhibitors during VV infection. Studies have suggested that asparagine endopeptidase (AEP) is an acidic protease capable of catalyzing the earliest steps in Ii proteolysis [16]. The addition of an AEP inhibitor or AENK during VV infection of APCs, slowed Ii processing consistent with a role for AEP in Ii proteolysis and further suggested that the early stages of Ii processing were conserved during virus infection. To rule out any effect of these Ii processing inhibitors on protein synthesis, our [^{3}H]-amino acid incorporation assay was performed. For example, PriessGAD cells were cultured with BFA or Leu in the absence or presence of CHX. No significant effect on protein synthesis was observed in either BFA or Leu treated samples with or without CHX (Fig. 19D).

To investigate whether serine proteases or the cytoplasmic proteasome play a role in Ii processing, the irreversible serine protease inhibitor-phenylmethyl-sulphonylfluoride (PMSF) [228] or proteasome inhibitors-lactacystin (LAC) [229] and epoxomicin (EPO) [230] were tested for their effects on Ii steady state levels in cells infected with VV or cells treated with CHX. However, no change in Ii abundance was observed upon treatment with either of these inhibitors. LIP was not detected. VV and CHX treatment decreased Ii levels to about of 50-60% of non-treated samples in the presence or absence of these same inhibitors (Fig. 19E and F). The level of viral Ags detected in cells exposed

to these inhibitors was similar to cells exposed to VV alone, suggesting these compounds did not diminish virus infection.

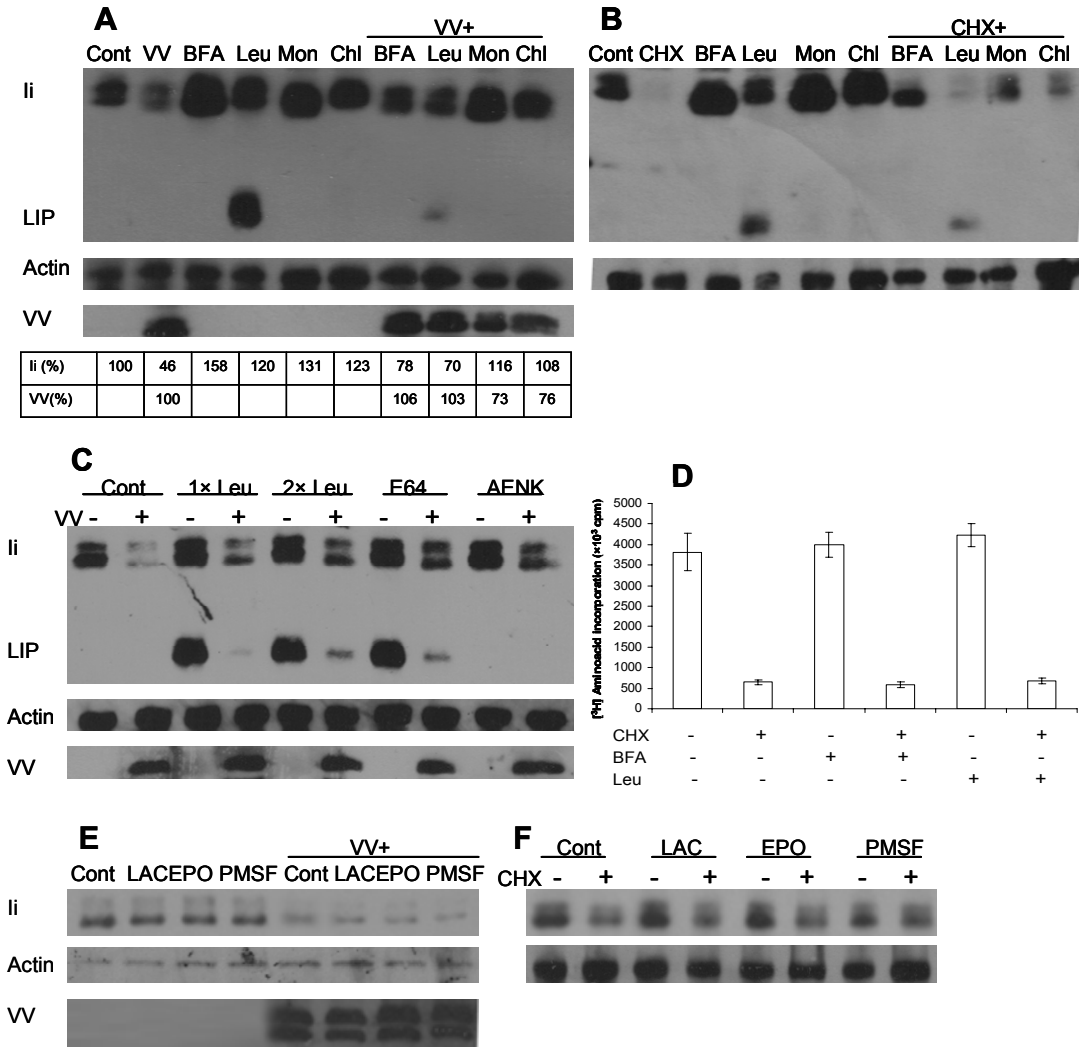
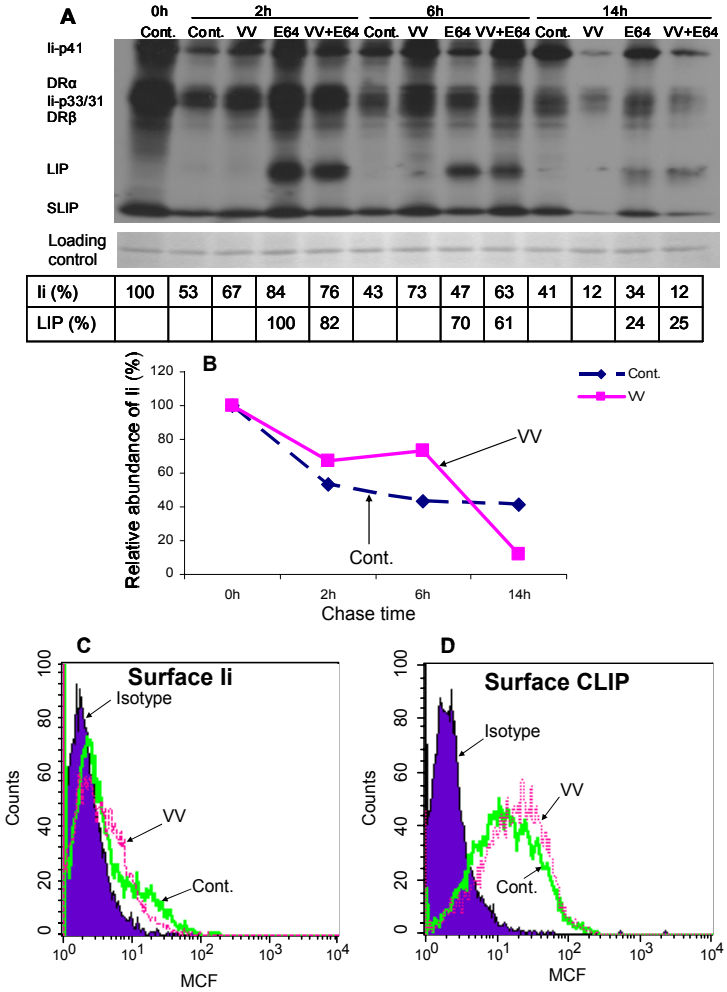


Fig. 19 Ii abundance and degradation in cells exposed to VV or CHX. A. Ii abundance and LIP accumulation in VV infected B cells. PriessGAD cells were incubated +/- VV (MOI=10) for 2 h, then brefeldin A (BFA, 50 ng/ml), leupeptin (Leu, 500 μM), monensin (Mon, 1 μM) or chloroquine (Chl, 0.3 mM) were added to cells for an additional 12 h. Cells were harvested and analyzed by Western analysis using an Ii-specific mAb. VV proteins in infected cells were monitored using a VV specific polyclonal Ab. Actin expression levels were used as the sample loading control. Full length Ii (31-33 KD) and LIP fragment (21 KD) were detected with the PIN 1.1 Ab. Relative abundance of Ii and VV normalized to actin is listed below the blots. Control (no treatment) and VV infected alone samples were set equal to 100% for Ii and VV respectively. B. Ii and LIP abundance in B cells exposed to CHX. PriessGAD cells were incubated with BFA, Leu, Mon or Chl for 1 h then 10 μg/ml CHX was added for an additional 4 h followed by Western analysis for Ii or actin.

Fig. 19 (Cont') C. Effect of cysteine proteases and AEP on Ii proteolysis within VV infection. PriessGAD cells were incubated +/- VV (MOI=10) for 2 h, then Leu (500 μM), E64 (500 μM) and AENK (1 mg/ml) were added for an additional 12 h. For 2×Leu samples, an additional dose of Leu was added to cells after 8 h incubation to ensure the active form of this inhibitor was present during the extended culturing. Cells were harvested and analyzed by Western Blotting. D. Effect of BFA and Leu on host protein synthesis. PriessGAD cells were co-incubated with BFA (50 ng/ml) or Leu (500 μM) +/- CHX (10 μg/ml) in media containing [^{3}H]-amino acid for 6 h prior to harvesting and quantitative analysis of [^{3}H]-amino acid incorporation. E. Effects of inhibitors of serine proteases and the proteasome on Ii abundance during VV infection. Phenylmethyl-sulphonylfluoride (PMSF, 50 μM), lactacystin (LAC, 250 nM) and epoxomicin (EPO, 200 nM) were added to PriessGAD cells which had been previously infected with VV for 2 h. After an additional 12 h incubation, cells were harvested for immunoblotting. F. Effect of inhibitors of serine proteases and the proteasome on Ii abundance during CHX treatment. PriessGAD cells were incubated with LAC, EPO or PMSF for 1 h followed by the addition of CHX for an additional 4 h prior to Western analysis for Ii or actin. Data is representative of at least three separate experiments.

To monitor the degradation of Ii, we employed pulse-chase analysis with [^{35}S]-methionine. Here, the proteolysis of Ii was analyzed following VV infection and culturing +/- E64. As shown in Fig. 20A and B, the levels of full length Ii dropped to 53%, 43% and 41% after 2 h, 6 h and 14 h periods respectively. At 2 h and 6 h VV infection appeared to slow Ii degradation as detected by the abundance of radio-labeled Ii while increased Ii degradation was observed at 14 h. This result was noted in several studies by the change in total Ii abundance for control and VV treated samples. E64 blocked Ii degradation and induced LIP production as expected in uninfected cells. VV infection of cells treated with E64 at 2 h and 6 h showed LIP levels less than cells treated with E64 alone. By 14 h, following VV and E64 exposure, Ii levels were greatly reduced. Equal loading of samples from immunoprecipitates was demonstrated by Coomassie Blue staining of gels prior to drying. The increased degradation of Ii at later stages of VV infection was verified by flow cytometric analysis. Using an Ii C-terminal specific Ab-LN2 which detects Ii but not LIP, it was observed that surface full length Ii levels were reduced after 14 h VV infection of PriessGAD cells (Fig. 20C). While the majority of Ii is found within cells, low levels of Ii transit to the cell surface as shown in Fig. 20C. A terminal fragment of Ii processing is called CLIP. Flow cytometry and Ab staining for CLIP revealed slightly enhanced levels of this peptide upon VV infection (Fig. 20D).

These results were consistent with enhanced degradation of Ii during the later stages of VV infection.



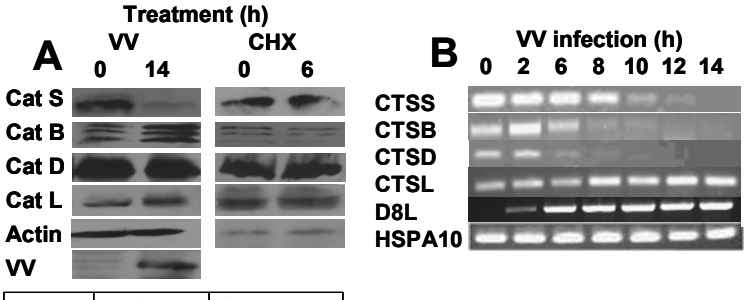
Ii (%)	100	53	67	84	76	43	73	47	63	41	12	34	12
LIP (%)				100	82			70	61			24	25

Fig. 20 Ii processing upon VV infection. A. Proteolysis of Ii upon VV and E64 treatment of B cells. PriessGAD cells pulse radio-labeled with [^{35}S]-methionine were cultured in media with excess unlabeled methionine +/- VV and E64 for 0, 2, 6, 14 h. Cell lysates were immunoprecipitated with Pin 1.1 and the precipitated proteins resolved on SDS-PAGE before Coomassie Blue staining and autoradiography. Relative abundance of Ii and LIP fragments is listed below. 0 h and 2 h E64 alone samples were set as 100% for Ii and LIP respectively. B. Degradation of Ii +/-VV. Data was replotted from the quantification analysis of Ii bands in panel A. C. Decreased surface Ii expression upon VV infection. PriessGAD cells were infected +/- VV (MOI=10) for 14 h before staining with FITC-LN2 Ab followed by flow cytometry. D. Enhancement of surface CLIP levels upon VV infection. PriessGAD cells were incubated +/- VV for 14 h before staining cells with FITC-CLIP-Ab followed by flow cytometric analysis. For panels C and D: The shaded histogram, dark line and dash line represented isotype control, non-infected and infected cells respectively. Experiments were repeated at least five times.

Hence, VV infection appears to alter Ii processing. Our result that an additional dose of Leu or the irreversible inhibitor E64 was more potent in blocking LIP degradation suggest that a cysteine protease may be induced or elevated to enhance Ii proteolytic processing during late stages of VV infection.

2.5 VV infection changed cellular lysosomal protease expression at both the protein and mRNA levels

The changes in Ii degradation during virus infection (Fig. 19 & 20), prompted us to look at the expression of cathepsins required for Ii and Ag proteolysis. Cathepsins are acidic proteases found in the endosomal-lysosomal network, important for processing both Ii and Ag [195, 231]. Among the cathepsins tested, Cat S plays a key role in the later stages of Ii cleavage in B cells [18, 32]. Cat L mediates Ii processing in cortical thymic epithelial cells to regulate positive selection [17, 232]. Cat B and D, on the other hand, take a more important role in processing Ag but not Ii in B cells [231, 233]. As shown by immunoblotting in Fig. 21A, Cat S levels were greatly reduced, Cat B and L increased and Cat D protein levels remained constant after 14 h VV infection. CHX, in contrast with VV, did not change the expression of these cathepsins. These results correlated with our previous data that only VV but not CHX accelerated LIP degradation (Fig. 19A and B). To determine if the same trend occurs at the mRNA level, RT-PCR was performed using specific primers. The alterations in CTSS and CTSL (encoding Cat S and L respectively) mRNAs were similar to that observed for protein expression, i.e. Cat S mRNA decreased while Cat L mRNA increased during VV infection in a time dependent manner (Fig. 21B). The mRNAs for CTSB and CTSD (encoding Cat B and D respectively), however, both diminished progressively upon VV infection. The discrepancy between mRNA and protein expression levels for Cat B and D upon VV infection may be due to the relative stability of these proteins even during VV infection. Alterations in cathepsins at both the protein and mRNA levels may lead to aberrant Ii and Ag processing. Specifically, reduction in Cat S may lead to the decreased Ii degradation during the early to mediate stages of infection. The increased Cat L, on the other hand, may accelerate Ii degradation during later stages of infection (Fig. 20A and B).



Protein (%)	VV infection (h)		CHX treatment (h)	
	0	14	0	6
Cat S	100	16	100	93
Cat B	100	145	100	91
Cat D	100	90	100	101
Cat L	100	151	100	102

Fig. 21 VV infection altered protein and mRNA expression of cellular lysosomal proteases. A. Protein expression of host cathepsins after VV infection or CHX treatment. PriessGAD cells were treated with VV (MOI=10) (left column) or CHX (10 μg/ml) (right column) and harvested for analysis by Western analysis using cathepsin specific Abs. VV proteins were detected to confirm viral infection. Actin expression levels were used as the sample loading control. Relative abundance of each cathepsin normalized to actin is listed below the blots. No treated control samples were set equal to 100%. B. Alteration in cellular cathepsin mRNAs upon VV infection. PriessGAD cells were infected with VV (MOI=10) for up to 14 h. mRNAs were isolated for semi-quantitative RT-PCR using primers for cathepsins, HSPA10 (encoding HSC70), and the viral late gene D8L. Analysis was carried out for multiple cycles of amplification as indicated: CTSS (32); CTSB (32); CTSD (32); CTSL (40); HSPA10 (32); and D8L (28). Viral D8L gene transcripts were monitored to confirm virus gene expression. Host HSPA10 expression was maintained during infection. Data is representative of at least three experiments.

The increase in Cat L and the decrease in Cat S upon VV infection were observed at both the mRNA and protein levels (Fig. 21). At the same time, LIP degradation was enhanced during the late stages of VV infection (Fig. 19 and 20). We proposed that the increased levels of cellular Cat L during virus infection could substitute for Cat S to drive the terminal stages of Ii processing. The existence of Cat L in B cells is controversial. A recent study confirmed Cat L expression in some human B cell lines, while others failed to detect this protease [18, 31, 234-238]. Thus, additional studies were carried out to confirm Cat L expression in B cells with or without VV infection. CTSL specific primers were used to amplify mRNA from PriessGAD B cells as cDNA and sequenced. The sequencing results and nucleotide blast software analysis confirmed the presence of human CTSL mRNA in B cells (Fig. 22A). Using the ClustalW software, the most

abundant amplified mRNA sequence in virally infected cells was also identified as human CTSL. The predicted proteins derived from these cDNA sequences were further validated as human Cat L. Taken with the Western analysis results using Cat L specific Ab (Fig. 21A), we concluded Cat L was expressed in human B-LCL and its abundance was enhanced upon VV infection. Moreover, in another human B-LCL, Frev, Cat L protein was also detected although it was slightly less abundant than in PriessGAD cells. Cat L protein increased after VV infection of Frev cells (Fig. 22B). Furthermore, when murine B cell tumors were tested, Cat L protein increased in both TA3 and 1153 cells during VV infection. Similar to results with PriessGAD cells, Cat D protein levels remained constant up to 14 h of VV infection (Fig. 22C). The absolute mRNA levels of Cat L or Ctsl in those murine cell lines appeared to either drop a bit (as in 1153) or did not change (as in TA3). However, when normalized with Gapdh as the endogenous control, Ctsl mRNAs either remained constant or increased in 1153 and TA3 respectively (Fig. 22D). CTSL mRNA levels in primary cells-human PBMCs were also measured upon *in vitro* VV infection. As shown in Fig. 22E, CTSL mRNA remained constant in these cells upon VV infection up to 14 h.

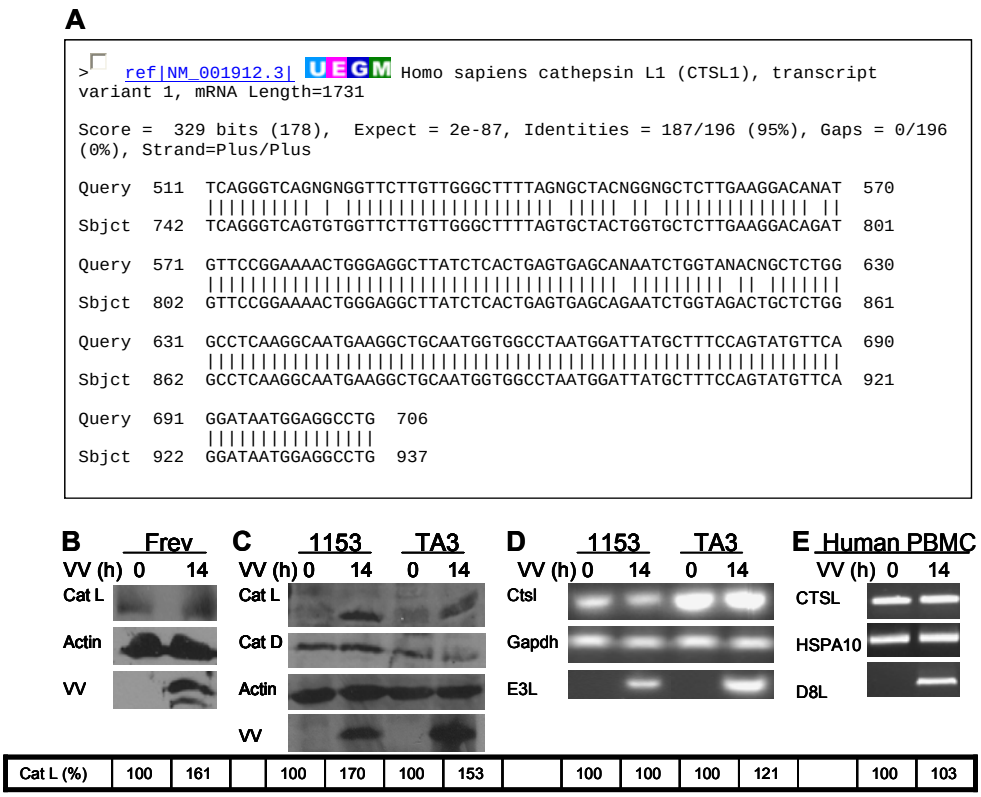


Fig. 22 Cat L expression in human B-LCL, murine B cell tumors and human primary PBMCs upon *in vitro* VV infection. A. Nucleotide blast of amplified mRNA from PriessGAD cells using CTSL specific primers. RNA were extracted, converted to cDNA, amplified with CTSL specific primers and sequenced. The nucleotide blast results confirmed the presence of human CTSL mRNA in human B cells. B. Enhanced Cat L protein levels in human B-LCL upon exposure to VV. Frev cells were infected with VV (MOI=10) for 14 h before Western analysis. C. Increased Cat L protein expression in murine B cell tumors during VV infection. 1153 and TA3 cells were incubated with VV (MOI=10) for 14 h prior to Western Blotting assays. For panels B and C, actin was used as the loading control. VV protein expression was probed as evidence of infection. D. Ctsl mRNA expression in murine B cell tumors +/- VV. Cells were infected as in panel C before RT-PCR analysis. Gapdh was used as the endogenous control. Early viral gene E3L was used as a marker of infection. E. CTSL mRNA levels in human PBMCs+/-VV. PBMCs were depleted of T cells then infected with VV (MOI=5) for 14 h prior to RT-PCR analysis. HSPA10 remained constant while viral late gene D8L expression increased. For panels B-E, quantification data for Cat L expression normalized to loading controls is listed below corresponding data. 0 h samples were set as 100%.

The expression of Ctsl mRNA in APCs upon *in vivo* VV infection was also studied. Here, C57BL/6 mice were i.p. infected with VV (10^6 pfu/mouse) up to 2 d before harvesting of spleens and the isolation of different APC populations. Ctsl mRNA was amplified from

each individual type of APCs using RT-PCR. As shown in Fig. 23A and C, Ctsl mRNA increased in a time dependent manner in DCs and macrophages. This enhancement was also apparent in a shorter course of VV infection using DCs (Fig. 23D). In primary B cells, Ctsl mRNA abundance was lower than in the other populations and seemed not to increase during infection (Fig. 23B).

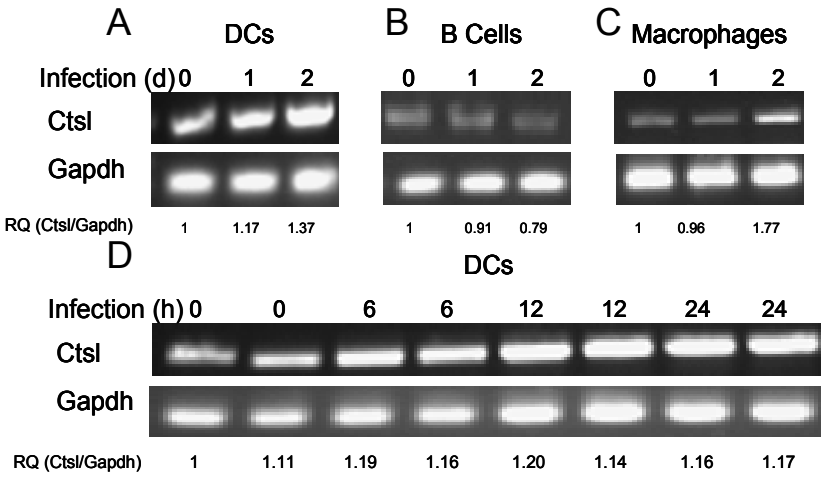


Fig. 23 Enhancement of Ctsl **mRNA abundance upon *in vivo* infection of APCs.** A. Progressive increases in Ctsl mRNA was observed with DCs upon *in vivo* VV infection. C57BL/6 mice were infected before harvesting of spleens and the isolation of DCs as described in Fig. 8C. RT-PCR was performed with specific primers. B. Ctsl mRNA in B cells detected after *in vivo* VV infection. B cell mRNA was isolated before RT-PCR was performed. C. Enhanced Ctsl mRNA levels in macrophages during *in vivo* VV infection. Macrophage mRNA was isolated prior to RT-PCR analysis. D. Ctsl mRNA abundance in DCs up to 24 h of *in vivo* VV infection. DCs were isolated as in panel A. mRNA samples from two mice for each time point were amplified and analyzed side by side. For panels A-D, relative quantification (RQ) for each sample is listed below corresponding data. RQ of Ctsl mRNA levels was normalized to Gapdh as the endogenous control. Ctsl mRNA from non-infected or 0 d/h sample for each APC population was set as 1. All other time points were calculated compared with the non-infected sample. In each time point for panels A-C mRNA was pooled from three mice.

Beside cathepsins, the expression of other enzymes contributing to Ag and Ii proteolysis was also investigated in cells exposed to VV. The protease, AEP not only initiates Ii proteolysis but also participates in Ag processing [31]. A significant reduction in LGMN (encoding AEP) mRNA was found during VV infection as shown in Fig. 24. mRNA levels for the early endosome marker-early endosome antigen 1 (EEA1) [239], however, did not change upon VV infection. EEA1 functions in endosome fusion by linking

phosphatidyl-inositol-3-OH kinase (PI(3)K) and the small GTPase Rab5. EEA1 may also help target Rab5-dependent endocytic transport [240]. GILT is required for processing Ags containing disulfide bonds [15]. Using a GILT specific Ab, it was observed that GILT protein levels remained constant up to 14 h of VV infection or 6 h CHX treatment (data not shown).

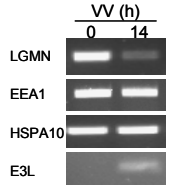


Fig. 24 Expression of LGMN and EEA1 mRNA upon VV infection. cDNAs were amplified from 0 and 14 h VV infected PriessGAD cells by RT-PCR using specific primers. HSPA10 was used as the loading control. Early viral gene E3L was detected confirming VV infection. Experiments were repeated three times.

These studies indicate cellular Cat S decreased while Cat L increased at both protein and mRNA levels upon *in vitro* VV infection of human and murine B cells. *In vivo* VV infection also resulted in enhanced Cat L expression in APCs. Reduction in the levels of mRNAs for Cat B, D and AEP upon VV infection may also result in abnormal Ii as well as Ag processing and thus contribute to defects in class II protein function and Ag presentation. CHX, on the other hand, did not change expression of these enzymes. Yet, the disruption of Ii synthesis during CHX treatment of cells may contribute to the defect in class II presentation.

2.6 Preservation of class II function by over-expression of Ii upon VV infection

Loss of Ii expression could in part contribute to class II dysfunction, thus we proposed enhancement of Ii expression in cells may reduce the inhibition of class II presentation observed with VV infection. Three methods to increase intracellular Ii levels were tried, i.e. 1) cell fusion to increase the Ii gene copy number; 2) transfection with an Ii-encoding plasmid; and 3) using VV as a recombinant vector to express the Ii gene. The results of these studies are presented here.

2.6.1 Enhancement of cellular Ii expression partially preserved class II function during VV infection of cells

Virus induced loss of Ii expression was most apparent in human B lymphoblasts, thus initial studies focused on these cells. Current human or murine B cells lacking Ii are not available, yet cell fusion can result in cell lines expressing multiple copies of Ii encoding genes. T2DR4DM cells were generated previously [185-187] by fusing human B and T lymphoblast cell lines *in vitro*. T2DR4DM cells express greater levels of Ii protein than a B-LCL 7C3DR4 (Fig. 25C). As shown in Fig. 25A, both T2DR4DM cells and 7C3DR4 cells presented the GAD peptide to T cells specific for this peptide and class II DR4. VV infection at MOI 10 disrupted GAD presentation in both cell lines. As in our prior studies, viral inhibition of class II function could be partially compensated by adding higher peptide concentrations to these assays. The perturbation of GAD presentation, however, was much less severe with T2DR4DM cells which express high levels of Ii compared with 7C3DR4 cells which express wild type levels of Ii. Using 1 μM GAD, VV infection inhibited GAD presentation 60% in 7C3DR4 cells while blocking only 20% of GAD presentation by T2DR4DM cells (Fig. 25B). Similar to our prior results with PriessGAD cells (Fig. 10A), alterations in cellular Ii after 6 h of VV infection were not observed. The over-expression of Ii in T2DR4DM cells compared with 7C3DR4 cells was confirmed by Western analysis using actin as the loading control. Cellular infection by VV was confirmed by probing these blots with a VV specific Ab (Fig. 25C). Levels of viral protein expression in each cell line were similar. This was important in confirming similar amounts of virus likely entered each cell line during the infection period.

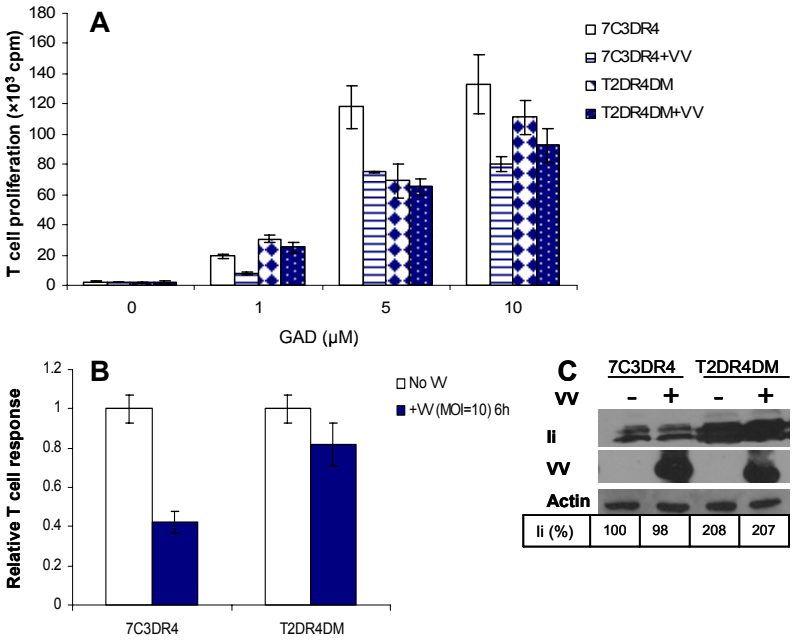


Fig. 25 Over-expression of Ii partially preserved GAD peptide presentation in human lymphoblasts upon VV infection. A. Differential inhibition of GAD peptide presentation by VV using lymphoblasts with high and wild type Ii expression. 7C3DR4 cells and T2DR4DM cells were co-incubated with GAD peptide (0-10 μM) and in some samples VV (MOI=10) was added for 6 h before fixation and GAD-specific T cell addition. T cell activation was then assessed. B. Relative T cell responses to different human lymphoblasts co-incubated with GAD and VV. 7C3DR4 cells and T2DR4DM cells were co-cultured with 1 μM GAD peptide +/- VV (MOI=10) for 6 h before fixation and T cell addition. Relative T cell proliferation was normalized to the samples with no VV, the latter were set to 1. C. Ii protein expression in lymphoblasts. 7C3DR4 cells and T2DR4DM cells were infected with VV (MOI=10) for 6 h before Western Blotting analysis. Ii, actin and VV proteins were probed with corresponding specific Abs. Relative abundance of Ii normalized to actin is listed below the data. 7C3DR4 sample without infection was set as 100%.

VV also disrupts class II mediated Ag presentation in non-professional APCs [97]. To construct comparable cell lines that differ only in Ii expression, M1DR4, a human fibroblastic cell line expressing class II DR4 molecules was transfected with a plasmid containing the human genes for Ii p31 and p33 proteins to generate M1DR4Ii cells. The heterologous expression of Ii genes was driven by a RSV promoter. The importance of Ii in chaperoning of class II was suggested here by the higher expression of surface class II DR4 molecules in M1DR4Ii cells compared with M1DR4 cells. About 60% of M1DR4 cells and 80% of M1DR4Ii cells expressed high levels of surface DR4 as detected by flow cytometry after cell selection for a co-transfected plasmid with a drug resistance

marker (data not shown). For functional assays, GAD peptide presentation +/- VV was tested. Here, M1DR4 cells and M1DR4Ii cells were co-incubated with GAD peptide (0-10 μM) +/- VV (MOI 0-10) for 6 h. As shown in Fig. 26A, both cell lines could present GAD peptide although M1DR4Ii appeared to be more effective and could induce higher T cell responses. Increased addition of GAD peptide to each cell type led to higher presentation and T cell activation. VV inhibited GAD peptide presentation in a dose dependent manner in both cell lines. However, viral inhibition of M1DR4 cells was greater than that observed for M1DR4Ii cells. For example, a greater than 75% decrease in GAD (5 μM) presentation was detected upon VV (MOI=10) infection of M1DR4 cells. Yet under the same conditions, a roughly 40% decrease in GAD presentation was found in M1DR4Ii cells exposed to virus. When the T cell responses to 10 μM GAD peptide in the presence of VV (MOI = 0-10) were compared, a similar trend was noted. I.e. at same concentration of virus, M1DR4Ii cells appeared consistently better as APCs during VV exposure (Fig. 26B). However, Ii expression only partially preserved class II-mediated Ag presentation, suggesting other mechanisms may contribute to VV-induced class II dysfunction. Ii was only expressed in M1DR4Ii cells but not in M1DR4 cells (Fig. 26C). Ii levels did not change at 6 h infection with VV at MOI 10. The expression of viral proteins in each cell line was comparable, suggesting similar levels of virus entry and gene expression for M1DR4 and M1DR4Ii cells.

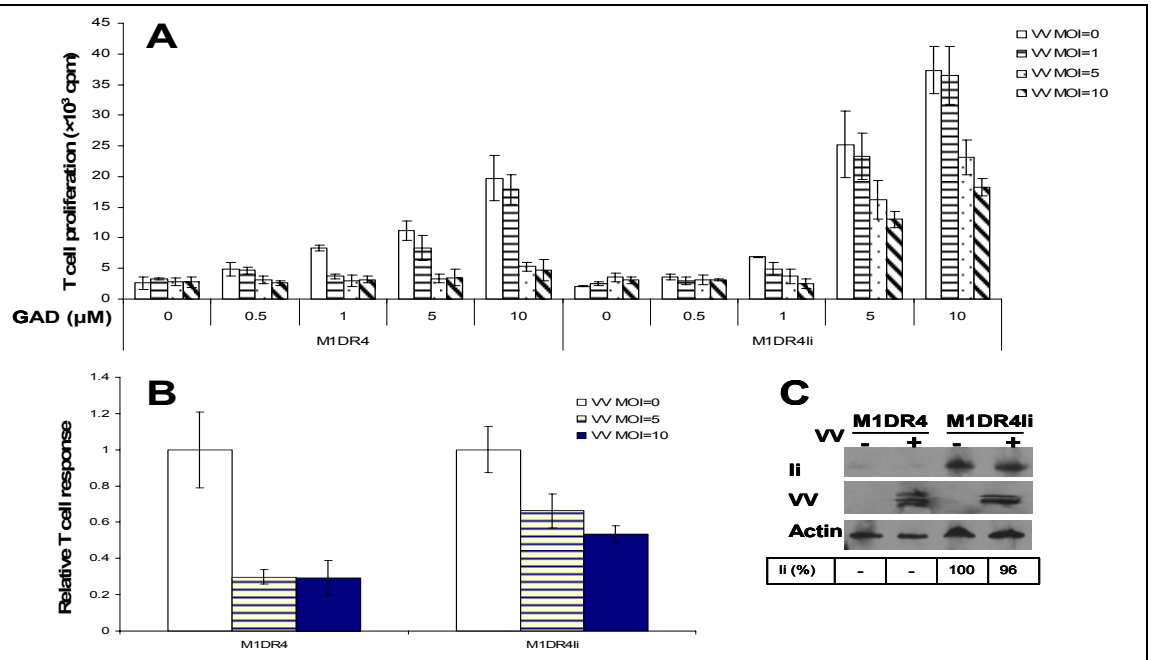


Fig. 26 VV disruption of class II function was greater in a human fibroblast cell line lacking Ii. A. GAD peptide presentation upon VV infection of M1DR4 cells and M1DR4Ii cells. M1DR4 cells were transfected with human Ii genes (CD74) yielding M1DR4Ii cells. Peptide presentation to T cells was tested using each of these cell lines pulsed with GAD peptide (0-10 μM) +/- VV (MOI up to 10) for 6 h. B. Relative T cell responses to human fibroblast cells co-cultured with GAD and VV. M1DR4 cells and M1DR4Ii cells were incubated with 10 μM GAD peptide +/- VV (MOI up to 10) for 6 h before fixation and GAD specific T cell addition. Relative T cell proliferation was normalized to the VV (MOI=0) sample, with these uninfected cells being set to 1. C. Ii protein expression in different fibroblast cells. M1DR4 and M1DR4Ii cells were infected with VV (MOI=10) for 6 h prior to Western analysis using specific Abs. VV protein levels was probed as evidence of infection. Actin was used as the loading control. Relative abundance of Ii normalized to actin is listed below the data. M1DR4Ii sample without infection was set as 100%. Data is representative of at least three experiments.

To test if class II function could be preserved by enhancing intracellular Ii expression in cells from other species upon VV infection, murine fibroblast cells +/- Ii were used as a model. L1DR4 are murine fibroblast cells transduced with the human class II molecule DR4. L1DR4 cells were co-transfected plasmids encoding human Ii p31 and p33 forms to yield L1DR4Ii cells. GAD peptide presentation, by L1DR4Ii cells was less sensitive to virus than L1DR4 cells (Fig. 27A). When relative T cell responses to 1 μM GAD peptide were compared, there was a 30% decrease in class II function for L1DR4 cells but no reduction in ability of L1DR4Ii cells to present this peptide upon VV infection (Fig.

27B). Only L1DR4Ii but not L1DR4 expressed Ii. Human Ii expressed in these cells seemed a little more unstable than observed using human fibroblasts, thus a slight reduction (about 24%) in Ii could be observed after 6 h VV infection. The infection of these two cell lines were similar as assessed by equal viral protein expression (Fig. 27C).

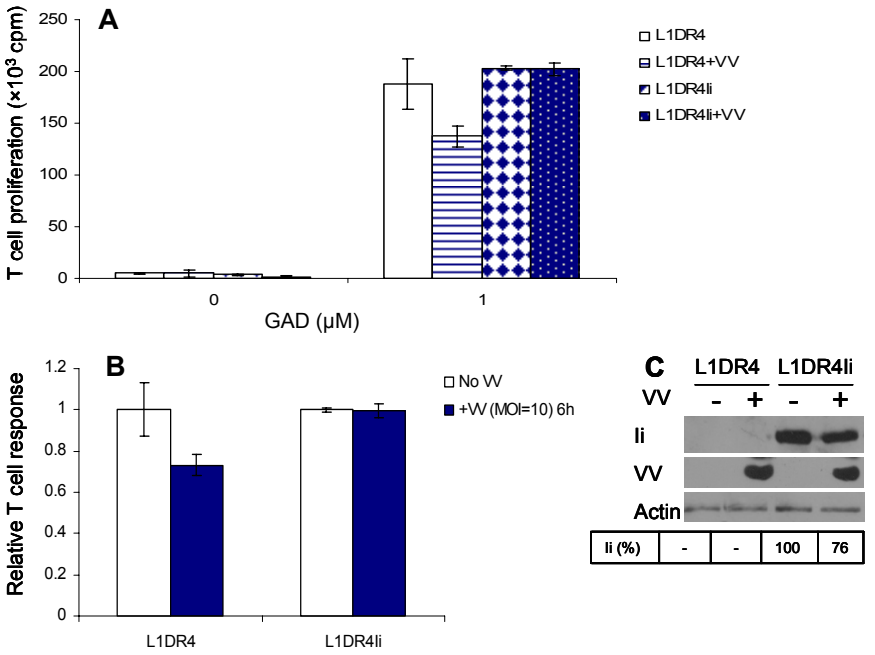


Fig. 27 Class II dysfunction induced by VV was less severe in murine fibroblast cell lines containing Ii. A. Presentation of GAD peptide upon VV infection of L1DR4 and L1DR4Ii cells. These cells were co-incubated with GAD peptide (1 μM) +/-VV (MOI=10) for 6 h prior to fixation and GAD specific T cell addition. B. Comparison of T cell responses to murine fibroblast cells +/- Ii. L1DR4 and L1DR4Ii were co-cultured with GAD peptide (1 μM) +/- VV (MOI=10) for 6h. Relative T cell proliferation was normalized to uninfected cells which were set to 1. C. Ii protein expression in murine fibroblast cells. L1DR4 and L1DR4Ii cells were infected with VV (MOI=10) for 6 h prior to Western analysis. Actin remained constant upon VV infection. Relative abundance of Ii normalized to actin is listed below the data. L1DR4Ii sample without infection was set as 100%.

Thus, over-expression of Ii by multiple methods could partially reverse the inhibitory effect of VV on class II function. Class II function in both professional and non-professional APCs was preserved by Ii over-expression upon VV infection. The species of host APCs did not appear to matter. These results confirmed the importance of Ii in class II-mediated Ag presentation upon VV infection. Notably, this preservation of class Ii function was observed at early stages of VV infection prior to viral disruption of Ii

expression. We have postulated at early stages of infection, a viral protein or gene product may directly disrupt class II function with the loss of Ii at late stages of infection, class II may be even more susceptible to this virus gene. This would be consistent with the observed protection of class II by Ii throughout infection. The incomplete preservation of class II function by over-expression of Ii upon VV infection also suggested that other mechanisms besides Ii reduction may contribute to VV-induced disruption of class II function.

2.6.2 Improved class II function in B-LCL infected by recombinant VV encoding Ii

As an alternate method to increase intracellular Ii levels, a recombinant VV encoding the cDNA for Ii was developed. To construct a recombinant VV expressing Ii cDNA, the full length human Ii (hIi, CD74) cDNA encoding the p31 form of Ii was amplified from mRNA of PriessGAD cells. This cDNA was inserted into a transfer vector to add proximal EcoRI recognition sites that could be used to excise the cDNA and facilitate insertion into another vector. The hIi cDNA with EcoRI recognition sites was ligated into the pSC59 vector to yield pSC59-hIi. The pSC59-hIi plasmid could be detected upon electrophoresis migrating at the correct size (i.e. about 4700bp) on an agarose gel. Two fragments were generated after HindIII cutting of the pSC59-hIi plasmid, one fragment was about 4400 bp, while the other was about 300 bp. Detection of the larger fragment which migrated above the uncut plasmid, was likely due to the fact that the linear forms always migrate slower than circularized form (Fig. 28A). The sequence and orientation of the human Ii insert were confirmed by DNA sequencing (data not shown). The recombination of pSC59-hIi with VV was performed as described in Material and Methods. After three rounds of selection with BUdR, the recombinant VV was harvested, titered (about 1×10^7 pfu/ml) and used to infect CV1 cells. VSC, a recombinant VV encoding β-Gal was used as a control virus for these studies. The hIi protein could not be readily detected following exposure of CV1 cells to the rVV encoding the human Ii cDNA. Even after incubation of CV1 cells for 24 h with the rVV at MOI 10, the expression of human Ii in these cells was questionable (Fig. 28B). Another recombinant VV expressing the murine Ii cDNA (Cd74) or mIi-VV was therefore tested as a measure

to promote mIi expression in cells. Since the homology between human and murine Ii protein sequences is about 80%, the specificity of the two Ii Abs: one specific for murine (In-1 Ab) and one detecting human Ii (Pin1.1) was tested. As shown in Fig. 28C, Pin1.1 Ab recognized only human Ii while In-1 bound specifically to murine Ii. Thus, these Abs could be used to monitor mouse Ii abundance even in cells expressing the human Ii. Murine Ii was detected in cells exposed to the rVV encoding this cDNA (Fig. 28D). Therefore, mIi-VV was therefore used in the following biochemical and functional assays as a method to increase intracellular Ii.

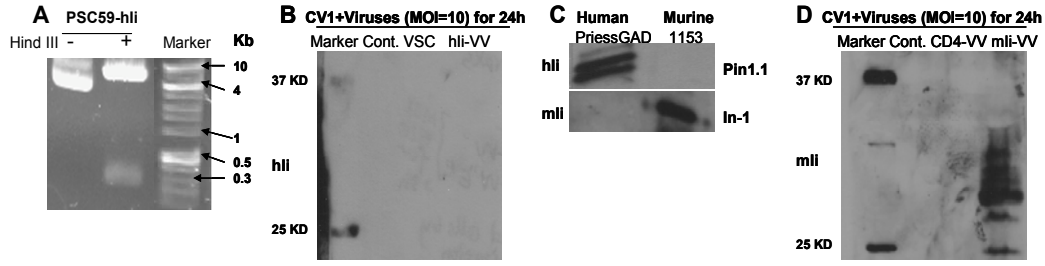


Fig. 28 Expression of Ii (CD74) cDNA with a recombinant VV vector. A. The treatment of plasmid pSC59-hIi +/- HindIII. 0.5 μg of pSC59-hIi was cut with 20 units of HindIII at 37˚C for 3 h. 0.5 μg of uncut and HindIII cut pSC59-hIi were electrophoresed on a 1% agarose gel, stained with SYBR® safe DNA gel stain, and detected with UV transillumination. DNA markers and corresponding sizes are listed on the right. B. Expression of hIi following cell infection with the hIi-VV. CV1 cells were uninfected (Cont.) or infected with VSC (recombinant VV expressing β-Gal derived from the vector pSC59) or hIi-VV at MOI=10 for 24 h. Western analysis was performed using hIi specific Ab Pin1.1. C. Specificity of human Ii Ab Pin1.1 and murine Ii Ab In-1. Lysates from human B-LCL PriessGAD and murine B cells 1153 were loaded side by side on a 10% PAGE gel. Western blots from this gel were probed with Pin1.1 and In-1 respectively. D. mIi expression by mIi-VV. CV1 cells were uninfected (Cont.) or infected with CD4-VV (recombinant VV encoding human CD4 derived similar to mIi-VV) or mIi-VV at MOI=10 for 24 h. Western analysis was performed using the mIi specific Ab In-1. A series of broad bands were detected with cells exposed only to mIi-VV, and these proteins displayed a molecular mass close to murine Ii (31 KD). Glycosylation of Ii typically results in the appearance of multiple forms of Ii.

To test the effect of mIi-VV on class II function, PriessGAD cells were infected with mIi-VV, CD4-VV or wild type VV. The ability of these APCs to present endogenous GAD Ag to T cells was measured. As shown in Fig. 29A, exposure of PriessGAD cells to each virus resulted in diminished GAD presentation. Of the three viruses, CD4-VV disrupted GAD presentation comparably to wild type VV. Thus, insertion of a gene into the virus did not significantly alter viral infection or its ability to inactivate class II function. mIi-

VV perturbed GAD presentation less severely compared with the other two viruses at 14 and 24 h after infection. Therefore, expression of Ii via a rVV partially preserved class II mediated Ag presentation at late stages of infection. Consistent with these findings, we could detect more hIi protein in mIi-VV infected cells compared to cells infected by wild type or CD4-VV (see 24 h in Fig. 29B). The cross-recognition of hIi and mIi by Abs was ruled out by our studies in Fig. 28C. It is notable that the mIi was expressed behind a viral early-late promoter, yet expression of mIi was slow and apparent as a late gene product (Fig. 29C). Yet even with delayed expression, the mIi appeared to substitute for hIi and preserved hIi abundance during infection. Cellular infection by each VV was similar with detection of similar levels of VV protein expression. Equal infection using these three viruses was also confirmed by identical E3L mRNA levels in infected PriessGAD samples as detected with RT-PCR (data not shown).

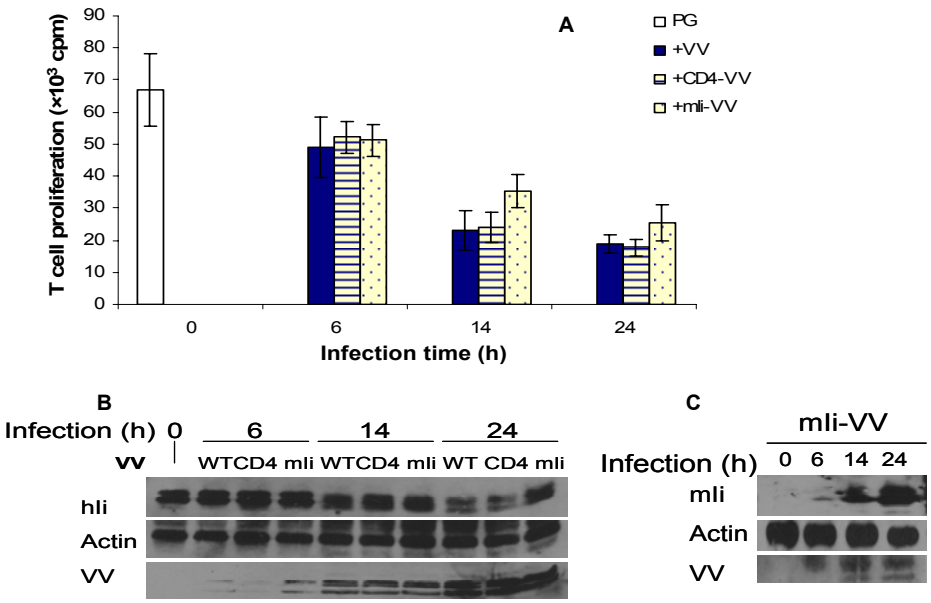


Fig. 29 Changes in class II function with rVV including mIi-VV. A. Endogenous GAD presentation by PriessGAD cells exposed to rVV. PriessGAD cells were infected with wild type VV, CD4-VV or mIi-VV (MOI=10) for up to 24 h prior to cell fixation and GAD specific T cell addition. T cell proliferation was assessed as described in the Material and Methods. B. Relative preservation of hIi following infection with mIi-VV but not wild type or CD4-VV. PriessGAD cells were infected with wild type VV, CD4-VV or mIi-VV (MOI=10) (marked as WT, CD4 and mIi respectively) for up to 24 h prior to Western Blotting analysis with hIi, actin and VV Abs. Actin levels remained constant up to 24 h infection. C. Expression of mIi during mIi-VV infection. PriessGAD cells were infected with mIi-VV (MOI=10) for up to 24 h before Western analysis with mIi, actin and VV Abs. VV proteins was probed to confirm cell infection. Actin was used as the loading control.

Overall, increasing cellular Ii levels both early and late during VV infection, served to partially preserve class II function. These results support our prior conclusion that viral infection may destabilize class II proteins and that the loss of cellular Ii during later stages of infection, further contributes to an overall loss of class II function.

Chapter 3 The presentation of VV viral Ag by class II and I molecules

The reduction in cellular Ii expression correlated with the late stages of VV infection, yet class II dysfunction and destabilization was observed at the earliest stages of virus infection. Thus, we considered mechanisms which might account for the apparent reduction in class II Ag presentation at the early stages of virus infection. While prior studies suggested VV entered cell by direct fusion at the plasma membrane, more recent efforts suggest VV efficiently enters cells by delivery into acidic endosomes [78, 79]. Ag and some peptides also enter APCs by endocytosis where proteolytic processing and binding to class II molecules take place. Thus, we tested whether VV Ags are processed and presented by class II molecules within infected APCs. Prior studies had demonstrated VV specific CD4, CD8 T cells and Abs in human vaccinated subjects as well as murine models after virus inoculation [83, 91, 241]. The detection of virus specific CD8 and CD4 T cells indicates viral Ags are presented by class I and class II molecules. Yet, this might take place after the release of virus Ags from dying infected cells. Such an indirect pathway, or cross presentation has been reported for VV Ags and MHC class II molecules [242]. Here, we attempted to study the direct presentation of viral Ags in the context of class II following APC infection. For these studies CD4 T cells specific for VV Ags were generated by *in vivo* murine infection. Cytokine production by CD8 T cells isolated from *in vivo* VV infected mice followed by *in vitro* restimulation was also investigated. The overall goal here was to gain insight into mechanism of VV immune evasion while also better understand the pathogenesis of VV infection-induced immunity.

3.1 Primary adaptive immune responses to VV

Primary adaptive immunity is most efficiently initiated after triggering of innate immune responses. There is typically a lag phase (about 6 d after pathogen invasion) prior to the initiation of primary adaptive immune responses [6]. APCs present processed Ag to T cells which can differentiate into effector T cells to induce both cellular and humoral immunity. VV-specific CD4 T cells are important for CD8 T cell activation and memory [243, 244]. They are also critical for VV specific Ab production [81]. Consequently, the

partnership of CD4 T cells and APCs is an essential component in the development of VV-induced immunity in both human vaccinees and mouse models.

Our goal was to understand whether virus infected APCs could activate virus-specific CD4 T cells and whether viral disruption of class II function interferes with the development of cellular immune responses to VV. To test whether virus-specific CD4 T cells were elicited during a VV infection, C57BL/6 mice were i.p. infected with 1×10^6 pfu VV/mouse for 1, 2 or 7 d before harvesting their lymph nodes and spleens. The CD4 T cells from these VV-infected animals (termed VV-CD4 T cells) were isolated and co-cultured *in vitro* with VV infected primary splenic cells. These splenic cells served as APCs and contain predominantly B lymphocytes but also macrophages and DCs. Spleen cells were harvested from uninfected C57BL/6 mice, then were irradiated to prevent proliferation and *in vitro* infected by VV (MOI up to 1 for 24 h). Primary APCs are more sensitive to VV infection and cell death increased upon exposure to virus at MOI>1. VV-CD4 T cells were co-cultured with these spleen cells for 3 d. Finally, [^{3}H]-thymidine was added to these cultures to detect T cell proliferation in response to the splenic APCs and virus. VV-CD4 T cell responses were very low in the first 2 d after *in vivo* VV infection, this is consistent with T cell responses to Ag or other pathogens during a primary immune response. However, after 7 d of infection, VV-CD4 T cell responses increased dramatically (Fig. 30A). Therefore, 7 d was used as the *in vivo* infectious period in our analysis of primary T cell responses to virus. The viral MOI used for *in vitro* infection of APCs also influenced our detection of VV-CD4 T cell responses. At a MOI of 0.01, the dose of viral Ag was too low to induce significant CD4 T cell activation. At a MOI of 0.1, a measurable T cell response to virus and splenic APCs was observed. Further increasing the MOI to 1, however, suppressed CD4 T cell responses. The immunoevasive properties of VV may account for this observed suppression at MOI=1, consistent with our studies that virus infection diminishes CD4 T cell recognition of protein Ags and peptides (Chapter 1 and 2).

To further investigate the responses of VV-CD4 T cells (from 7 d infected mice) to VV viral Ags, primary splenic cells were *in vitro* infected with VV (MOI=0, 0.01, 0.1, and 1)

from 2 to 36 h (Fig. 30B). T cell responses to VV infected cells (MOI=0.01) were barely detectable at this low dose of virus. At an infectious MOI 0.1, viral Ags presented by splenic APCs induce measurable T cell responses. Notably, the presentation of viral Ags resulted in very rapid activation of VV-CD4 T cells, i.e. incubation of spleen cells with VV for 2 h was sufficient to induce significant T cell responses. T cell responses to VV (MOI=0.1) increased in a time dependent manner. By contrast, VV at MOI=1 induced significantly lower T cell responses which appeared to plateau by 6 h post spleen cell infection. Again this latter result was similar to our prior findings demonstrating virus infection at MOI 1-10 blocked class II presentation of protein Ags such as GAD (Fig. 3). Thus, viral suppression of host immunity at VV (MOI=1) likely affects class II presentation of viral Ags to CD4 T cells.

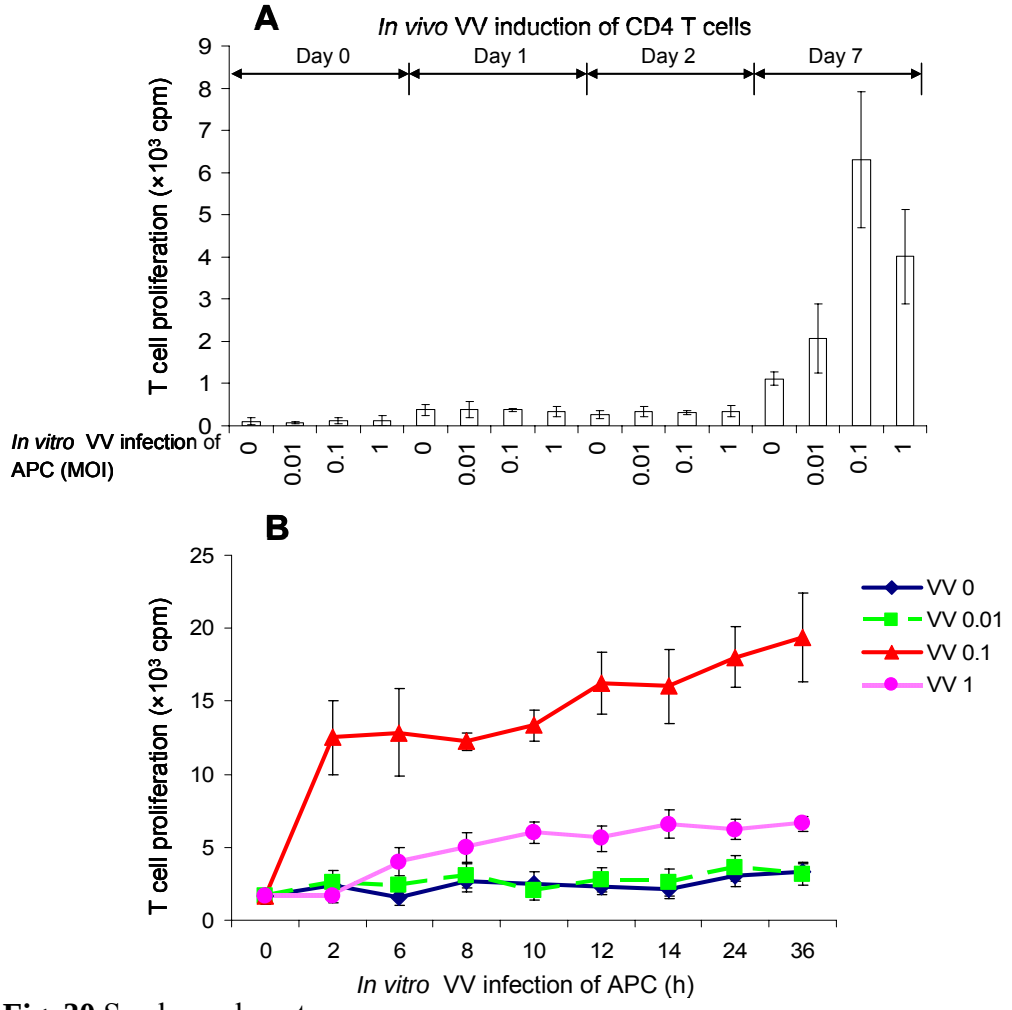


Fig. 30 See legend next page.

Fig. 30 Primary CD4 T cell responses to VV. A. Response of VV-CD4 T cells to viral Ags presented by splenic APCs. CD4 T cells were harvested at 0, 1, 2, 7 d following VV i.p. inoculation of C57BL/6 mice (VV-CD4 T cells). Primary splenic cells from uninfected C57BL/6 mice were used as APCs and irradiated followed by *in vitro* infection with VV (MOI=0, 0.01, 0.1, and 1) for 24 h. VV-CD4 T cells from virus infected mice and APCs exposed to VV *in vitro*, were co-cultured for 72 h before [^{3}H]-thymidine was added to detect T cell proliferation. B. Time course of VV-CD4 T cell responses to *in vitro* infected APCs. Primary splenic cells from uninfected mice were harvested, irradiated, *in vitro* infected with VV (MOI up to 1) for up to 36 h prior to the addition of VV-CD4 T cells harvested from 7 d *in vivo* infected mice. T cell proliferation was monitored by [^{3}H]-thymidine incorporation. APC exposure to different viral MOI is indicated. Individual experiments were repeated at least five times.

The suppression of T cell responses to viral Ag at MOI=1 (Fig. 30), suggested that viral infection of APCs at higher MOI could disrupt class II presentation of viral Ags. Our prior studies of class II-mediated Ag presentation had shown that the inhibition of class II function was less severe with UV-treated virus [97] and was not observed with heat treated virus (Fig. 4B). Therefore, we examined the effects of these two modified forms of virus relative to replication competent wild type VV on viral Ag presentation. Splenic APCs were exposed to UV treated VV (UV-VV) or heat inactivated VV (HI-VV) at MOI=0.1 and 1 with untreated VV serving as a control. As shown in Fig. 31A, UV-VV at MOI=0.1 induced T cell responses comparable to untreated VV at same MOI. UV-VV at MOI=1 induced higher T cell activation. Again, at MOI=1, viral Ag presentation was suppressed by untreated VV. Exposure of APCs to HI-VV induced significant VV-CD4 T cell responses. At MOI=0.1, HI-VV and untreated VV were similarly presented by APCs. Yet, unlike untreated VV, exposing of APCs to a dose of HI-VV (MOI=1) measurably enhanced T cell responses (Fig. 31B). This result is consistent with our prior data that heat-treatment of VV ablates its ability to inhibit the class II pathway. Data from our lab suggested that D8, a late viral membrane protein, could bind to class II molecules (P. Li, unpublished). Thus, we reasoned that mutation of the D8L gene within VV might thwart the ability of VV to disrupt class II Ag presentation. Experimentally, we tested a virus expressing a truncated form of the D8L gene (BSSH) which results in virus production of a soluble form of D8. Studies in the literature suggested this virus was replication competent and could readily infect cells [177]. Thus, we predicted that

exposure of APCs to the BSSH virus might lead to better class II presentation of viral Ags and high T cell proliferation than wild type VV. Indeed, when BSSH was tested, MOI of 0.1 and 1 induced higher T cell responses than wild type VV at MOI=0.1 (Fig. 31C).

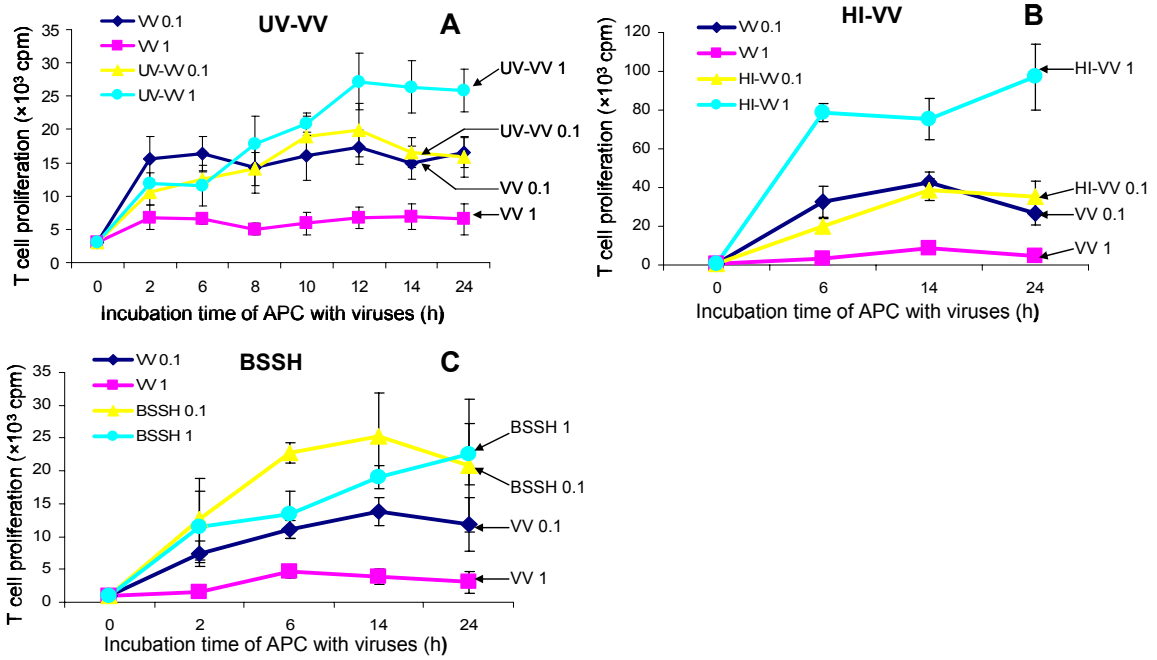


Fig. 31 Presentation of UV-, heat-inactivated or D8L truncated VV compared with untreated, wild type VV. A. T cell proliferation induced by UV-VV. VV was irradiated at 254 nm of UV for 20 m. Splenic cells from uninfected C57BL/6 mice were used as APCs and irradiated before incubation with UV-VV and VV at MOI 0.1 or 1 respectively for up to 24 h. VV primed CD4 T cells (VV-CD4) were isolated from 7 d infected mice and added to washed APCs. Proliferation of VV-CD4 T cells was monitored as in Fig. 30. B. Presentation of HI-VV. VV was heated at 60°C for 1 h before addition to the irradiated APCs for up to 24 h. VV-CD4 T cells were isolated and co-cultured with washed APCs for 72 h before [^{3}H]-thymidine addition. C. Presentation of BSSH. D8L defective VV (BSSH) or wild type VV were added to APCs at MOI=0.1 or 1 for up to 24h prior to the addition of VV-CD4 T cells.

Fig. 31 demonstrated that viral Ag presentation could be observed using modified or genetically altered VV, and that in some cases these modified viruses induced higher CD4 T cell responses than wild type VV. Thus, we wondered if these modified viruses could be used as vaccines to promote stronger immune responses than wild type virus. Our rationale is that since these defective VV partially preserve class II presentation, they may induce immune responses more effectively than untreated, wild type VV. Thus, these defective VV were used to prime mice and compared with untreated, wild type VV

in terms of inducing CD4 T cells recognizing viral Ags. After 7 d *in vivo* inoculation, CD4 T cells were isolated from mice primed with either the modified viruses or wild type VV. Here, the CD4 T cells were denoted as UV-CD4, HI-CD4 and BSSH-CD4 respectively according to the corresponding viruses used for *in vivo* inoculation. When the spleens and lymph nodes were harvested from UV-VV or HI-VV primed mice, these organs were not swollen or enlarged as typically observed with VV infected mice suggesting less induction of lymphocyte proliferation after inoculation. Consistent with this, neither UV-CD4 nor HI-CD4 could respond to APCs even after re-stimulation with HI-VV at MOI=1 (Fig. 32A and B). HI-VV was used because it induced the highest stimulation of CD4 T cells from mice infected with wild type virus (Fig. 31). UV-VV and HI-VV can not replicate in cells. This may be one reason why they failed to prime and stimulate the proliferation and expansion of CD4 T cells *in vivo*. When plaque forming assays were performed with these two viruses, neither of them could form plaques (data not shown). Thus, replication competent VV was required to induce effective CD4 T cell responses *in vivo*. This failure of inactivated VV to promote virus specific T cell development *in vivo* was consistent with several previous studies [206]. However, by using some modified methods such as increasing inoculation time, defective VV could be used to induce immune response [245, 246]. BSSH, unlike UV-VV and HI-VV, induced effective CD4 T cells responses *in vivo*. The BSSH infected mice had enlarged spleens and lymph nodes similar to VV infected mice. The BSSH-CD4 T cell responses were measurable after *in vitro* re-stimulation with HI-VV at MOI=1 although less than wild type VV (Fig. 32C). Thus, *in vivo* priming with BSSH can induce responding CD4 T cells. *In vitro* presentation of BSSH appeared better than wild type VV (Fig. 31C). Thus, one would have predicted BSSH would generate a T cell response stronger than wild type virus during *in vivo* infection. Whether BSSH replicates to the same efficiency as VV upon animal inoculation is unknown, and could provide one explanation. Still, these studies suggest BSSH is a good candidate vaccine. Possibly adding adjuvants to BSSH or complete deletion of the D8L gene may help to improve its antigenicity.

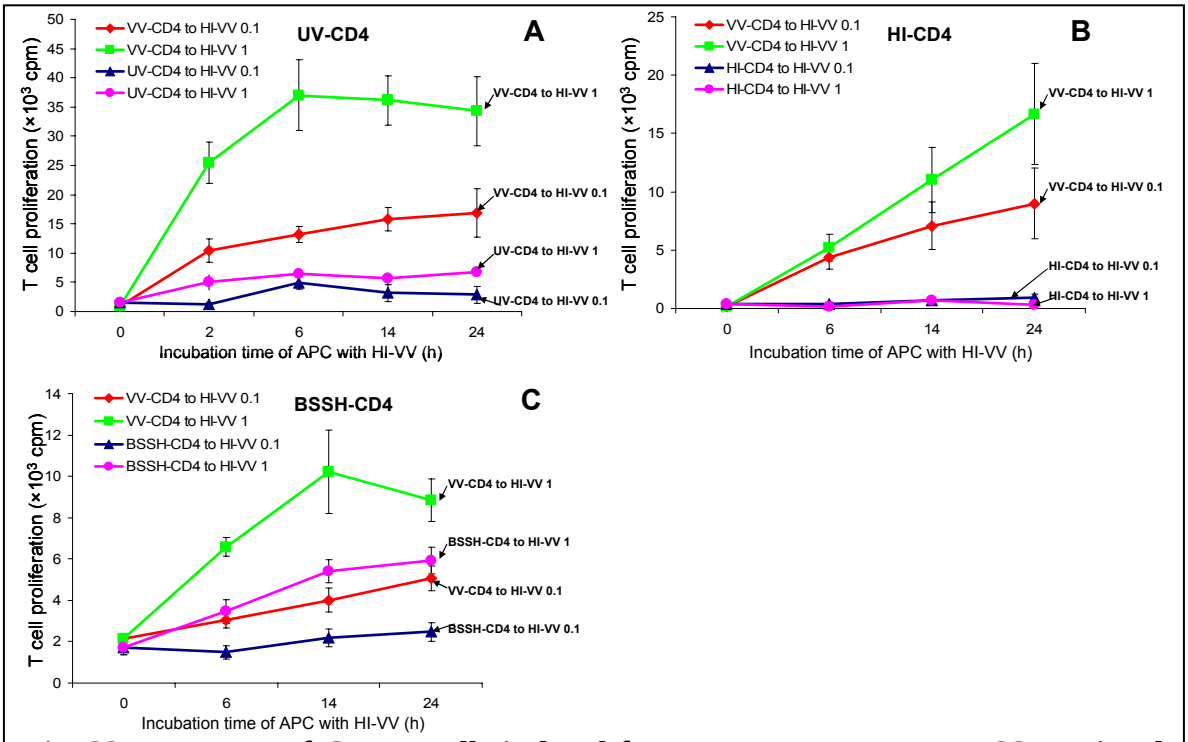


Fig. 32 Responses of CD4 T cells isolated from UV-VV, HI-VV or BSSH primed mice to *in vitro* HI-VV treated APCs. A. CD4 responses from UV-VV primed mice. C57BL/6 mice were i.p. inoculated with UV-VV (1×10^6 pfu/mouse) for 7 d before lymph nodes and spleens were harvested. CD4 T cells were isolated and co-cultured with irradiated APCs pretreated with HI-VV (MOI=0.1 and 1, up to 24 h). After 72 h, [^{3}H]-thymidine was added to measure T cell proliferation. B. HI-VV failed to induce CD4 responses. Mice were injected with HI-VV (1×10^6 pfu/mouse) for 7 d before CD4 isolation. APCs were treated as in panel A and co-cultured with CD4 T cells from HI-VV or VV inoculated mice. C. CD4 responses after *in vivo* BSSH infection. Mice were infected with BSSH (1×10^6 pfu/mouse) for 7 d before CD4 T cell harvesting. In parallel studies, another set of animals were infected with VV. CD4 T cells from each set of mice were isolated and incubated with APCs that had been treated with HI-VV. T cell responses were monitored. For all the three panels, the responses of VV primed CD4 T cells (VV-CD4) were tested parallel as the positive controls.

One of the primary cellular cytokines detected by the T cell proliferation assay used in our laboratory is IL-2. To more specifically examine cytokines produced by virus primed CD4 T cells, ELISAs were performed. Here, mice were *in vivo* infected with wild type VV or BSSH for 7 d before CD4 T cells (VV-CD4 and BSSH-CD4 T cells) were isolated from the animal spleens and lymph nodes. These CD4 T cells were then co-cultured with APCs (splenic cells from uninfected mice) that had been incubated with HI-VV (MOI=1, 24 h). Culture supernatants were harvested and analyzed by ELISA using IFN-γ, TNF-α, IL-4 and IL-17 specific Abs. Both VV-CD4 and BSSH-CD4 T cell produced significant

amount of IFN-γ upon incubation with APCs + HI-VV compared to CD4 T cells cultured with APCs alone. BSSH-CD4 T cells secreted slightly more IFN-γ than VV-CD4 in this assay (Fig. 33). This result may again support the use of BSSH virus as a vaccine candidate. ELISAs for TNF-α, IL-4 or IL-17 failed to detect the production of any of these cytokines by CD4 T cells from VV or BSSH inoculated animals under the conditions tested (data not shown). Thus, we primarily detected Th1 cytokines, i.e. IL-2 (assayed by proliferation assay) and IFN-γ (by ELISA) upon *in vitro* re-stimulation of VV or BSSH primed CD4 T cells. Similar results were obtained by using a multi-analyte ELISArray kit for mouse Th1 / Th2 / Th17 Cytokines (data not shown). Among the 12 cytokines we screened, only IL-2 and IFN-γ levels were significantly above background (i.e. using CD4 T cells co-cultured with APCs alone as a background control).

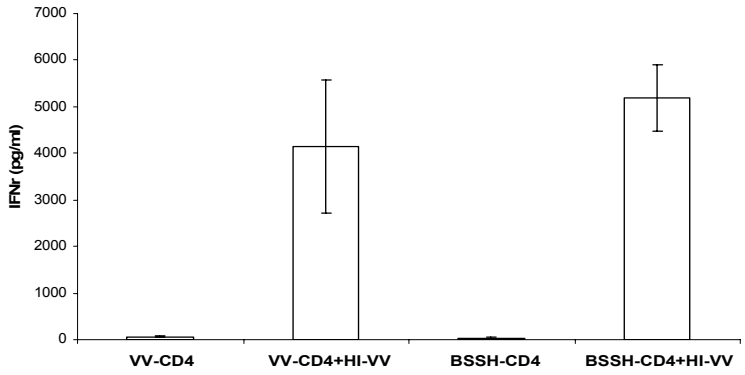


Fig. 33 IFN-γ production by virus specific CD4 T cells upon exposure to HI -VV and APCs. APCs were incubated with HI-VV (MOI=1) for 24 h before addition of CD4 T cells from VV or BSSH primed mice. After co-cultured for 72 h, culture supernatants were harvested and analyzed by ELISA using IFN-γ specific Abs. CD4 T cells co-cultured with APCs but no virus were set up as a control (labeled as VV-CD4 or BSSH-CD4).

During these studies to analyze VV specific responses of CD4 T cells, we observed that the wild type, untreated VV was the best *in vivo* inducer or activator promoting the expansion and development of virus-specific CD4 T cells. The HI-VV at MOI 1 was observed to maximally re-stimulate CD4 T cells *in vitro*, possibly due to its lack of immunoevasive properties and its relative preservation of class II function. BSSH, a mutant virus, appears to hold promise as a potential vaccine candidate due to its ability to induce significant CD4 T cell responses *in vivo* and could be presented by class II molecules *in vitro*.

3.2 Class II presentation of VV peptides

Compared with viral Ags or proteins from intact VV, viral peptides may need less intracellular processing and be presented more efficiently. In collaboration with Dr. Alessandro Sette (La Jolla Institute for Allergy and Immunology San Diego, CA, USA), we examined the ability of class II molecules to bind and present several VV peptides to CD4 T cells (listed as Table 3). Peptides were named according to the corresponding gene names. Using co-immunoprecipitation assay, our laboratory has shown that the H3 and D8 viral proteins can bind to the class II molecules. We proposed that peptides from these viral Ags might thus be presented to virus-specific CD4 T cells. Depending on their binding affinity for class II, viral peptides could compete with other peptides for class II molecules and potentially diminish the presentation of other Ags.

Table 3 Viral peptides tested for class II presentation.

Peptide/ Encoding Gene Name	VACV-WR Protein Name	Time of Expression	Viral Protein Function	Function Category	Peptide Sequence
F15L	VACWR054	/	unknown	unknown	TPRYIPSTSISSSNI
E1L	VACWR057	late	poly(A) polymerase, large subunit	Genome regulation	VLTIKAPNVISSKIS
E9L	VACWR065	early	DNA polymerase	Genome regulation	PSVFINPISHTSYC Y
I1L	VACWR070	late	virion core DNA binding protein	Genome regulation	LKAYFTAKINEMV DE
L4R	VACWR091	late	core DNA-binding protein, VP8	Genome regulation	ISKYAGINILNVYS P
J4R	VACWR096	early	DNA-dependent RNA polymerase subunit rpo22	Genome regulation	DDDYGEPIIITSYL Q
H3L	VACWR101	late	IMV heparin binding surface protein	Virion structure	PGVMYAFTTPLISF F
D8L	VACWR113	late	IMV membrane protein	Virion structure	GEIIRAATTSPARE N
D13L	VACWR118	late	rifampicin target associates with inner surface immature virus membrane	Virion structure	PKIFFRPTTITANV S
A18R	VACWR138	early	DNA helicase; effects elongation and termination of postreplicative viral transcription	Genome regulation	PKGFYASPSVKTS LV
A20R	VACWR141	early	DNA polymerase processivity factor	Genome regulation	DNIFIPSVITKSGK K
A24R	VACWR144	early	DNA-dependent RNA polymerase subunit rpo132	Genome regulation	IHVLTTPGLNHAFS S
A28L	VACWR151	late	IMV protein	Virion structure	FFIVVATAAVCLL FI

The pool of viral peptides was divided randomly into 3 groups for testing. The peptides at various concentrations (from 1-20 μg/ml) were added to the 1153 murine B cell line for 24 h prior to paraformaldehyde fixation of these APCs. 1153 cells express class II I-A^b molecules. Similar to the primary APCs used by Sette and colleagues in their studies to identify VV peptides binding class II [81], VV-CD4 T cells (harvested from 7 d VV infected mice) were co-cultured with virus peptide treated 1153 cells for 72 h prior to measuring T cell proliferation. APCs incubated with VV (MOI 0.1) were set up as the

positive control to stimulate virus-specific CD4 T cells. The absolute values of T cell proliferation were plotted in Fig. 34A to C. Typically, the 10 to 20 μg/ml of each peptide induced the highest response in terms of T cell activation or proliferation. As shown in Fig. 34D, the relative response of virus-induced CD4 T cells to each peptide was compared following normalization (i.e. VV-CD4 T cells co-cultured with 1153 with no peptide or virus addition, set as 1). Those viral peptides that induced responses above 1 were considered antigenic. Similar to published studies with these peptides, we found differences in the relative ability to activate T cells. Based on our results, we concluded that J4R, H3L, F15L and L4R peptides displayed the highest relative simulation of T cells, followed by I1L, E1L, D8L, D13L, A20R, E9L and A24R derived peptides. Two peptides-A18R and A28L appeared unable to activate T cell responses using 1153 cells as APCs. Interestingly, peptides from D8L and H3L viral gene products do appear to bind class II molecules and activate virus-specific CD4 T cells. In their published study, Sette and colleagues directly measured the binding of these viral peptides to class II. While binding was observed in their study, using an assay to measure T cell IFN-γ production, this group also found not all these peptides can stimulate T cell cytokine secretion [81].

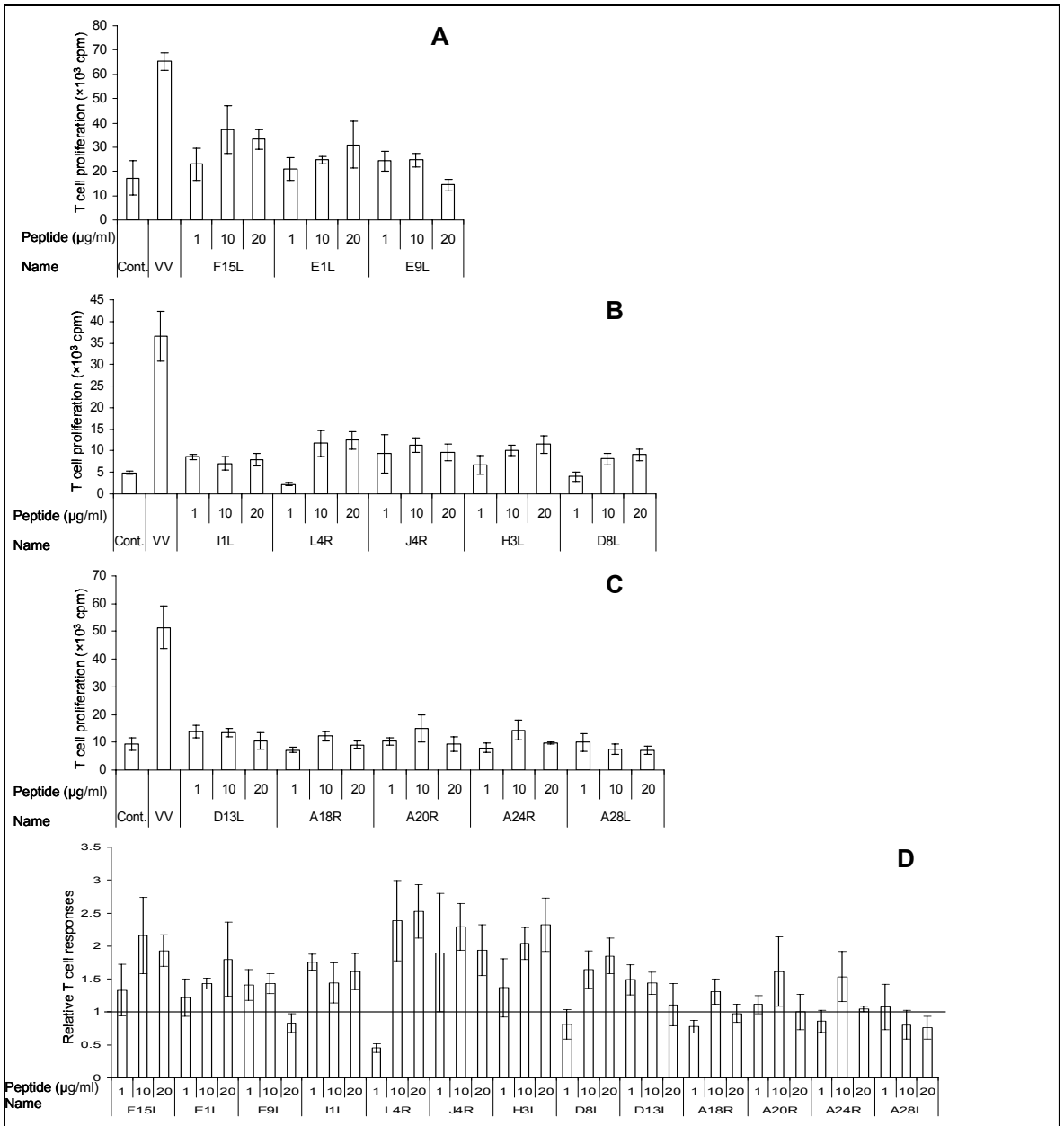


Fig. 34 Presentation of VV peptides by class II molecules. A. Presentation of F15L, E1L and E9L synthetic peptides by class II I-A^bon 1153 cells. Peptides at various concentration (1, 10, 20 μg/ml) were added to 1153 cells for 24 h followed by cell fixation. VV-CD4 T cells isolated from 7 d *in vivo* infected mice were co-cultured with peptide-treated 1153 for 72 h before measuring T cell proliferation by [^{3}H]-thymidine addition. VV-CD4 T cell responses to VV (MOI=0.1) and 1153 were monitored as a positive control. B. Presentation of I1L, L4R, J4R, H3L and D8L peptides by 1153 cells. C. D13L, A18R, A20R, A24R and A28L peptide presentation by I-A^b expressing on 1153 cells. For panels B and C, 1153 were incubated with the corresponding peptides and treated as described in panel A. D. Relative T cell responses induced by these peptides and 1153 APCs. CD4 T cell responses to 1153 cells and each virus-derived peptide as plotted in panels A-C were normalized. For each set of assays, T cell proliferation in response to 1153 cells alone was set equal to 1.

3.3 The effects of VV infection on class II presentation of a model Ag *in vivo*

Our prior studies demonstrated that *in vitro* infection of APCs by VV diminished the ability of these cells to present both peptide and proteins to CD4 T cell. This work was expanded here to test whether *in vivo* virus infection could influence the ability of APCs to stimulate T cell responses to a protein Ag in a mouse model of induced asthma. We selected a murine model of ovalbumin (OVA) induced asthma for these studies which were carried out in collaboration with the laboratories of Drs. David Wilkes, Mark Kaplan and Randy Brutkiewicz. The asthma model was also selected for these studies as the natural route for poxvirus infection is via the respiratory track. Respiratory infectious viruses such as respiratory syncytial virus (RSV) and influenza virus may trigger and exacerbate asthma [247, 248]. The study of host immune responses to VV in healthy and asthmatic animals had not been studied in depth and offered an important model to further investigate how VV infection influences host immunity *in vivo*. OVA induced asthmatic mice as well as healthy mice were mock or intratracheally infected with VV for 4 d, followed by an analysis of virus-induced lung pathology. Histological analysis of animal lungs and cytokine analysis of splenocytes from these mice were performed. Tissue sections were stained with haematoxylin/eosin (HE) to detect cellularity and immune cell infiltration. Mucous production was detected by PAS staining of tissue sections and served as a hallmark indictor of asthma. VV infection of mice typically results in the activation of T cells producing pro-inflammatory cytokines like IFN-γ (Fig. 33). Asthma, on the other hand, is mediated by the production of cytokines like IL-4, IL-5 and IL-13 in the lungs. We speculated that virus infection and pre-existing asthma might antagonize each other. Therefore, the inflammation and pathology associated with asthma and VV infection might be ameliorated in asthmatic mice challenged with VV compared to asthma or infection alone mice. Indeed, VV infection of mice appeared to diminish asthma related inflammation as shown by the histological analysis (Fig. 35). In control or untreated animal groups, no obvious lesion or PAS^+ mucous producing cells were observed (Fig. 35A and E). Asthma alone induced peribronchiolar and perivascular infiltrates and bronchus-associated lymphoid tissue (BALT) production (Fig. 35B). Many PAS^+ cells showed up in mice exposed to asthma only (Fig. 35F). Mice with VV

infection only demonstrated peribronchiolar and perivascular inflammation as well as a few PAS^+ cells (Fig. 35 C&G). As expected, inflammation in VV-infected asthma mice was less severe and in the lungs PAS^+ cells were less frequently detected (Fig. 35D and H) compared with asthmatic mice alone.

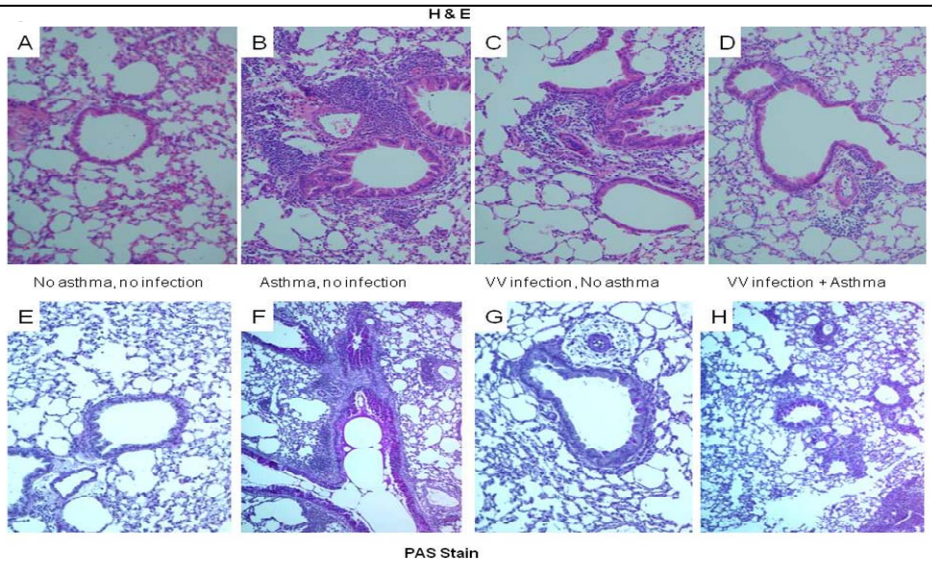


Fig. 35 Histochemistry of lungs from mice +/- asthma and/or VV. Panels A to D were lung samples stained with H&E while panels E to H were lung tissue stained with PAS. Panels A and E were samples from non-asthmatic, non-VV infected control mice. B and F were from asthmatic mice without VV infection. C and G were from VV infected healthy mice. D and H were from both asthmatic and VV infected mice. Representative images from each group of animals (5 per group) were shown using a magnification of 20X. Tissue histopathology was analyzed by Dr. David Wilkes, IUSM.

The analysis of cytokine production by splenocytes correlated with our histological data. Splenocytes including APCs and T cells were *in vitro* re-stimulated +/- OVA for 72 h before ELISA analysis were performed. Among the cytokines we tested, IL-5 and IL-13 are two important Th2 cytokines induced during asthma. IL-5 plays a role in the development, maintenance and activation of eosinophils [249]. IL-13 initiates B cells to secrete IgE [249] and induces mucous secretion [250]. As shown in Fig. 36A and B, both IL-5 and IL-13 were detected only upon re-stimulation of spleen cells from asthmatic but not healthy mice. The production of cytokines in these assays was dependent on OVA addition to splenocyte cultures, strongly suggesting that cytokines were secreted by T cell recognizing OVA peptides presented by class II molecules on APCs. Viral infection of asthmatic mice resulted in reduced IL-5 and IL-13 production during *in vitro* re-

stimulation assay. This could reflect with reduced class II OVA Ag presentation upon viral infection of the asthmatic mice, which would lead to less T cell activation *in vivo*. Consistent with this, we observed less cytokine production when we attempted to re-stimulate T cells from these mice *in vitro* (Fig. 36). The reduction in T cell responses and IL-5 and IL-13 secretion may explain the amelioration of the asthmatic condition in these mice with VV inoculation (Fig. 35). Induction of asthma alone or VV infection induced IFN-γ production in mice. Combining these treatments enhanced T cell IFN-γ production synergistically (Fig. 36C). The increased production of IFN-γ in the asthmatic mice upon virus infection could promote VV clearance. Indeed, VV titers from animal lung tissue were consistently lower in asthmatic mice exposed to virus compared with VV infected mice (data not shown). IL-17, another pro-inflammatory cytokine, was produced in both asthma alone and asthma + VV groups of mice. Although infection alone did not induce IL-17 production in these studies, VV inoculation increased IL-17 expression dramatically in the presence of asthma (Fig. 36D). IL-17 appears to trigger inflammation in several autoimmune diseases and is associated with asthma [251]. However, our histological data (Fig. 35) suggests that this enhanced IL-17 in the combined treatment group did not exacerbate asthma. One recent report suggests that IL-17 is partially involved in host immunity against VV infection [252]. Thus, enhanced IL-17 production may also contribute to the reduced VV titers in the combined treatment group. Together these studies suggest virus infection *in vivo* alters the generation of cytokines secretion profile of T cells responsive to a protein Ag, OVA. Future studies will be necessary to address whether this is specifically due to changes in class II function upon virus infection of resident APCs. But it is known that the level of Ag presentation by APCs can shift T cell development from producing Th1 cytokines like IFN-γ to Th2 cytokines like IL-4 [253].

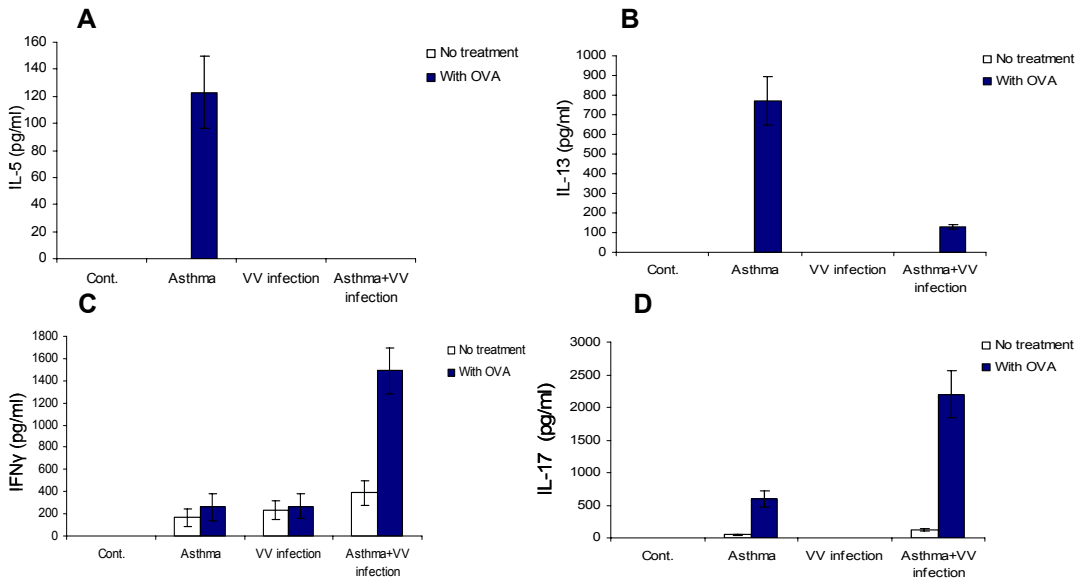


Fig. 36 Cytokine production by splenocytes from mice +/- asthma and/or VV infection. A. IL-5 production in mice +/- asthma and/or VV infection. Splenocytes were harvested and *in vitro* restimulated +/- OVA (100 μg/ml) for 72 h prior to being analyzed with ELISA using IL-5 specific Abs. B, C, D: Quantity of IL-13, IFN-γ and IL-17 production detected with corresponding Abs for ELISA analysis. Data was averaged from five mice in each group. These ELISA analysis were performed by Dr. Gourapura Renukaradhya.

3.4 Recall VV specific responses of T cells

Primary adaptive immune responses always contract with the disappearance of Ag. However, some Ag-specific T and B cells persist and become the bases for immune memory. When the body encounters the same Ag again, the lag period for immune responses is shorter and the secondary immune response is greater [6]. Here, we examined whether similar T cell responses were observed during secondary challenge of animals with VV. Booster immunization of C57BL/6 mice was performed by i.p. injection of VV (1×10^6 pfu/mouse) 3 weeks after a primary inoculation with virus. Since re-activation is faster than the primary response, CD4 T cells were isolated 4 d after the 2^{nd} injection. These CD4 T cells were termed 2×VV-CD4 T cells. 2×VV-CD4 T cells were co-cultured with virus incubated APCs (i.e. APCs treated with VV at MOI 0.1 or HI-VV at MOI 1 for 24 h). T cell cytokine production was analyzed by ELISA using IL-2, IFN-γ and IL-17 specific Abs. As in primary responses, only IL-2 and IFN-γ could be

detected (Fig. 37A and B). IL-17 production was not observed (data not shown). Similar to primary responses (Fig. 31), HI-VV stimulated higher amount of IL-2 and IFN-γ production than untreated VV. The production of IFN-γ but not IL-17 by 2×VV-CD4 T cells was also confirmed by intracellular staining after *in vitro* polyclonal re-stimulation with PMA and ionomycin (done by Norman Yeh at Dr. Mark Kaplan's laboratory, data not shown).

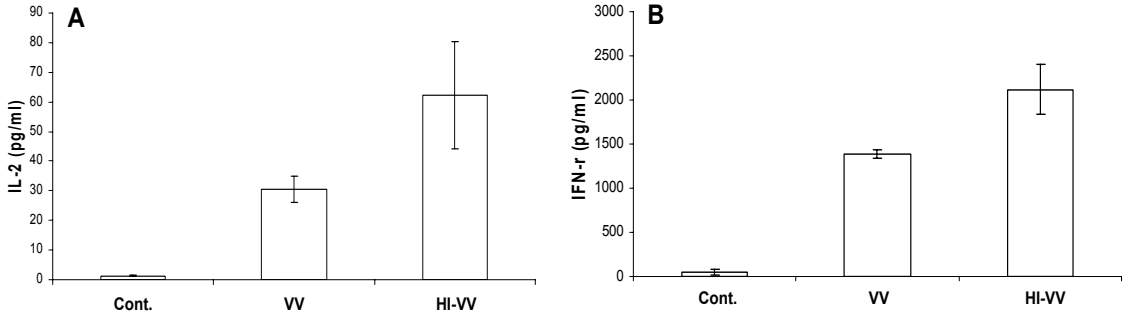


Fig. 37 Cytokines produced by T cells after *in vivo* VV rechallenge. A. IL-2 secretion by 2×VV-CD4 T cells. 2×VV-CD4 T cells were isolated from lymph nodes and spleens of mice after a 2nd *in vivo* virus inoculation. 2×VV-CD4 T cells were co-cultured *in vitro* with APCs pre-incubated with VV (MOI=0.1) or HI-VV (MOI=1). After 72 h incubation, culture supernatants were harvested and analyzed by ELISA using IL-2 specific Abs. B. Production of IFN-γ by 2×VV-CD4 T cells. Cell culture was same as described in panel A. Supernatants were detected by ELISA using IFN-γ specific Abs.

In addition to CD4 T cells, CD8 T cells also play an important roll in VV specific immune memory. To test CD8 T cell function in VV recall responses, CD8 T cells from twice inoculated mice (2×VV-CD8 T cells) were isolated with microbeads coated with CD8 specific Abs. After *in vitro* re-stimulation of these T cells with PMA and ionomycin, intracellular staining with granzyme B and IL-17 Abs was performed. Granzyme B is a caspase like serine protease released by CD8 T cells to lyse infected cell targets [254]. Granzyme B is considered a marker to detect cytolytic CD8 T cells. As expected, mice infected twice with VV contained a greater percentage of granzyme B expressing CD8 T cells (Fig. 38A). These results correlated with previous literatures which had shown that VV infection can induce CD8 T cell responses in human [241, 255]. Interestingly, the expression of IL-17 was consistently detected in 2×VV-CD8 T cells from infected animals (Fig. 38B). IL-17 can be produced by multiple cell types including CD4, CD8, γδ

and NK T cells [256-259]. IL-17 production by CD8 T cells has been linked to pathogenesis in autoimmunity and infection [256, 260-262]. The induction of IL-17 expression by CD8 T cells may be one of the mechanisms the host uses to fight VV infection. Collaborative studies are underway with Dr. Kaplan and Dr. Brutkiewicz's laboratories to test this hypothesis.

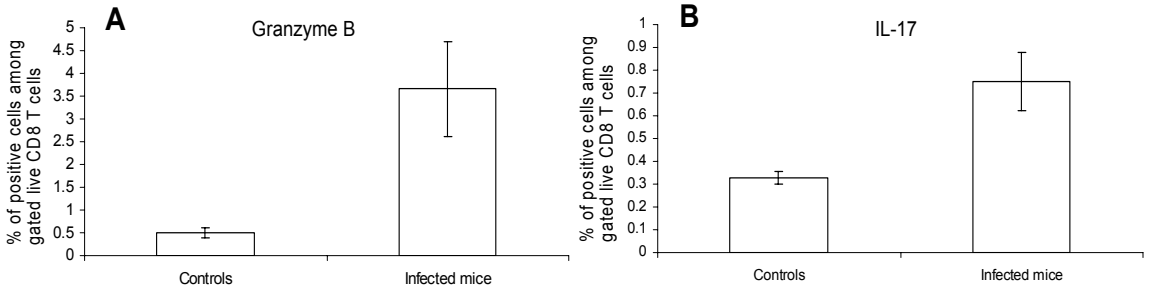


Fig. 38 Production of granzyme B and IL-17 by VV-induced CD8 T cells. A. Granzyme B expression in 2×VV-CD8 T cells. CD8 T cells were isolated from mice after a 2nd inoculation with VV followed by *in vitro* re-stimulation with PMA and ionomycin. Intracellular staining of these cells using granzyme B specific Ab was performed 4 h after this re-stimulation. B. IL-17 secretion by 2×VV-CD8 T cells. The isolation and re-stimulation CD8 T cells were the same as in panel A. Intracellular staining of CD8 T cells was done using an IL-17 specific Ab. *In vitro* re-stimulation and intracellular staining were done by Norman Yeh.

To further investigate which APC populations could promote T cell IL-17 production, CD4 and CD8 T cells were isolated from uninfected mice and activated *in vitro* under IL-17 polarizing condition [201] in the presence of various populations of APCs. Splenic cells and lymph node cells from untreated C57BL/6 mice were harvested and depleted of T cells with CD4 and CD8 Ab coated microbeads to isolate total APCs. Individual APC subsets were isolated with CD11c, CD11b and B220 Ab coated microbeads sequentially. Splenic CD4 and CD8 T cells were also harvested from uninfected C57BL/6 mice by isolation with corresponding Ab coated microbeads. CD4 and CD8 T cells were activated *in vitro* with anti-CD3 and anti-CD28 in the presence of various APC populations (total, DCs, B cells and macrophages) and then cultured under conditions to skew these cells for differentiation into IL-17 producing T cells for 5 d before intracellular staining with an IL-17 specific Ab. For CD4 T cells, B cells followed by DCs were most effective in inducing T cell IL-17 production compared with total APCs or macrophages (Fig. 39A). For CD8 T cells, DCs appeared to be best while both B cells and macrophages increased

T cell IL-17 secretion greater than total APCs did, although the differences are slight (Fig. 39B). When APCs from lymph nodes were tested compared with APCs from spleen, IL-17 production was much lower for both CD4 and CD8 T cells (data not shown).

Taken together, VV inoculation of mice can induce immune memory via activation of CD4 and CD8 T cells. The main cytokines produced by these CD4 T cells include IL-2 and IFN-γ. CD8 T cells, on the other hand, produce granzyme B, IL-17 and IFN-γ (Fig. 38 and data not shown). *In vitro* DCs, macrophages and B cells served as APCs to induce T cell IL-17 production for both CD4 and CD8 T cells. For activation of IL-17 producing CD4 T cells, again each APC type appeared to activate these cells although B cells and DCs appeared more potent than macrophages.

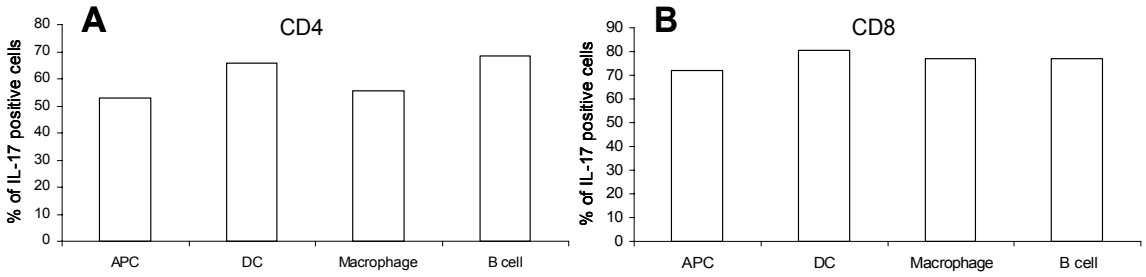


Fig. 39 The effect of various APC populations on T cell-induced IL-17 production. A. The effect of APC populations on IL-17 secretion by CD4 T cells. CD4 T cells isolated from C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 Abs in the presence of various APC subsets under conditions to promote IL-17 production for 5 d before intracellular staining with an IL-17 specific Ab. B. Differential ability of various APC populations to promote IL-17 production by CD8 T cells. CD8 T cells were isolated from mouse spleens. Other steps were same as in panel A. *In vitro* culture and intracellular staining were done by Norman Yeh.

In summary, VV induced both primary and secondary acquired immune responses. Inactivated or genetically defective VV could be presented better than wild type VV. This may be due to preservation of the class II pathway for Ag presentation upon APC exposure to these modified viruses. However, the overall antigenicity of wild type, untreated VV was higher than that of the defective VV as demonstrated by the ability of wild type, replication competent virus to induce greater numbers of VV-specific T cells *in vivo*. The main cytokines producing by VV specific CD4 T cells were Th1 cytokines

(IFN-γ and IL-2). Accordingly, VV induced Th1 polarization and antagonized Th2 cytokine-mediated asthma. A secondary infection induced CD8 T cells to secrete granzyme B and IL-17. IL-17 production by CD8 T cells could be enhanced by *in vitro* co-culturing with APCs such as B cells and DCs

Chapter 4 Development of humoral immunity to VV membrane proteins

A key goal in our studies of VV and its interaction with host immunity is to generate new ideas or approaches for safer vaccines. Immunoevasion represents a major obstacle for safe vaccination and the development of protective immunity. VV-induced disruption of class II Ag presentation has been demonstrated and could compromise cellular immune responses [96, 97]. The two viral envelope proteins - D8 and H3 bind cell surface chondroitin sulfate and heparan sulfate respectively, implicating that these viral Ags involved in cell attachment, entry and infection [193, 194]. Using radio-labeled virus, we previously observed that D8 and H3 proteins could bind to class II molecules. Peptides from these two viral proteins induced CD4 T cell responses (Fig. 34). Thus, these two viral proteins are potential targets for amelioration of the current VV vaccine. Ab responses to H3 and D8 are detected in humans and mice after VV inoculation [263, 264]. Here, a polyclonal Ab specific for an H3 epitope (eH3) was demonstrated to be effective in blocking VV entry and preservation of class II function during VV infection. These results potentially support a role for Ab in protecting immune cell function during subsequent VV infections and may offer an alternative for vaccine-induced human immunoglobulin (VIG) being used to treat infections in immune compromised individuals.

4.1 Expression of viral D8 and H3 mRNAs and proteins in infected B-LCL

To demonstrate that D8L and H3L gene products could be detected in VV infected cells, PriessGAD cells were infected with virus (MOI=10) for up to 14 h before Western Blot and RT-PCR analysis. As shown in Fig. 40, expression of two viral late gene products increased progressively during VV infection. As a viral late gene produce, D8 protein was weakly detectable at 2 and 6 h presumably due to its presence in infectious virions which enter cells (Fig. 40A). By 8 h, D8 protein was readily detected. H3 protein is also considered a viral late Ag and present in virions. H3 protein was detected at a similar level at 2 and 6 h, with increased protein expression after 8 h. Differences in mAb recognition may account for our ability to more readily detect the H3 *vs.* the D8 protein

in virus infected cells. As expected for a late viral gene, D8 mRNA was just detectable at 2 h post infection and plateaued by 8 h (Fig. 40B). A similar time course for viral H3L late gene mRNA was also observed. In studies of viral early gene product E3L, we noted strong expression at 2 h followed by decreased viral mRNA at later times (Fig. 8A). The increase in mRNA abundance for D8L and H3L suggest transcription was rapidly increased between 2-6 h, which just proceeded a detectable increase in each protein's expression. Thus, both D8L and H3L gene products are readily detected at both early and late stages of virus infection, with likely new protein synthesis commencing between 6-8 h.

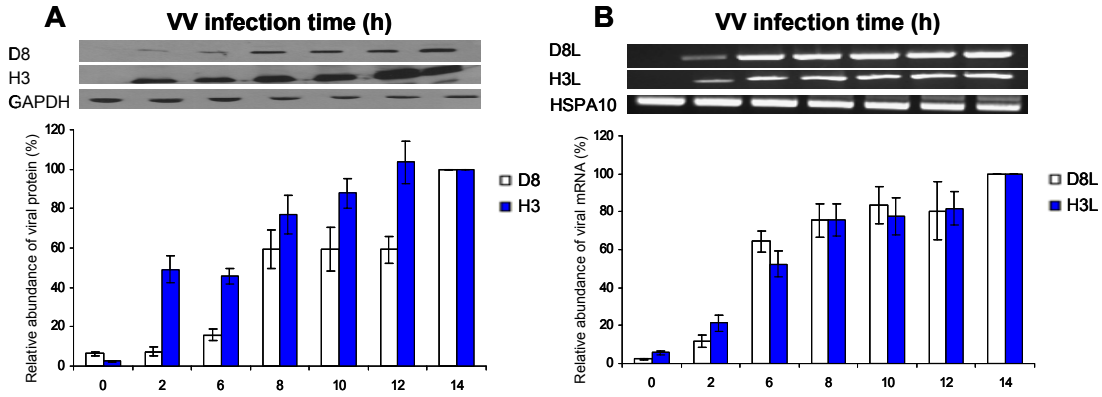


Fig. 40 Expression of D8L and H3L viral gene products in VV infected B-LCL. A. D8 and H3 proteins in VV infected cells. PriessGAD cells were infected with VV (MOI=10) for 0-14 h before cell harvesting and lysis for Western analysis using D8 or H3 specific Abs. Host GAPDH was detected in samples as the loading control. Quantification data from three separate experiments was averaged and plotted. B. mRNA from D8L and H3L in VV infected B-LCL. PriessGAD cells were infected as described in panel A. Cells were lysed for RT-PCR analysis using D8L or H3L specific primers. Host HSPA10 (encoding HSC70) mRNA was used as a loading control. Quantification of RT-PCR data was averaged from three individual experiments.

4.2 Synthesis of recombinant D8 and H3 proteins

Studies in our laboratory suggested rabbit Abs for H3 and D8 proteins could be generated using specific peptides from these viral proteins as immunogens. To confirm that the polyclonal rabbit anti-sera raised against an H3 peptide could detect viral H3 Ag, we synthesized recombinant H3 protein *in vitro*. H3 was expressed in *E. Coli* and purified with the QIAexpressionist kit. Recombinant D8 protein was synthesized as well.

Plasmids containing truncated D8L or H3L genes with histidine tag were generously provided by Dr. W. Chang (Institute of Molecular Biology, Academia Sinica, Taiwan). Bacterial expression of D8 and H3 was confirmed using D8/H3 specific Abs and His-Ab (Fig. 41 A and B). The D8/H3 Abs were raised using the viral Ags as immunogens and provided by Dr. Chang.

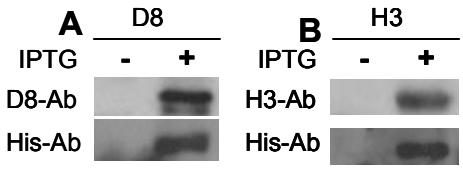


Fig. 41 *In vitro* synthesis of D8 and H3. A. Bacterial expression of histidine tagged D8 protein was detected with D8 protein specific Ab and His-specific Ab. Bacteria were grown with (+) or without (-) IPTG to induce the expression of the recombinant viral Ag. *In vitro* expressed D8 protein was purified in a nickel chromatography column, fractionated by SDS-PAGE, transferred to membranes and probed with D8 protein specific Ab or a His-specific Ab. B. Expression of H3 protein with a histidine-tag. H3 protein was isolated from bacteria +/- IPTG induction and detected by Western analysis with Abs to H3 and Abs to the His-tag. For panels A and B, samples without IPTG induction were used as negative controls.

4.3 Recognition of the recombinant H3 Ag by Ab generated by a peptide immunogen

D8 and H3 proteins can bind to host surface molecules and may help VV attach or enter host cells [193, 194]. Abs of these two viral proteins might therefore be useful in blocking viral entry and thus help preserve class II function. The protective properties of Abs specific for poxviruses has been demonstrated in mice, monkeys and humans [91, 152, 265, 266]. Studies suggest the H3 protein is an immunodominant target of human anti-VV Ab, and Abs to H3 can effectively protect against lethal VV infection in mice [264]. Often in contrast with whole Ag, vaccines derived from antigenic peptides or epitopes induce fewer and/or milder side-effects and may be more readily prepared in the laboratory. Thus, two epitopes from H3 protein were conjugated to keyhole limpet hemocyanin (KLH) and used to immunize rabbits. One epitope - the $H3_{44\text{-}61}$ peptide is located in the extracellular domain of the H3 protein and likely exposed on virions. The other epitope - the $H3_{244\text{-}259}$ peptide is located in the transmembrane domain of H3.

Polyclonal Abs specific for these two epitopes were readily detected in the sera of immunized rabbits. The $H3_{44-61}$ specific antisera was named as eH3 while the antisera to $H3_{244-259}$ was termed eH3c. Biochemical and functional assays were performed to test the specificity and efficacy of these H3 epitope specific Abs in protection of cells from VV infection.

First, the specificity binding of eH3 and eH3c to the H3 protein was tested by Western analysis and ELISA analysis (Fig. 42). Both eH3 and eH3c recognized viral H3 protein but not host cellular proteins in CV1 cells as shown by Western analysis (Fig. 42A). These samples were also probed with a set of Abs generated to the H3 and D8 recombinant proteins as a positive control. ELISA using *in vitro* synthesized H3 protein also demonstrated both rabbit polyclonal Abs to the H3 peptides recognize purified viral H3 (Fig. 42B). The affinity of eH3 Ab binding to the H3 protein was higher than that of the eH3c Ab based upon the results of these ELISA. Together, these studies confirm that anti-sera to H3 peptides recognize the H3 Ag produced in virus infected cells as well as purified recombinant H3 protein.

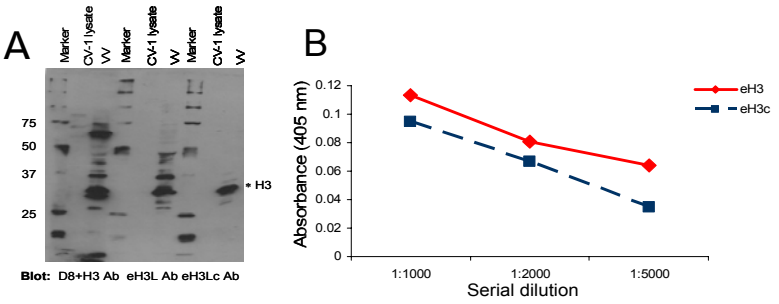


Fig. 42 Specific binding of eH3 and eH3c Abs to VV encoded H3 and recombinant H3 protein. A. Specific recognition of VV H3 protein by eH3 and eH3c Abs. Equal volumes of CV-1 cell lysate and VV purified from CV-1 cells were resolved on SDS-PAGE followed by immunoblotting with eH3, eH3c or a mixture of Abs to H3/D8 proteins. An H3 protein specific Ab and a D8 specific Ab were used as controls to confirm the migration of H3 protein on electrophoresis. Data is representative of five individual experiments. Monomers of H3 protein are marked by * at approximately 35 KD. A protein at 37 KD was also frequently detected by the H3 Abs and may represent a form of viral H3 with increased glycosylation. B. Binding of eH3 and eH3c Abs to purified recombinant H3 protein. *In vitro* synthesized H3 protein was coated on ELISA plates followed by the addition of serial diluted eH3 or eH3c Abs. Goat anti rabbit IgG Fc-HRP and ABTS were then added sequentially for detection in these ELISA. Data shown in panel B was ELISA done by Xiaoping Gu using recombinant H3 produced by the author.

To further investigate if these two Abs to H3 peptides were functional in blocking virus infection, flow cytometry analysis and T cell assays were performed. Vaccinia immune globulin or VIG, is a concentrated pooled plasma from recent VV human vaccinees potentially purified by serial ethanol precipitation [267]. VIG was used in our analysis as a positive control since VIG is known to neutralize VV and block host infection. Pre-immune sera (PI) harvested from rabbits before immunization with the $H3_{44-61}$ peptide was used as a negative control which failed to react with purified virions in ELISA (experiments by K. Toomey, not shown). To rule out non-specific binding of the test Ab to host cell Fc receptors (FcR), PriessGAD cells are treated +/- a commercial Ab which binds and blocks FcR from other Abs. VV association with human B cells in the presence of various Abs was tested *in vitro*. PriessGAD cells were exposed to the fluorescent EGFP-VV (MOI=10) in absence or presence of different Abs for 6 h followed by flow cytometric analysis to detect the green viral protein produced upon cell infection. Infection was calculated as the % of EGFP positive cells among total live cells. This data is summarized in Fig. 43A. When infected by EGFP-VV alone without pre-incubation with Ab, cellular infection efficacy was around 80%. eH3 Ab blocked VV infection as potently as VIG, i.e. only about 10% of the cells contained EGFP when virus was added plus these two Abs. eH3c or pre-immune sera (PI), on the other hand, could not protect cells from VV infection. The percent cellular infection was similar in the presence or absence of the FcR blocker. Ab treatment did not promote or block virus uptake by FcR. Next, class II function upon cell incubation with VV and the eH3 or eH3c Abs was tested using endogenous GAD as a model Ag. PriessGAD cells were infected with VV (MOI=10) +/- various Abs for 6 h. As shown in Fig. 43B, VV infection reduced class II mediated-GAD Ag presentation. Pre-incubation of VV with either VIG or eH3 reduced VV inhibition of GAD Ag presentation by blocking the ability of the virus to inactivate class II molecules. eH3c and PI, however, were both ineffective in protecting class II function. Again the FcR blocker did not affect GAD Ag presentation by cells in these assays.

A

% EGFP+ cells	FcR blocker	
	-	+
Control	1.03	0.58
EGFP-VV alone	77.09	82.51
EGFP-VV+VIG	7.2	8.13
EGFP-VV+eH3	12.87	9.61
EGFP-VV+eH3c	81.19	83.61
EGPF-VV+PI	84.15	75.97

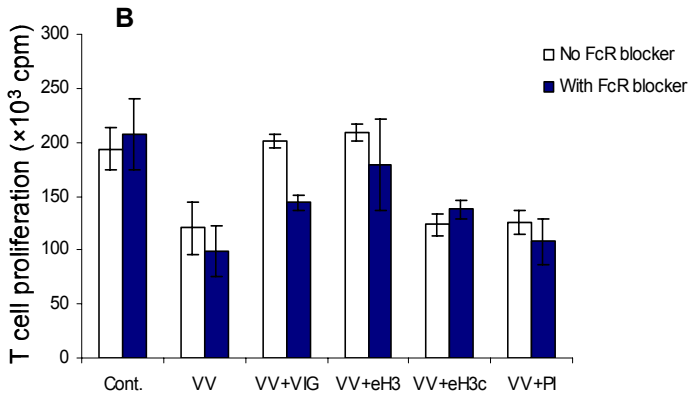


Fig. 43 eH3 Ab reduced VV infection and preserved class II function. A. Reductions in VV infection were observed in the presence of the eH3 Ab. PriessGAD cells +/- FcR blocker reagent were exposed to EGFP-VV (MOI=10) in the presence of different Abs for 6 h before cell fixation and flow cytometric analysis. Methods were described in detail in Materials and Methods section. The percent EGFP positive cells were detected only for live cells. B. Preservation of class II function by eH3 Ab. PriessGAD cells +/- FcR blocker were exposed to VV (MOI=10) +/-Abs for 6 h before GAD specific T cell addition. At least three separate experiments were carried out.

Thus, we demonstrated here that two viral envelope proteins D8 and H3 were expressed in VV infected cells. An Ab specific for the H3 extracellular epitope (eH3) bound to the intact H3 protein. This eH3 Ab blocked VV infection of host cells and preserved class II function upon exposure to VV.

DISCUSSION AND FUTURE DIRECTION

1. Mechanisms underlying VV disruption of MHC class II function

The goal of this thesis was to investigate the effects of VV infection on APC function and the activation of CD4 T cells. Perturbation of MHC class II function may represent an important mechanism of VV-induced immunoevasion, which influences the development of anti-viral immunity during VV infection. Correlating with previous investigations [94-96], studies here demonstrated that VV readily infected APCs but not T cells. VV-induced APC dysfunction and specifically altered class II presentation were demonstrated in both primary APCs and immortalized cell lines (Fig. 3). Virus-induced changes in class II presentation correlated with the level of cellular infection. Our prior studies [97] failed to detect a soluble mediator released from VV infected cells. Also, in blocking VV infection of cells using an Ab to viral H3 protein, we found class II presentation was preserved (Fig. 43). Thus, virus infection of APCs appears to be directly required for virus-induced alterations in class II presentation by these cells. Heat treatment (60˚C, 1 h) disrupts VV replication and gene expression, and heat treated virus no longer altered class II presentation by APCs (Fig. 4). UV treatment of virus, which blocks early gene expression and virus replication, did not ablate the effects of VV on class II presentation [97]. Yet, treating cells infected with VV with a viral polymerase inhibitor, AraC did partially overcome the loss of class II function at late stages of infection (Fig. 4). AraC blocks the expression of viral late gene products [202]. Together these results suggest a viral late gene product likely present in infectious virions may be responsible for altering class II presentation in APCs.

1.1 Inhibition of host protein synthesis

The precise events leading to virus-induced alterations in the class II pathway remain poorly defined. Multiple steps within this pathway may be perturbed by VV following virion entry into cells and the transition from early to late stages of virus infection. Yet few studies have addressed the temporal changes during VV infection that might

influence class II presentation by APCs. Class II dysfunction was observed within 1-2 h after VV infection with a progressive decrease in class II presentation as virus infection transitioned from early to late stages [97]. In epithelia and some tumor cells, VV infection was shown to disrupt host protein synthesis within the first 1-2 h of infection [106]. Using the well characterized protein synthesis inhibitor-CHX, it has also been demonstrated that inhibition of protein synthesis results in a loss of class II presentation [22, 44]. Thus, host protein synthesis inhibition was tested as one possible mechanism by which VV might induce class II dysfunction. Class II protein total cellular expression (by Western analysis, Fig. 7) and surface expression (by flow cytometric analysis, [97]) did not change even after 24 h infection of B cells. Synthesis of new class II subunits was detected at both 6-14 h after cell infection (Fig. 10). However, mRNAs encoding class II α subunit were found to significantly decrease during both *in vitro* and *in vivo* VV infection (Fig. 8). The relative long life of class II protein (> 24 h) in human B cell lines may account for the sustained presence of class II protein subunits, even as virus inhibition of class II gene transcription was observed [215].

Besides class II molecules, several other host mRNAs including ACTA1 (encoding actin) and GAPDH decreased in a time dependent manner upon VV infection of APCs (Fig. 8). Although reductions in host mRNA appeared to be a general phenomenon upon VV infection, some host genes were spared possibly so the virus could exploit these host proteins to survive and replicate or due to virus-induced cell stress. For example, host HSPA10 mRNA and corresponding HSC70 protein levels did not decrease with VV infection. Thus, this HSC was used as a control for our studies. HSC70 is closely structure-related to the stress-induced heat shock protein 70 kD (HSP70), the latter increases upon VV infection [268].

1.2 Loss of Ii and destabilization of class II molecules

While screening the expression levels for other components of the class II pathway, the relative abundance of an important chaperone for class II, Ii was observed to be significantly reduced at both mRNA and protein levels following VV infection of APCs

(Fig. 10-12). This reduction in Ii expression was temporally correlated with the later stage of virus infection when class II presentation heavily ablated (compared Fig. 3 and 10). Virus-induced reduction of Ii was a general phenomenon observed using several human B-LCL, human PBMCs and murine splenocytes upon both *in vitro* and *in vivo* VV infection. Levels of functional Ii (class II associated Ii) were diminished along with total Ii expression during VV infection (Fig. 13). Ii is important for class II αβ maturation and the presentation of endogenous and exogenous Ag as well as peptides. Thus, reductions in cellular Ii especially the class II associated Ii during VV infection likely contributes to a loss of class II function [22]. Notably, studies with replication-deficient virus also revealed decreased Ii expression in infected cells. For example, exposing cells to UV irradiated VV lead to both loss of class II function and a reduction in cellular Ii (reference [97] and Fig. 10). Viral replication inhibitor-AraC partially preserved class II-mediated Ag presentation and Ii expression simultaneously (Fig. 4 and 10). Consistent with our results, Ii loss induced either by genetic manipulation (antisense oligodeoxynucleotides) or inhibitors of protein synthesis (CHX) also significantly reduce MHC class II-mediated Ag presentation [20, 22].

On the other hand, enhancement of Ii expression by either transfection or knock-in is known to increase the function and folding of some class II alleles [21]. Similarly, we observed that over-expression of cellular Ii preserved class II-restricted Ag presentation in both professional and non-professional APCs upon VV infection (Fig. 25-27, 29). By cell fusion to increase Ii gene copy number or transfection of cells with class II +/- Ii plasmids, we demonstrated that higher Ii expression helped preserve class II presentation upon exposure to VV (Fig. 25 -27). Notably, we also found that cells transfected with Ii have higher surface class II expression and likely more stable class II molecules compared to cells without Ii (Fig. 26 and data not shown). Studies have shown Ii promotes class II folding and intracellular trafficking [269]. Enhancement of cellular Ii level by infection cells with recombinant VV encoding Ii also partially preserved class II function (Fig. 29). Together those studies suggest that Ii loss likely contributes in part to VV-induced class II dysfunction.

Ii chaperones class II from the ER to the endosomal network. Ii deficiency leads to misfolding for some class II alleles in the ER [34]. Class II molecules in the absence of Ii can associate with ER resident stress proteins such as GRP94, ERP72, GRP78/Bip and calnexin [270-272]. These ER chaperones binding to class II molecules could result class II accumulation in the ER and induced stress. Stress protein targeted class II molecules could also be retrotranslocated to the cytoplasm and degraded by the ubiquitin-proteasome system [273]. In our proteomics study, we observed that levels of stress proteins such as Bip and HSP70 were increased upon VV infection (data not shown). The accumulation of class II molecules in the ER also could prevent their transit to endosomes and the cell surface, steps required for function. Experiments are ongoing to examine class II association with ER stress proteins upon VV infection. Whether ER stress is induced during virus infection is unclear but could be linked to accumulation of host or viral proteins in this compartment.

Reductions in cellular Ii levels may disrupt class II function by comprising class II dimeric structure. Alterations in class II structure with VV infection of cells, are consistent with our previous finding that VV destabilizes class II-peptide binding [97]. Additionally, when compared the peptides bound with low or high affinity to class II molecules, we observed that class II presentation of the low affinity peptide was more dramatically reduced by VV infection (Fig. 9). Thus, class II destabilization caused by Ii reduction may further compromise the class II pathway in the context of VV infection.

Early studies demonstrated that Ii processing and expression regulates class II transport [274, 275]. Thus, although the steady total and surface class II expression remained constant up to 24 h of VV infection, it remains possible that the intracellular distribution or routing of class II molecules may be altered in cells exposed to virus. This hypothesis could be further tested using confocal or electron microscopy. A previous report demonstrated abnormal aggregation for some but not all class II alleles in Ii deficient cells [35]. Yet during VV infection, such high molecular mass aggregates of class II DR4 were not observed (Fig. 14). Another co-factor of the class II pathway chaperoned by Ii, DM may be destabilized, retained in the ER or degraded more quickly in the absence of Ii [35, 42, 43]. However, here we found no changes in DM protein levels in human B cell

lines infected with VV (Fig. 14). The differences between various species (human *vs.* mouse) or cell types (B cells *vs.* fibroblasts) may lead to this disparity.

Both mRNA and protein Ii expression were diminished upon VV infection, with the reduction in mRNA temporally preceding protein loss (Fig. 10). Thus, we concluded that inhibition of Ii synthesis was likely the primary cause of reduced cellular Ii levels. Decreased [^{35}S]-methonine incorporation into newly synthesized Ii protein upon VV infection was consistent with this conclusion. To further investigate the negative effects of disrupting host protein synthesis on Ii expression and class II presentation, CHX was used. CHX mimicked VV effects on Ii protein expression and class II function (Fig. 15-18). However, some differences were noted between the negative effects of CHX and VV. For example, only VV but not CHX altered Ii mRNA expression. This result was consistent with the fact that CHX works at the translational stage to disrupt protein synthesis. CHX treatment of cells reduced Ii protein levels much faster than cellular VV infection (Fig. 17). Furthermore, the inhibitory effect of CHX on class II peptide presentation was more selective than observed with VV infected cells (Fig. 18). In line with previous studies [22, 44], only the presentation of peptides which require intracellular processing, could be efficiently inhibited by CHX. While presentation of peptides that bind directly to cell surface class II molecules was more resistant to CHX treatment. In contrast with CHX, VV infection of cells potently inhibited the presentation of all peptides tested (Fig. 9, 18 and data not shown).

These observed differences in the effects of VV and CHX on class II function suggest that virus-induced host protein synthesis inhibition not likely the only factor contributing to cellular Ii reduction during VV infection. Another pathway potentially contributing to Ii loss during VV infection i.e. enhancement of Ii degradation, was also investigated. Using the [^{35}S]-methonine pulse-chase assay, we observed decreased degradation of Ii during the early stages of VV infection while Ii degradation increased at later stages of infection (Fig. 20). Using the cysteine protease inhibitor leupeptin [195], we detected reduced expression of the Ii fragment, LIP upon later stages of VV infection (Fig. 19). This may suggest faster or more efficient Ii processing. This more rapid degradation of Ii

might be due to cellular catabolism or export of Leu, the protease inhibitor, due to virus infection. Indeed, VV encodes a cysteine protease I7, important for core protein cleavage [276]. Virus derived I7 could bind Leu that otherwise would act on cellular cathespins to limit Ii processing. Consistent with this, either increasing the amount of Leu in culture media or using an irreversible cysteine protease inhibitor, E64, resulted in increased LIP fragment accumulation. These results confirmed that cysteine protease(s) sensitive to Leu catalyze the late stages of Ii processing in VV infected cells (Fig. 19). Accelerated LIP degradation during the late stages of virus infection was also demonstrated by elevated surface CLIP levels on VV infected APCs (Fig. 20). CLIP is a terminal fragment formed during Ii degradation by cysteine proteases such as Cat S or Cat L. CHX treatment, by contrast, resulted in equally decreased cellular Ii and LIP levels in the presence of Leu (Fig. 19). These latter results indicate that disruption of host protein synthesis did not directly alter Ii processing.

To further investigate the mechanisms leading to altered Ii processing in virus infected cells, the expression of cathepsins as well as other proteases required for Ii processing was studied. AEP is a peptidase reported to initiate Ii processing in human B cells [16, 31]. Decreased AEP mRNA (LGMN) levels were observed upon VV infection (Fig. 24). Cat S controls the late stages of LIP processing in many APCs including B cell [32]. Upon VV infection, cellular Cat S expression decreased at both the mRNA and protein levels (Fig. 21). With reductions in both cellular AEP and Cat S levels, Ii processing was expected to slow or halt. Indeed, at early stages of VV infection, Ii processing did slow (Fig. 20). Remarkably however, LIP degradation accelerated at late stages of VV infection (Fig. 19 and 20). Even after treatment with a 2nd dose of Leu or an irreversible cysteine protease inhibitor E64, LIP processing appeared to become faster. These results suggested another protease may have been induced or activated to replace Cat S and to facilitate Ii processing during late stages of VV infection. In thymic epithelial cells, Cat L substitutes for Cat S to proteolyze LIP and promote class II maturation [17, 232]. Both Cat L mRNA (CTSL) and mature, proteolytically active form of Cat L increased upon VV infection (Fig. 21). This result suggests Cat L may be responsible for the accelerated Ii degradation at late stages of VV infection. However, other cellular cysteine proteases

and/or viral counterparts like the I7 protease, may also function to speed Ii processing. Further screening to map changes in cellular and viral protease expression by Western analysis will be required to further address this question. The observed increase in Cat L expression was not limited to *in vitro* VV infection of human B-LCL. Similar results were detected upon *in vitro* and *in vivo* infection of murine cells (Fig. 22 and 23). The explanation for enhanced Cat L expression during VV infection remains to be determined. Several possibilities exist. Viral induction of host Cat L may facilitate infection, as has been observed for mouse hepatitis virus type 2 and acute respiratory syndrome coronavirus [277, 278]. Or VV could use Cat L to promote extracellular matrix degradation like tumor cells [279, 280]. Alternatively, elevated Cat L may represent a stress response by the host during infection, accelerating processes such as apoptosis [281, 282]. Correlated with our data that CHX treatment of APCs did not change Ii processing (Fig. 19), inhibition of host protein synthesis alone failed to alter cellular Cat S or Cat L protein levels at short times of exposure (Fig. 21).

Besides processing of Ii, AEP, Cat S and Cat L are also involved in Ag proteolysis [31, 283]. Reduced AEP and Cat S may lead to aberrant Ag processing and consequently disrupt class II presentation. Even increased Cat L, may not be beneficial for Ag proteolysis. Like Ii, these class II-restricted Ags may be degraded faster than they should be. Thus, Ag presentation would be down regulated. mRNAs of other proteases mainly involved in Ag processing, such as Cat B and D, both decreased upon VV infection (Fig. 21). The reduction in Cat B and D expression may also contribute to abnormal Ag proteolysis. Whether VV-induced changes in cathepsin expression can really influence Ag processing remains to be determined. Interestingly, the peptide content of class II was altered during cellular infection with an attenuated VV, MVA [284]. Changes in the type of peptides as well as their terminal residues were found upon comparing class II DR ligands from MVA or uninfected cells. However, not all proteins involved in Ag processing are changed by VV. For example, the disulfide bond reductase-GILT protein remained constant up to 14 h of VV infection. Remarkably, EEA1 mRNA did not change upon VV infection (Fig. 24). EEA1 is an early endosome marker involved in endosome fusion and endocytic transport [240]. The recent discovered acidic entry pathway for VV

[78] requires endosome. That's perhaps the reason for VV to keep EEA1 expression constant.

Besides serving a chaperone function in class II pathway, Ii also plays a role in CD1d-restricted lipid presentation. Ii associates with CD1d, redirects CD1d to endosomes to increase CD1d-mediated lipid presentation [285]. CD1d function is also perturbed by VV infection of APCs [89, 90]. A reduction in cellular Ii expression during VV infection may also influence CD1d presentation. Studies are ongoing to examine whether over-expression of Ii preserves CD1d function upon VV infection.

Thus, we conclude that VV induced Ii reduction likely contributes to loss of class II function during late stages of VV infection. Both a reduction in Ii synthesis and an alteration in Ii processing together contribute to the observed loss of cellular Ii protein (Fig. 44). Alterations in cellular lysosomal proteases during virus infection may influence Ag processing and degradation. This could be further examined in the future. Over-expression of cellular Ii preserved class II function, confirming the importance of Ii in maintaining class II function. Whether viral proteins associate with class II molecules during infection, and whether the loss of cellular Ii facilitates these interactions are important questions for future studies.

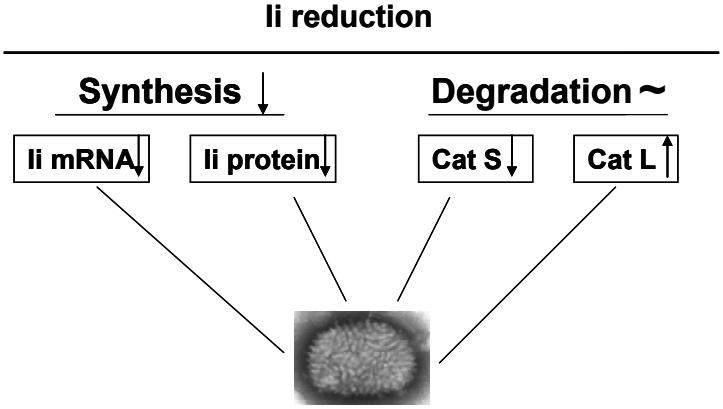


Fig. 44 Mechanisms underlying Ii reduction upon later stages of VV infection. See details in the text.

1.3 Viral Ag presentation

Inhibition of class II presentation was detected within 1-2 h of VV infection [97]. Ii reduction, however, was not obvious until 6-8 h after VV infection (Fig. 10). Therefore, Ii loss likely contributes to class II dysfunction during to the late stage of the virus life cycle. Other mechanism(s) that could contribute to the loss of class II function at early stages of infection were investigated using both *in vitro* and *in vivo* approaches. Our published studies revealed destabilization of class II molecules which resulted in diminished peptide binding [97]. Consistent with this, we found that peptides which form less stable complexes with class II molecules, were more susceptible to VV inhibition (Fig. 9). Although not investigated here, competition by VV for other resources required by the class II presentation pathway may represent another mechanism, contributing to defects in class II function. Thus, the virus might sequester cellular chaperones or components of intracellular trafficking which are required by class II molecules.

VV enters the cell by both fusion at neutral and low pH [78, 79]. The latter pathway is also the route for internalization and proteolytic processing for exogenous Ag which are presented by class II. Thus, viral proteins delivered into the endosomal pathway may be readily processed and yield peptides which can be presented by class II molecules for recognition by CD4 T cells. VV specific CD4 T cells and Ab have been detected in human vaccinated subjects as well as in mouse models, confirming viral proteins are processed and likely presented by class II molecules [75, 83, 91, 286], that's also the basis for VV being used as a vaccine. The importance of MHC class II presentation during VV infection is also demonstrated by increased lethality of MHC class II deficient mice upon VV challenge [83]. Using an *in vivo* mouse model of VV infection, we demonstrated that VV specific CD4 T cells (VV-CD4 T cells) recognize viral peptides bound to class II molecules after *in vitro* infection of APCs by wild type VV or treatment of APCs by mutant rVV (Fig. 30-33). These virus specific T cells, VV-CD4 T cells also recognized VV derived peptides added to class II^+ APCs (Fig. 34). This successful induction of VV specific CD4 T cells after *in vivo* VV inoculation of mice was consistent with the detection of virus specific T cells in human vaccinees' blood samples [287] and

primed murine splenic cells [81]. The activation of VV-CD4 T cells by APCs was dependent on the *in vitro* dose and the incubation time of added virus. Treatment of APCs for longer incubation times and at low MOI (i.e. MOI=0.1) using wild type VV, induced higher CD4 T cell responses. Immunosuppression and disruption of class II function by VV were considered as likely reasons for the reduced CD4 T cell responses observed after APC exposure to high MOI (i.e. MOI=1) using wild type VV. Inactivation of VV by UV or heat resulted in higher CD4 T cell responses compared with untreated replication competent VV. Compared with untreated virus, these inactivated viruses disrupt class II function less efficiently ([97] and Fig. 4). The greater CD4 T cell responses induced using inactivated VV support our hypothesis that viral immunoevasion is responsible for the altered class II function at high viral MOI. A recombinant virus BSSH expressing truncated D8 (a viral envelope protein that binds to class II), also induced higher CD4 T cell responses than wild type VV. These studies with the BSSH rVV suggest that late viral protein D8 may play a role in perturbing class II function. However, T cell activation by APCs infected with the BSSH virus was still lower than that induced by APCs incubated with heat inactivated VV. There are two possible explanations for this observation. Truncated D8 may still bind to class II and disrupt presentation more severely than heat treated virus. Or heat treated virus likely contains unfolded viral proteins which are more easily processed to peptides for presentation by class II, leading to stronger T cell activation. When heat inactivated VV was used to immunize mice, no VV specific CD4 T cells were detected, suggesting *in vivo* the inactivated virus was less immunogenic (Fig. 32). This inability of heat treated VV to induce VV specific immune response was in agreement with several previous studies, showing that heat-inactivated VV is ineffective in protecting hosts from orthopoxvirus infection [206].

Class II presentation of synthetic VV peptides was also evaluated and demonstrated a hierarchy of CD4 T cells were activated *in vivo* after virus inoculation (Fig. 34). Similar to a published report [81], VV specific CD4 T cells isolated from virus infected mice typically targeted viral late gene products, the majority of which are viral structural proteins not associated with virulence. While the published study used IFN-γ to monitor T cell activation, here T cell IL-2 production was readily detected in response to viral

peptide added APCs. Notably, VV epitopes identified using murine T cells here are similar to those identified using human T cells [287], indicating conservation of VV specific CD4 T cell responses between different mammals.

The main cytokines VV-CD4 T cells produced were Th1 cytokines, IL-2 and IFN-γ (Fig. 30-33). Similar Th1 cytokine production was observed using wild type or recombinant VV infection [288-292]. Interestingly, a study with Ii deficient mice found CD4 T cell responses were biased towards Th1 differentiation and Th1 cytokine production [293]. Thus, VV-induced Ii reduction may in part contribute to this Th1 polarization. Using recombinant VV encoding IL-2 (rVV-IL2), IL-2's function in VV infection has been studied. IL-2, working together with IFN-γ, increases VV clearance by activation of NK and CD4 T cells in both immunocompetent and immunodeficient mice [151, 294]. Induction of local immunity and chemotaxis by rVV-IL2 has been reported [295]. This immunostimulatory function of IL-2 has been extensively used in infection and cancer research.

The importance of IFN-γ in host immunity to VV is demonstrated by the increased susceptibility to virus infection in IFN-γ receptor deficient mice compared to wild type mice [296]. It has been long established that VV infection induces IFN-γ production in CD4 T cells [288]. IFN-γ activates cellular PKR; 2'-5' oligoadenylate synthetase (OAS); and inducible nitric oxide synthase (INOS) to fight against virus infection [297]. On the other hand, VV has evolved several mechanisms to block IFN-γ action. VV produces a decoy receptor to disrupt IFN-γ anti-viral function [52]. Deletion of the viral gene B8R which encodes the decoy IFN-γ receptor, significantly reduces VV virulence [298]. Blocking of the B8R encoded decoy receptor by a murine IFN-γ mimetic helped C57BL/6 mice survive a lethal VV challenge [299]. VV also encodes a phosphatase, VH1. VH1 inactivates signal transducers and activators of transcription (Stat) 1, an important component within the IFN-γ signal transduction pathway [300]. The viral gene E3L produces ds-RNA binding proteins to prevent IFN-induced PKR activation [60]. The E3L gene product also counteracts OAS activation [61]. The K3L viral gene product blocks PCR activation by mimicking its substrate eIF-2a [62]. PKR- induced host mRNA

translation inhibition and OAS-mediated host RNA degradation are therefore blocked by VV. This may favor infected cell survival and offer an environment for VV replication. As for IL-2, IFN-γ has been tested as a therapeutic for infection and cancer.

Another potential role for Th1 cytokines is to antagonize Th2 cytokine responses [301]. Th2 cytokines are mainly involved in allergy and parasite infection. A previous study showed that increased apoptosis of Th1 cells led to Th2 predominance in atopic diseases [302]. Thus, we proposed that VV infection would antagonize a Th2-mediated allergic disease. Indeed, using an OVA-induced asthmatic mouse model, we observed significantly decreased inflammation in mice co-infected with VV compared to uninfected mice (Fig. 35). The observed allergic airway inflammation reductions in the eosinophilic inflammatory infiltrate and mucus production with VV infection of asthmatic mice may be due to the decreased IL-5 and IL-13 production respectively (Fig. 36). Although atopic dermatitis has long been established as a contraindication for VV vaccination [48], these studies using an asthma model suggest VV inoculation or immunization may not be detrimental in patients with airway allergic diseases. In fact, the amelioration of pre-existing asthma suggests that exposure to some viruses or rVV might be useful as a treatment for asthma. These studies also suggest that VV or related attenuated viruses could be considered as a vaccine for individuals with airway allergic diseases. Additional research is required using mouse models before testing VV as a prevention and/or treatment for asthma in humans.

Th2 cytokine production was greatly reduced in animals with asthma that had been co-infected with VV. However, expression of pro-inflammatory cytokines, IFN-γ and IL-17 was significantly enhanced in the VV infected asthmatic mice compared to healthy animals exposed to VV alone (Fig. 36). Although not severe, inflammation and mucosal production could be observed in mice inoculated with VV alone. In animals treated to induce asthma, these small abnormal lesions were not observed upon VV inoculation (Fig. 35). In addition, Th2 cytokines antagonize Th1 cytokine-mediated anti-VV responses [303], thus, the reduction of Th2 cytokine production in VV infected asthmatic mice may also help VV clearance. Accordingly, VV titers in the lungs of inoculated

mice were lower in the asthmatic mice compared with healthy animals challenged with virus. The enhanced VV clearance was likely due to the increased IFN-γ production. IL-17, also a pro-inflammatory cytokine could not be detected in mice exposed to VV alone. Pre-existing asthma dramatically increased IL-17 expression (Fig. 36). IL-17 may play important roles in inflammation and pathogenesis during asthma via recruitment of neutrophils [251]. Since the asthmatic lesions were mitigated upon VV infection, we would propose here IL-17 production was not detrimental to animals. IL-17 may decrease OVA-induced bronchial eosinophilia by reducing IL-5 levels [304]. The role played by IL-17 in OVA-induced asthma model may depend on the timing of events such as virus challenge. The OVA mediated asthma model involves Ag priming (by i.p.) followed with Ag boost (by i.n. or inhalation) to induce allergic sensitization. Before the OVA inhalation boost, IL-17 promotes bronchial neutrophilic influx. During inhalation, however, IL-17 becomes an asthma inhibitor through blocking IL-5 expression [304]. In our model VV was administered after OVA inhalation, thus, VV-induced IL-17 production in asthma progression, should be inhibitory. Thus amelioration of asthma was observed (Fig. 35). Besides mediating autoimmunity, IL-17 is also involved in protective immunity against various infections caused by bacteria, viruses, and fungi [252, 257, 262, 305-307]. We proposed that similar to a Gram negative bacterium *Klebsiella pneumoniae* infected mouse model [308], IL-17 was involved in clearance of VV from the lung. Correlating with our results on IL-17 and VV, a recent study supports a role for IL-17 in VV clearance [252]. Here the authors demonstrate the reduced virulence of recombinant VV encoding murine IL-17 compared to wild type VV. However, using a different vaccinia viral vector with a leader sequence, an earlier report drew the opposite conclusion that a recombinant VV expressing murine IL-17 was more virulent than wild type VV [154]. More detailed comparisons of these two different murine IL-17 expressing VV are required before a conclusion can be drawn. Nevertheless, more recent literature [252] showed that IL-17-deficient mice were more susceptible to wild type VV infection than control mice. This result suggests IL-17 may function in protection against viruses. Interestingly, IFN-γ and IL-17 do not act independently. IL-17 induces IFN-γ production [307]. On the other hand, IFN-γ limits IL-17 production to contain inflammation [309]. Some studies also suggest T cells producing IL-17 are plastic and

can shift to produce IFN-γ [310]. Thus, we conclude that IFN-γ and IL-17 might work together to increase VV clearance in an asthma plus VV infection model.

The diminished Th2 cytokines may promote asthma mitigation and cellular immunity against VV, as well as decreasing VV specific humoral immunity. The titration of VV specific Abs would address this possibility. Besides secreting cytokines, Th1 cells also induce the production of IgG2b and IgG2c Abs while Th2 cells elicit the secretion of IgG1 and IgE Abs [311, 312]. These Th-related Ab isotypes have important implications in autoimmunity as well as infectious diseases. One essential mechanism by which Ab help to clear pathogens is via activation of complement system to lyse the infected cells. Thus Ab production, Ab isotype switching as well as complement activation are potentially interesting future directions that could be pursued in this virus-asthma model.

Besides the antagonism between two types of Th cytokines, VV infection may block asthma development by directly disrupting a residual OVA Ag presentation by APCs. Using splenocytes taken from asthmatic mice +/- VV infection as APCs and CD4 T cells isolated from those mice as responders, class II presentation of OVA in the presence or absence of VV could be tested using Ag presentation assays. The presentation of OVA and/or VV by APCs from healthy mice to treated/control CD4 T cells could be tested in parallel. OVA presentation by class I could also be tested using CD8 T cells and assays to monitor cytokine production. Such studies would directly investigate the function of APCs and T cells from asthmatic mice in the context of a virus infection.

The profile of cytokines produced by CD4 T cells from twice inoculated mice (2×VV) were similar to those secreted by CD4 T cells after the primary exposure to VV. The main cytokines secreted by the 2×VV-CD4 T cells were IL-2 and IFN-γ (Fig. 37). Also as in primary response, no detectable IL-17 was produced by 2×VV-CD4 T cells. Investigations using CD8 T cells from mice inoculated twice with virus (2×VV-CD8 T cells) revealed significant granzyme B and IL-17 production (Fig. 38). Granzyme B is a marker for CD8 T cell activation. Although first discovered in activated memory $CD4^+$ T cells, IL-17 is produced by $CD8^+$ memory T cells after stimulation [313]. IL-17

producing by CD8 T cells is important for immune defenses against *Klebsiella pneumoniae* infection [256, 260-262]. A recent report demonstrated IL-17 producing CD8 T cells protected naïve recipients from lethal influenza A infection [10]. We inferred IL-17 expressing in CD8 T cells might also play a role in VV specific T cell recall responses. IL-17 is repressed by Th1 cytokines [314], perhaps limiting the amount of IL-17 detected in the present study.

IL-17 exerts its pro-inflammatory function mainly by expansion and recruitment of myeloid lineages (monocytes and neutrophils) with the production of growth factors (such as granulocyte colony-stimulating factor) and CXC chemokines respectively. It also activates T cell responses through the induction of the co-stimulatory molecule intercellular adhesion molecule (ICAM). Other IL-17 linked mechanisms regulating immunity include enhancement of the local inflammatory environment by expression of IL-6 and PGE2 [251]. Damaging inflammation is the basis for IL-17 mediated autoimmune diseases. Yet this cytokine also promotes the destruction of invading pathogens like the Gram negative bacterium *Klebsiella pneumoniae* and possibly viruses. The role IL-17 plays in cancer progression may depend on the angiogenic activity of IL-17 and tumor immunogenicity. For example, IL-17 promotes tumor growth by enhancing angiogenesis in a murine fibrosarcoma model [315]. While this cytokine also inhibits murine hematopoietic tumors by increasing cytotoxic T cell generation [316]. By adding different APC populations to *in vitro* polarized IL-17 producing CD4 and CD8 T cells, we detected the abilities of these cells to promote IL-17 production (Fig. 39). B cells followed by DCs, were better at promoting CD4 T cell IL-17 production. For CD8 T cells, DCs were most effective among the three types of APCs. We inferred that the IL-17 promoting ability of APCs could be used to control production of this cytokine *in vivo*. As for autoimmunity or certain cancers, IL-17 production could be decreased focally by blocking the activity of distinct APC type. For other cancers or infections, IL-17 production may be enhanced by activation of specific APC type. Thus, IL-17 production could be manipulated to regulate disease and inflammation.

In summary, we conclude that VV-derived peptides are presented by class II molecules. Yet the efficiency of even viral Ag presentation may be reduced due to virus-induced changes in the class II pathway. Whether viral peptides compete to fill the class II ligand binding groove and act as competitive inhibitors remains possible. Yet the observed reduction in viral Ag presentation suggests the virus may have evolved other mechanisms to regulate class II function such as manipulating cellular Ii levels. Class II presentation of VV epitopes correlated temporally with the inhibition of exogenous or self Ag presentation (Compare Fig. 3 and 30). This was interesting as it suggests very rapid proteolysis of viral proteins to yield peptides for class II, again supporting virus entry into endosomes containing class II. Notably, heat treated virus was also processed and presented by class II again likely due to endocytic uptake of the virus. However, heat inactivated VV failed to induce VV specific CD4 T cell activation and proliferation *in vivo*. By contrast, animal inoculation with infectious, replicating VV lead to the development of VV specific CD4 T cells which secrete cytokines IL-2 and IFN-γ. These Th1 type cytokines are important in further mobilizing host defenses to virus infection as well as potentially antagonizing Th2 mediated diseases such as asthma. Consistent with the induction of Th1 cytokines in response to virus, in animals with induced asthma VV intra-tracheal inoculation significantly ameliorates bronchial eosinophilia inflammation and mucosal secretion by abrogating IL-5 and IL-13 expression respectively. IL-17 was also detected in spleen cells after VV infection of asthmatic animals. VV clearance was slightly improved in asthmatic mice compared with mice infected with VV only. IFN-γ together with IL-17 may be important in viral clearance. Interestingly, IL-17 secretion was detected in CD8 T cells after virus inoculation, suggesting a role for these cells in viral clearance or associated inflammation.

2. Candidate viral genes linked to VV-induced class II dysfunction

To define potential viral or host gene products which may be important in disrupting class II presentation pathways, it is important to consider the biology of this virus. Using virus infected Hela cells, it was demonstrated that the expression of VV genes is programmed and divided into at least three stages. Viral proteins involved in the early

stages of transcription are synthesized late in infection and packaged in the virions. Early gene transcription initiates within 20 m of infection and peaks at 1.5 h. Early mRNAs are translated into growth factor such as secreted epidermal growth factor-like protein (encoded by C11R), immunoevasive molecules such as soluble IFN-γ receptor-like protein (encoded by B8R), as well as enzymes and factors for DNA replication such as DNA polymerase (encoded by E9L) or intermediate gene transcription such as capping enzyme (encoded by D1R) and VITF 1 (vaccinia virus intermediate transcription factor 1, encoded by E4L). Intermediate transcription starts 1 h after infection and peaks at 2 h which products are required for late gene expression such as VLTF 1 (vaccinia virus late transcription factor 1, encoded by G8R). The transcription of late genes begins about 2 h of infection and continues to increase [1]. Some of the late gene products are structural proteins including envelope proteins (such as D8 and H3, encoded by D8L and H3L respectively) and core proteins (such as P4a, encoded by A10L). Late genes also encode factors or enzymes for early transcription such as VETF (vaccinia virus early transcription factor, encoded by A7L and D6R) and RAP94 (a 94 KD viral polypeptide which associate with RNA polymerase, encoded by H4L). Viral DNA replication occurs 1-2 h after infection and is essential for intermediate and late gene transcription [1, 317].

Using UV irradiated VV, we demonstrated that virus replication is not essential for disruption of class II presentation [97]. Similar results were obtained with the virus specific DNA polymerase inhibitor AraC (Fig. 4). These suggest a possible role for viral late proteins in regulating class II presentation. Consistent with this, viral early genes B1R and H5R likely do not play a role in disrupting the class II pathway (Fig. 5). At later times of infection, AraC treatment of cells could partially preserve class II function, supporting a role for viral late gene product(s) in VV disruption of class II presentation. Indeed, the products of two late viral genes, D8L and H3L bind to class II molecules (P. Li, data not shown). VV encoding truncated D8L partially preserved class II presentation (Fig. 31). Thus, future studies should explore the role of these two viral genes in both *in vitro* and *in vivo* experiments for their immunosuppressive properties. If class II function could be preserved by a deficiency in either of these two genes, this again would support hypothesis that specific viral proteins contribute to VV-induced class II dysfunction.

Using I-A^b restricted VV specific CD4 T cells from C57BL/6 mice, peptides derived from 13 different viral proteins were identified as immunodominant in mice (Table 3 and [81]). Most of these peptides are derived from late gene products. Peptides derived from D8 and H3 proteins are included among these immunogenic epitopes. Using CD4 T cells derived from vaccinees' PBMCs, viral genomic wide screening of peptides that could induced human VV specific CD4 T cell response, was carried out [287]. Similar to those viral peptides that could induce murine CD4 T cell responses, immunodominate peptides from virion structural proteins expressed during late stage of VV infection were recognized by human CD4 T cells. For instance, the six epitopes most frequently recognized by human CD4 T cells include products of A3L (encoding p4b precursor of core protein 4b) and A10L (encoding core proteins), D13L, H3L, L4R and WR148 (encoding an inclusion-forming protein). It has also been demonstrated that A24R and D1R (mRNA capping enzyme large subunit) could be presented by class II DR1 to human CD4 T cells [318]. Gene products of I1L, A20R and A28L have also been proposed as candidates yielding epitopes which might bind class II DR1 based on predictive algorithms [319]. Thus, there may be considerable overlap between human and murine CD4 T cell specificity for VV proteins. Whether the presentation of these viral peptides influences class II presentation of other non-viral Ag has yet to be tested. It might also be informative to examine class II presentation using rVV lacking some of these immunodominant viral Ags.

3. H3 peptide specific Ab in the preservation of class II function upon VV infection

Circulating Abs can exert immune function by inactivating the inoculum and reducing pathogen transmission. Several studies have shown the importance of Ab for immunity against VV infection [91, 152]. Passive immunization by transferring Abs is efficient in protecting a host from poxvirus including VV infection [265, 266]. Administration of sera from vaccinees (VIG) has been successful in treating VV infection and preventing additional complications. However, since routine smallpox vaccination has been stopped after eradication of smallpox, the supply of VIG is limited. One goal is to find an alternative for VIG. H3, an envelope protein involved in viral entry, has been tested as an

immunogen. One paper demonstrated that recombinant H3 could be recognized by vaccinees' sera [287], indicating H3 specific Ab was induced after vaccination. Another paper showed that mice passively immunized with anti-H3 protein sera survive a lethal VV challenge [264]. Notably, Ab to H3 protein appears to be an important component of VIG [320]. Using a peptide located within the extracelluar domain of H3 to immunize rabbits, Ab to H3 was generated. As expected, this Ab to H3 (eH3) is effective in blocking VV infection and preservation of class II function (Fig. 43). Specific host cell receptors for VV have not been identified, yet virion binding to the cell surface appears to precede virus endocytosis and fusion [78]. The eH3 Ab may block viral entry by disrupting H3 binding to host cell surface heparin sulfate.

Our data suggests that Ab to a single epitopes within the viral H3 protein can block VV infection and consequently maintain class II function in APCs. Potentially peptide specific Ab could be used as an alterative for VIG. Ongoing experiments in our lab are testing the protective effect of Ab to H3 in an i.n. VV mouse model. Here, H3 peptides are being tested as immunogens to induce active immunity against VV in a mouse model. Whether this peptide will induce protective or virus neutralizing Abs in mice remains to be determined. It may be possible to enhance immunity by coupling H3 peptides that induce T cell responses and Ab production.

4. Design of attenuated VV

The ultimate aim for viral research is to design effective vaccine or therapeutics. For VV, a safer vaccine is essential for protection of immunodeficient patients and hypersensitive individuals from poxvirus-induced pathogenesis. Since VV has been used as a vector to express foreign genes from tumor antigens and pathogens, an improved VV vaccine may have broad implications. Both innate and adaptive immunity are important for VV clearance. Yet VV has evolved several mechanisms to evade host immunity. To design an improved attenuated VV, the focus must be on increasing host immunity while reducing viral factors which disrupt host defenses. Ideas on such improved vaccine strategies are offered here.

4.1 Enhancement of host immunity

In this thesis, we observed a reduction in Ii expression correlated with the late stages of virus infection and class II dysfunction. Over-expression of Ii partially preserved class II function. Recombinant VV encoding Ii (mIi-VV) inhibited class II function less severely than wild type VV (Fig. 29). Thus, we propose Ii encoding rVV could be used as an attenuated VV to preserve class II function. This recombinant virus must be tested *in vivo* in a mouse infection model to determine whether enhanced cellular and humoral immunity can be induced. In the long term, Ii encoding rVV may be useful for promoting defenses in some immunodeficient individuals.

VV infection induces Th1 cytokine production. Studies suggest expression of IL-2 by VV (rVV-IL2) may be useful in boosting host vaccination. For example, rVV-IL2 inoculation helped to inhibit SIV/HIV progression by promoting the induction of mucosal as well as systemic humoral and cellular immunity [321]. This recombinant virus also deterred head and neck carcinoma growth by increasing cellular immunity [322]. Notably, co-expression of mucin-1 (MUC-1) and IL-2 by a recombinant virus led to metastatic breast tumour regression in advanced stage patients [323]. Recombinant VV encoding IFN-γ (rVV-IFNγ) has also been shown to have improved potency in infection and cancer therapy. rVV-IFNγ blocked infection of cells and mice by various viruses including VV itself [324, 325]. rVV-IFNγ inoculation inhibits murine breast cancer [326]. Engineering recombinant VV to express cytokines and Ii may offer a novel vaccine vector.

4.2 Reduction of virulent genes

As another approach to design better VV-based vaccine, one could try deletion of virulence or immune suppressive factors. D8, a viral envelope protein that binds cell surface chondroitin sulfate [194] also appears to bind class II molecules (data not shown). VV with a truncated D8L gene (BSSH virus) induced higher VV specific CD4 T cell responses than wild type VV (Fig. 31), suggesting this modified virus was less suppressive. However, when BSSH was used to prime mice, CD4 T cell responses were

not as robust as those observed with wild type VV (Fig. 32). Thus, further analysis of the key domains within D8 and their role in blocking class II function is needed. Alternatively, addition of an adjuvant may be needed to increase the antigenicity of BSSH. Another envelope protein, H3 was also found to bind to class II molecules (P. Li, data not shown). Thus, VV with mutations in the H3L gene should also be tested to see if this restores class II presentation during virus infection.

Inactivation of VV by either UV irradiation or heat, led to improvements in class II molecule presentation compared with replication-competent VV, likely due to reduced immune suppression by the modified viruses (Fig. 31). However, these modified viruses failed to induce VV specific CD4 T cells *in vivo* (Fig. 32). The observation that inactivated VV provides little to no protection against orthopoxvirus infection, has been confirmed in several studies [206]. However, the antigencity of these inactivated VV could be increased by several methods. It has been demonstrated that multiple inoculations of heat inactivated VV protects rabbits from lethal challenge using rabbitpox by inducing host production of neutralizing Ab [245]. VV inactivated by strictly controlled UV irradiation can induce humoral immunity in rabbits and monkeys [246]. VV inactivated by antimicrobial nanoemulsion of soybean oil and detergent is effective in inducing both humoral and cellular immunity against VV [327]. Thus, by applying multiple inoculation [245], carefully controlling UV exposure [246] or using different methods to modify virus [327], improved non-replicating vaccines could be achieved.

Taken together, VV may disrupt class II presentation by reduction of Ii at late stages of infection. At early times of virus infection, destabilization of class II molecules and possibly competitive binding of class II to viral Ag may contribute to class II dysfunction. Other possibilities may also explain the early stages of VV-induced class II dysfunction (Fig. 45). The results here have implications in terms of our understanding of both VV pathogenesis and host immune responses to poxvirus infection. Finally, these studies may offer new insights into the design of attenuated vaccines in the future.

Early stages	Late stages
1. Viral Ag/peptide competition for class II?	Ii reduction
2. Viral Ag/peptide binding to class II molecules?	1. Synthesis inhibited by reduction of Ii at both protein and mRNA levels.
3. Disruption of class II association with rafts?	2. Degradation altered by regulation of cathepsin S and L.
4. Changing in class II sorting?	

Fig. 45 The effects of VV on class II-mediated Ag presentation.

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Conferences Attended:

1. Viral Modulation of MHC class II Mediated Antigen Presentation (Abstract, Oral presentation and Poster), **Wang, N.**, Blum, J. S., *37th Autumn Immunology Conference* (Chicago, IL, November 21-24, 2008)
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Publications:

1. **Wang, N.**, Weber, E, and Blum J. S. (2008) Loss of invariant chain expression with vaccinia virus infection of APC (revision)
2. Li, P., **Wang, N.**, Yao, Y., Alkhatib, G., Brutkiewicz, R.R., Chang, C.H., and Blum J. S. (2008) Preservation of antigen presenting cell function: role of vaccinia virus H3 antigen specific antibodies (submission)
3. **Wang, N.** and Blum J. S. (2008) Protective effects of invariant chain during vaccinia virus induced MHC class II dysfunction (preparation)
4. Li, P., **Wang, N.**, Zhou, D., Yee, C. K., Chang, C. H., Brutkiewicz, R. R. and Blum, J. S. (2005) Disruption of MHC class II-restricted antigen presentation by vaccinia virus.

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