A Secreted Viral FcγR-Binding Protein Contributes to Immune Evasion by Cowpox Virus

by

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Abstract

Cowpox virus (CPXV) is a member of the genus *Orthopoxviridae* (OPXV), which have been evolving alongside the mammalian immune system for millennia. CPXV possesses the largest genome and number of potentially immune-modulating proteins amongst the OPXV. Our laboratory began studying CPXV immune evasion molecules in the hope that it would reveal new mechanisms for immune evasion and control. Previous studies conducted in our laboratory characterized two proteins (CPXV 012 and CPXV 203) that CPXV uses to limit the presentation of viral peptides by MHC Class I in infected cells, thereby evading the $CD8^+$ CTL immune response. Prior studies demonstrated that the related monkeypox virus (MPXV) is able to inhibit the activation of human T cells *in vitro* following anti-CD3 stimulation. This inhibition is mediated by the transmembrane B22R protein encoded by MPXV 197, which is an ortholog of CPXV 219. However, CPXV 219 is only effective in inhibiting human, but not mouse, T cells since murine T cell stimulation is restored in the absence of the MHC Class downregulating ORFs CPXV 012 and 203. We extended our studies to determine if there were other T cell inhibitors encoded by CPXV. The intent of these studies was to potentially identify new therapeutics for the treatment of T cell-mediated diseases such as multiple sclerosis.

As detailed within this work, we identified the secreted immune modulating protein, CPXV 014, by its ability to inhibit naive murine splenic T cell activation by plate-bound anti-CD3 and anti-CD28 antibodies. With further study, it became evident that CPXV 014 does not act directly on T cells, but instead possesses an FcγR-binding activity that

blocks the binding of agonist antibody to neighboring “accessory cells.” Blocking binding of agonist antibodies to FcγR on non-T splenocyte cells prevents the augmentation of T cell activation.

Our current working hypothesis is that CPXV 014 is functioning as a specific agonist for the inhibitory Fc receptor, CD32B, which is expressed by various antigen presenting cells and, notably, B cells. CPXV 014 appears to limit the proliferative response of B cells *in vitro*, and may therefore play a role in limiting the humoral response against the virus, which is critical for effective control and protection against disease. Furthermore, our T cell studies have demonstrated a largely-overlooked facet of *in vitro* antibody-driven T cell activation assays - the augmentation of T cell responses by neighboring FcγR-bearing non-T cells. This reliance of antibody-driven T cell activation on FcγR-bearing cells should be taken into consideration by anyone working with these assays. The ability of FcR-bearing cells to bind a wide range of antibodies from multiple species and affect their function has significant bearing on the design and activities of agonist and blocking antibodies used as laboratory reagents and clinical therapies.

While we initiated this project to search for potential T cell inhibitors, it seems that we have discovered a B cell inhibitor which may potentially lead to new therapies for other autoimmune diseases characterized by a dysregulated humoral immune response, such as rheumatoid arthritis or systemic lupus erythematosus.

Preface

This dissertation encompasses my original work but research is a collaborative process, so there is work within which was performed either by or with the direct assistance of several people. The initial T cell assays identifying CPXV 014 (Figures 6, 7, 8 and 10) were performed by myself together with David Edwards of the Früh laboratory. *In vivo* experiments using the Δ14 CPXV and recombinant CPXV 014 proteins were performed by either David Edwards (Figures 17, 18 and 30) or the laboratory of Dr. Jeffrey Nolz, OHSU (Figure 19 and 31). The human T cell activation assay (Figure 27) was performed by Erika Hammarlund in the laboratory of Dr. Mark Slifka, OHSU. Genomic sequencing of viral DNA was performed by the core facilities at OHSU and the analysis of the sequences was performed by Dr. Daniel Malouli, Früh laboratory. Deletion mutant viruses were produced by the laboratory of Dr. David Pickup, Duke University. Plasmids for the His-tagged recombinant protein constructs were a gift from the laboratory of Dr. Daved Fremont, Washington University. Further thanks are also offered to the Fremont laboratory for sharing their unpublished data demonstrating the binding of CPXV 014 and its orthologs to FcγR. I have endeavored to credit these individuals for their contributions throughout this work, and without their efforts it would not have been possible.

Acknowledgements

This work would simply not exist without the help and support of many amazing people over the years and I owe all of them a debt of gratitude. First off, I need to thank Klaus for allowing me to work and learn in his laboratory, for all of his invaluable advice and direction during the ups and downs of this project, and for helping to teach me the wide range of skills that being a good scientist entails, from choosing experimental controls to selling your story. Thanks to my thesis advisory committee, for all their support and direction. A huge thanks goes to all members of the Früh lab throughout the years for their help, support and encouragement. In particular, I wish to thank Dina Alzhanova, whose assistance in all things poxviral was of immeasurable value, and for always being available for help with troubleshooting tricky cloning dilemmas. Marieke Verweij, Elizabeth Rowland and Jennie Womack all provided much-needed editing help with this dissertation, as well as advice and discussions in all things scientific and far, far beyond. There are so many members of other labs in the VGTI and OHSU community that have provided help and advice it would be impossible to list them all. Of them I especially want to thank, in absolutely no particular order, Scott Wong, Ann Hill, Jeff Nolz, David Parker, Mark Slifka, Erika Hammarlund, Daniel Malouli, Sjoerd van der Worm, Damir Alzhanova, Minsha Manoharan, Jen Gardell, Kati Marshall and Haley Wolford down in the vivarium, the Thursday immunogroupies and every single mouse I euthanized.

Thanks to my mother and father, who raised me to love learning and instilled me with the sense of awe and wonder with the natural world that I still experience every

day. Thanks to Dr. Shawn Skerrett of the University of Washington for giving me my first opportunity as a researcher; if he had not taken the chance of hiring me as an unexperienced laboratory technician I probably would never have realized that I wanted to pursue immunology as a career. Thanks to the innumerable friends and family without whose support I probably would not have found the daily fortitude to continue, you know who you are. Thanks to all the folks at Blackbird pizza and the Reel M'Inn for feeding me. And my cat Smudge, whose destructive, narcissistic and irrational behavior has kept me sane for thirteen years.

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A Note on pox viral gene terminology:

The standard nomenclature for pox viral genes is in a seeming state of constant redefinition, an area of frustration for students of the field. Initially, genes from VACV and VARV were classified based on the HindIII fragments resulting from genomic digest, with additional designations coming from the direction of transcription (present in

the gene name but omitted from the protein name) and a number representing the gene's position in the fragment. In recent years as more poxviruses are identified and sequenced, genes have been referred to more by their order in the genome. In this work, VACV genes are referred to using the classical terminology, while CPXV genes are referred to using their short gene number as listed on vipbrc.org.

Selected abbreviations:

APC – antigen presenting cell

BCR – B cell receptor

BFA- brefeldin A

CPXV - Cowpox virus

Crm - Cytokine response modifier

CTL – cytotoxic T lymphocyte

DAG – diacylglycerol

ECTV - Ectromelia virus

ER – endoplasmic reticulum

EV - enveloped virus

FcR – Fc receptor

ICS - Intracellular cytokine staining

IP_3 – inositol triphosphate

ITAM – immunoreceptor tyrosine-based activation motif

ITIM - immunoreceptor tyrosine-based inhibitory motif

MCMV - murine cytomegalovirus

MHC – major histocompatibility complex

MPXV - Monkeypox virus

MV - mature virus

ORF - open reading frame

PBMC – peripheral blood mononuclear cells

PHA - phytohaemagglutinin

PIE - poxviral immune evasion domain

PMA - phorbol myrsistate acetate

PTK – protein tyrosine kinase

SCP-2 - SECRET-containing protein 2

SECRET - Smallpox encoded chemokine receptor

SH2 – Src homology 2

TCR – T cell receptor

VACV - Vaccinia virus

VARV - Variola virus

WT - wild type

ZAP-70 - zeta-associated protein kinase 70

Chapter One: Introduction

I. Cowpox and *Orthopoxviridae*

I A. Background

The genus *Orthopoxviridae* (OPXV) is a group of large, complex, double-stranded DNA viruses. All of the OPXV are antigenically similar such that immunity against any particular member affords cross protection against the others [1]. The most infamous member of the OPXV is Variola virus (VARV). VARV infects only humans and caused the ancient blight of smallpox. The World Health Organization spearheaded a global program of vaccination using the Vaccinia virus (VACV) and declared smallpox to be eradicated from the natural environment in 1979. Since its elimination from the human population and the halt of universal vaccination, global immunity to Variola and to other orthopoxviruses which the vaccine protects against has waned. If smallpox were to be reintroduced via laboratory accident or bioterrorism, humanity today is largely unprotected. Additionally, other orthopoxvirus species cross over into humans via zoonotic infection, such as occurred during the US monkeypox (MPXV) outbreak in 2003 [2]. Cowpox (CPXV) virus is one such zoonotic member of the OPXV occurring naturally throughout western Europe. Despite its name, CPXV is primarily a pathogen of rodents, though it sometimes makes the jump into humans or other species demonstrating a broad range of potential hosts [3-5].

Poxviruses are relatively large, brick-shaped viruses typically measuring around 200 x 300nm, at the size limit of what can be discerned by light microscopy.

They are surrounded by one or two lipid membranes, depending on the method of cell egress. Within the virion is a dumbbell-shaped core containing genomic DNA with two lateral bodies on either side. Numerous viral proteins are carried in the virion itself [6].

Even though the poxviruses, like all viruses, are obligate intracellular pathogens, they possess much of their own machinery for viral replication. Their large linear genomes (often >200 kilobase pairs and containing hundreds of open reading frames) are organized in such a way that the highly conserved genes necessary for replication are located within the midsection of the genome. The open reading frames near the terminal flanking regions of the genome encode a vast array of genes used for immune evasion and species or tissue tropism [7]. The genomic termini themselves are covalently closed hairpin structures containing terminal inverted repeats [6]. Presumably having immune evasion and tropism genes clustered towards the flanks of the genome enables the poxviruses to duplicate, rearrange and mutate these genes at a greater frequency in order to adapt to different hosts and conditions [8]. One such mechanism documented describes transient accordion-like multiplication and expansion of viral genes in response to host antiviral responses, such that the extra copies generated may acquire beneficial mutations without the risk of completely disrupting the existing gene [9].

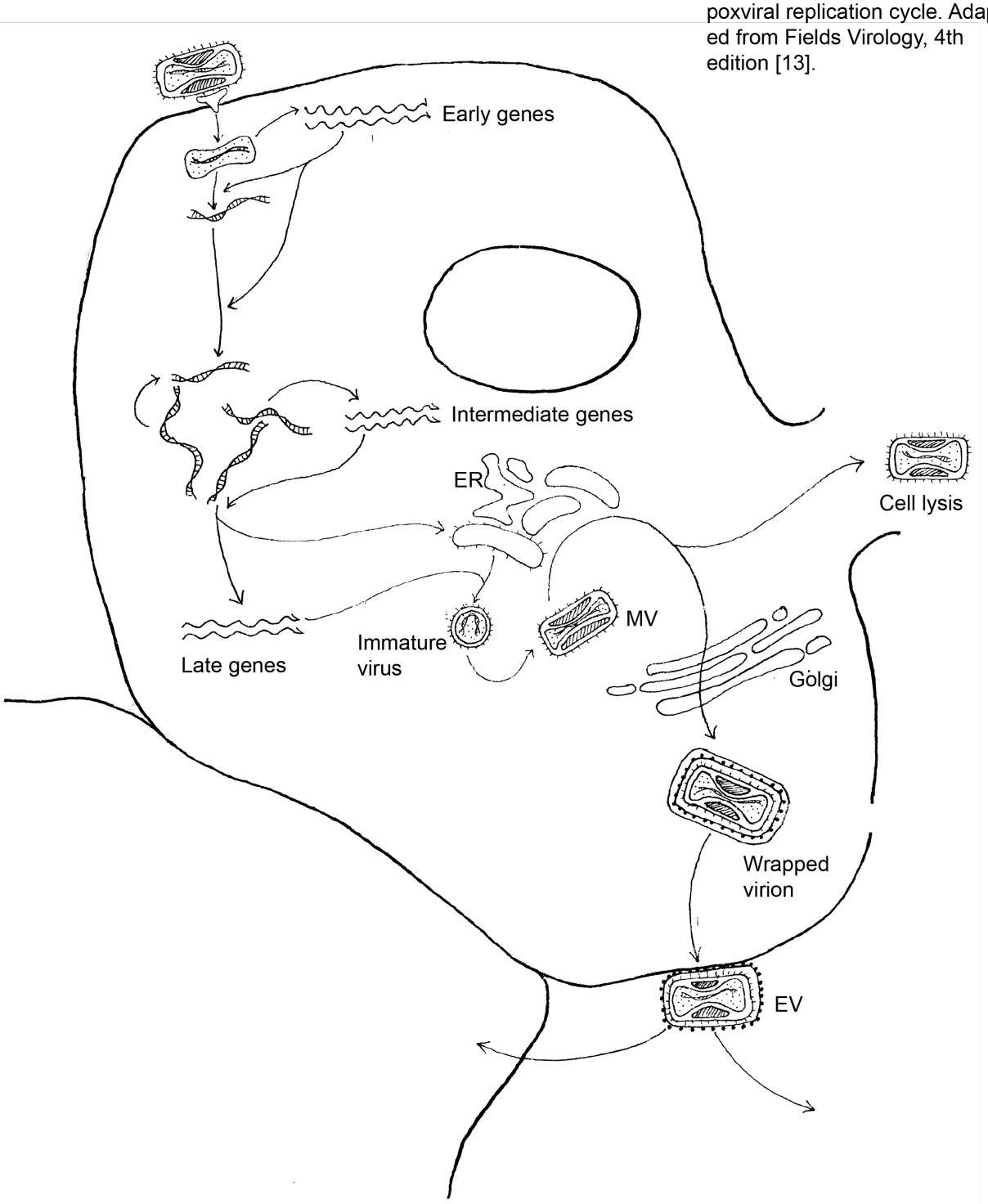
Among the OPXV, Cowpox virus possesses the largest number of these immune modulating and tropism "accessory genes," many of them uncharacterized. Even though CPXV is not a major human pathogen, its possession of so many novel immune modulating genes makes it worthy of study. Cowpox has been adapting to mammalian immune systems for millennia, far longer than the science of immunology has existed.

We embarked on this project with the idea that the study and characterization of CPXV immune evasion genes will bestow a greater understanding of immunity, and potentially lead to new therapies for immune and inflammatory disorders. There are multiple precedents for the development of poxviral proteins as treatments for immune disorders. Several of these drugs have been tested in animals and clinical trials (such as the SERP-1 protein from myxoma virus), indicating that this is a potentially rich area of research for new therapies [10-12].

I B. Replication and life cycle

Poxviruses replicate in a complicated process occurring in the cytoplasm **(Figure 1)**, unlike the replicative cycles of most double-stranded DNA viruses which occur in the nucleus. Most knowledge of OPXV replication is based on research into the VACV lifecycle and is discussed here in terms of that virus, though the overall strategy is the same for CPXV and other OPXV [14]. The inability of VACV, CPXV and other related poxviruses to successfully replicate in certain cell and host types largely seems to occur not at the levels of cell attachment and entry (as they are able to get inside of most cells), but rather by blocks to permissive infection (such as type 1 interferon responses) within the cell while the virus is attempting to complete its cytoplasmic replication cycle [15].

Figure 1 Poxvirus life cycle Diagram representing typical poxviral replication cycle. Adapted from Fields Virology, 4th edition [13].



I B1. Attachment:

No single cell receptor has yet been identified as being necessary for poxvirus attachment to host cells, although several glycosylaminoglycans on the surface of various cell types appear to be used [16]. Unlike many viruses which rely on just one or two, the poxviruses utilize a large complement of proteins to attach to target cells. Given the broad range of host and cell types which they are able to infect, it appears that multiple and/or ubiquitous receptors are used. The field of poxviral attachment and entry is further complicated by the fact that infectious virus exists in multiple forms, differentiated by the presence or absence of an additional viral envelope in a portion of viral progeny (enveloped virus, EV), which attach to cells differently [17, 18]. The majority of infectious particles possess only a single viral envelope and are termed mature virus (MV). There are known to be at least 20 proteins in the MV membrane, several of which seem to mediate cell binding, and there are more within the EV outer envelope [6]. Most of what we have learned about poxviral entry is restricted to MV as EV virions are fragile and prone to losing their outer envelope by standard methods of viral purification [17, 18]. In the specific case of VACV as the model OPXV, the D8 protein can bind chondroitin sulfate and the A27 and H3 proteins (H3 being highly conserved among poxviruses) [19] are reported to bind heparin sulfate [20, 21]. The A26 protein binds cell surface laminin [22]. Heparin can inhibit viral entry, but not completely and is likely dependent on the type of cell [16]. All of this evidence supports a model in which a multitude of poxviral proteins possess an overlapping repertoire, enabling binding to many host cell types via multiple potential entry receptors. No one

viral protein appears to be completely necessary for VACV infectivity [23-25] though some are also involved in MV and EV formation.

I B2. Cell entry:

Once successfully attached to the target cell surface, the primary method of entry (at least for VACV) appears to be via a low-pH-dependent endocytic pathway. Another mechanism involves lipid rafts and fusion at the plasma membranes [14, 19, 26].

A group of at least 8 proteins associated with VACV MV (including A21, A28, H2 and L5) [27-29] interact together to form an entry-fusion complex which has been shown to be needed for cell entry but dispensable for attachment [29]. Regardless of the specific receptors and mechanism, the MV membrane must fuse with either the plasma or an endosomal membrane at some point in order to allow the viral core to enter the cytoplasm and for viral gene expression to occur. Since VACV entry is facilitated by low pH and inhibited when endosomal acidification is prevented, it seems that this endosomal route is preferred [30]. VACV EV sheds its outer membrane via a ligand-dependent and non-fusogenic mechanism using the viral glycoproteins A25 and B5 before it can fuse the inner viral membrane with cellular membrane and deliver the viral core to the cytoplasm [31].

VACV, and presumably CPXV and the other poxviruses, also prevent superinfection of previously infected cells by blocking a step in between adsorption and early gene transcription [32]. Furthermore, the VACV A33 and A36 proteins are expressed at the surface of infected cells and induce actin polymerization upon contact with potentially superinfecting EV, which repels the virions from the surface of infected

cells and towards uninfected cells, allowing for more effective spread to uninfected cells [33].

I B3. Replication and egress:

Poxviruses are notable in that they encode most of their own machinery for gene transcription and genomic replication. Unlike most DNA viruses which typically use cellular proteins within the nucleus for gene expression and genomic replication, poxviral replication takes place within intracytoplasmic "virus factories." Within these factories crescent-shaped structures (thought to be derived from the ER) are processed into immature virus and then intracellular MV. MV represent the majority of infective particles and are released with cell lysis. A portion of the generated MV is further trafficked along microtubules and the endosomal or Golgi pathways where they acquire an additional lipid envelope, becoming EV. EV buds from the cell to either be released into the extracellular milieu or remain associated with the cell surface. Contact with the cell surface by EV causes the virus to induce actin polymerization, propelling itself outwards [34] and this actin-based motility is important for cell-to-cell spread [14, 35-39]. This cell-to-cell spread leads to the formation of syncytia in infected cultures in some strains and cell lines, particularly at acidic pH [30, 40-42].

II. Adaptive Immune Activation Pathways

In order to informatively discuss CPXV and immune evasion, some background into a few of the key adaptive immune cell types that are activated is required. Though the initial work of this project involved studying T cell activation and inhibition, it has

since led us to investigate the activation and regulation of other cell types, such as B cells. An overview of some of these relevant cell types and pathways follows.

II A. T cell activation

T cells are critical mediators of the adaptive immune response to infection. T cell precursors arise from hematopoietic stem cells in the bone marrow, and then migrate through the blood stream to the thymus where they undergo positive and negative selection based on the affinity of their antigen receptor for self peptides displayed by MHC complexes on the surface of thymic epithelial cells [43]. Upon leaving the thymus they circulate throughout the body to encounter cells bearing both self- and foreign-derived peptides nested in surface-expressed major histocompatibility complex (MHC) molecules. The T cells are activated to proliferate and carry out their effector functions upon encountering a cell bearing an MHC molecule with the foreign peptide epitope for which their T-Cell Receptor (TCR) is specific.

A T cell that has yet to encounter its specific cognate MHC:peptide is considered to be “naive.” For a naive T cell to become activated, binding of the TCR by MHC:peptide must be accompanied by an additional signal transduced through costimulatory receptors such as CD28. Costimulatory ligands are upregulated by activated or infected antigen presenting cells (APC). Binding of the TCR complex and co-receptors initiates a signal transduction cascade beginning with the recruitment of cytosolic tyrosine kinases and culminating with the activation of T cell effector functions.

There are many different T cell subsets that differ in their roles and functionality, including $CD4^+$ helper and $CD8^+$ cytotoxic T cells (CTLs), natural killer T cells and

Foxp3^{+} T regulatory cells (Tregs). Also, the state of T cell activation and differentiation varies within each population. As such, no single model or outcome of T cell activation will apply to all cells. Nevertheless, the basic signaling mechanisms are largely the same.

II A1. Early events.

The initial interaction in T cell activation is between the TCR and an APC bearing an MHC molecule carrying the antigenic peptide for which that TCR is specific. The TCR is a multi-protein complex including CD3 molecules bearing cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs). The variable region of the TCR which gives it specificity for a certain MHC:peptide complex is generated during T cell development via site-specific recombination events, and T cells leaving the thymus have been selected based on their low affinity for self peptide [43]. In addition to the TCR-MHC:peptide interaction, ligation of a TCR co-receptor is also required for activation. CD4 and CD8 perform this role by binding directly to MHC Class II (on the surface of APC) or Class I (on the surface of all nucleated cells), respectively. The Src-family protein tyrosine kinase (PTK) Lck is associated with their cytoplasmic domains. When these coreceptors bind to MHC, Lck is brought into the vicinity of the TCR complex. There Lck phosphorylates the CD3 cytoplasmic tail ITAMs. Once the CD3-ζ ITAMs are phosphorylated Zap-70, another key PTK, is recruited to them via its SH2 domain. There, Zap-70 phosphorylates at least two adaptor proteins, LAT and SLP-76. Phosphorylated LAT and SLP-76 nucleate multi protein complexes termed

"signalosomes" which serve to amplify and diversify the downstream activation signal [43].

II A2. Downstream signaling

Phosphorylated SLP-76 recruits and leads to the activation of PLCγ by inducible T cell kinase (Itk), which hydrolyzes PIP2 to produce the second messengers DAG and IP_3. DAG activates PKC and the MAPK pathway, leading to NFκB activation [43]. IP_3 serves to mobilize Ca^{2+} from the ER, the Ca^{2+} binds and activates calmodulin, which activates the phosphatase calcineurin. Activated calcineurin dephosphorylates NFAT which migrates to the nucleus and instigates transcription of IL-2 genes leading to proliferation [44].

II A3. CD28 costimulation

Early studies suggested that when naive T cells receive only signal one – the ligation of the TCR and coreceptor - they become anergic [45]. However, subsequent work has indicated that this model is overly simplistic and that the quality and duration of TCR engagement plays a major role in determining the fate of a naive T cell [46]. Nevertheless, in order for a naive T cell to become fully activated costimulation is also required. The best studied costimulatory receptor for T cells is CD28. CD28 is constitutively expressed on the surface of naive T lymphocytes and is the major costimulatory receptor needed to prime naive T cells [47]. CD28 binds to B7.1 (CD80) and to B7.2 (CD86) on the surface of APCs, and this ligation can deliver the coactivating signal necessary for naive T cell activation.

CD28 ligation contributes to T cell activation via a signaling pathway that overlaps with that of the TCR. The cytoplasmic tail of CD28 has a YMXM motif which, when phosphorylated, recruits PI3K via its SH2 domains [48]. PI3K converts PIP2 into PIP3 in the inner leaflet of the plasma membrane. Generation of PIP3 allows for the recruitment of PDK1 and Akt (PKB) by binding to their pleckstrin homology domains. AKT phosphorylates several downstream molecules which promote NFκB and NFAT activation [49]. CD28 also is required in the recruitment of PKCθ via a PYAPP motif (P187YAPA in mice) [50] and RASGRP, a GEF which feeds into the JNK and ERK pathways. When CD28 is absent, PKCθ is not efficiently localized to the immunological synapse which results in ineffective activation of NFκB and IL-2 genes [51, 52]. CD28 has also been shown to interact with Lck [53].

The IL-2 promoter contains a specific response element that binds transcription factors in a CD28-dependent fashion, demonstrating the influence of this coreceptor over this crucial pathway [44, 54]. Also, CD28 signaling has been shown to stabilize IL-2 mRNA (as well as that of other cytokines) [55, 56]. CD28 stimulation also upregulates antiapoptotic and prosurvival members of the (mitochondria-associated) Bcl family [57]. In these ways, CD28 signaling is important for preventing the induction of anergy during naive T cell stimulation.

Another important role of CD28 is to assist with the organization of lipid raft membrane architecture. Via motifs in its cytoplasmic tail, it orchestrates the immunological synapse (IS) between T cells and APC surfaces [58, 59], which is necessary for productive TCR signaling. Conversely, the fellow Ig family member

coreceptor cytotoxic T lymphocyte protein 4 (CTLA-4) has been demonstrated to inhibit the formation of lipid rafts [59].

II A4. CTLA-4 and inhibition.

The CTLA-4 gene encodes a coinhibitory receptor and is located next to CD28 on both human and mouse chromosomes [60]. It is upregulated upon T cell activation [61], (though Tregs constitutively express this receptor) [62]. CTLA-4, similarly to CD28, binds to B7.1 and B7.2. However, it binds B7.1 with a higher affinity than CD28 [63]. By delivering a negative signal to the cell and competing for CD28 ligands, signal transduction through CTLA-4 creates a negative feedback loop to control levels of T cell activation. Via its YVKM motif, CTLA-4 associates with the SHP2 tyrosine phosphatase [64] which dephosphorylates CD3, LAT and ZAP-70, countering TCR signaling. It also recruits the serine-threonine phosphatase PP2A, which may counteract PKB activity, or perhaps act as a feedback inhibitor to the inhibitory signal from CTLA-4 [65, 66]. Bi-directional signaling also occurs for some of the co-receptor:ligand interactions. The inhibitory coreceptor CTLA4 sends a signal via B7.1 or B7.2 on the surface of DCs to induce indoleamine 2,3-dioxegenase production, which then acts on the T cells to inhibit function by limiting bioavailable tryptophan [67].

Since the initial development of this "two signal model," it has become apparent that there is a whole kaleidoscope of costimulatory and coinhibitory receptors on T cells and ligands on APCs, which all serve to modulate the level and quality of T cell activation by numerous pathways. Several of these co-receptors interact with multiple ligands as well, providing more opportunity for competition and cross talk between

signaling pathways. The relative levels of costimulation and coinhibition, the cytokine milieu, and the affinity of the TCR-MHC:peptide interaction all come together to determine the extent and quality of T cell activation [68].

II A5. IL-2 signaling in T cells

In the absence of costimulation via CD28 or other means, TCR binding will often lead to a state of unresponsiveness (termed anergy) and eventual apoptosis for naive T cells. A major way in which this requirement may be circumnavigated is via signaling by the prosurvival/proliferative cytokine IL-2, which is produced by many different types of activated immune cells.

The receptor for IL-2 exists in two different forms depending on its subunit makeup. The β_c and γ_c subunits are capable of heterodimerizing to form the intermediate-affinity IL2R, and are necessary [69] and sufficient [70, 71] for signal transduction. The α subunit is dispensable for signal transduction but its inclusion in a heterotrimer forms the high-affinity IL-2R [72, 73]. The α subunit is upregulated upon TCR ligation [74].

There are multiple signal transduction pathways that can occur upon IL-2 binding to T cells, depending on the presence or absence of TCR ligation. Generally, if the TCR signaling pathway has been activated, IL-2 signaling will lead to both antiapoptotic and proliferative responses. In the absence of TCR ligation, the proliferative pathway remains inactive and only the prosurvival signal is transmitted. The mechanism for this appears to involve the presence or absence of Jak3 kinase, which is upregulated by TCR signaling and recruited to the IL2R complex [75, 76].

Current models postulate that when IL-2 binds the high-affinity IL2R heterotrimer in the context of TCR signaling, Jak3 is recruited to the γc subunit and Jak1 is recruited to a Ser-rich domain in the βc subunit of the IL2R [75, 76]. Transphosphorylation and activation leads to Jak1 and Jak3 phosphorylating other PTKs and adaptor molecules [75, 76]. One of these is Lck. Initial Lck activation requires the dephosphorylation of one tyrosine (Tyr) residue by a phosphatase that associates with the membrane proximal PROX domain of the γc subunit [76, 77]. Jak1 and/or Jak3 then phosphorylate Lck at other Tyr residues which renders Lck fully active [76, 77]. Lck then phosphorylates and activates PI3K. PI3K generates PIP3 in the inner leaflet, thereby recruiting pleckstrin-homology domain-containing proteins such as Akt, as well as providing substrate for the generation of second messengers DAG and IP_3 by PLCθ, which leads downstream to the activation of transcription factors NFAT and NFκB, leading to the further induction of IL-2 and other genes[75, 76]. Akt downstream signals for survival as well as actin cytoskeleton reorganization [76]. Another pathway induced by IL2 involves the Src-family kinase, Syk, and leads to induction of the c-myc gene, which is essential for a proliferative response [78]. Jak1 and Jak3 also mediate the phosphorylation, dimerization and activation of STAT1 and STAT5, which translocate to the nucleus and instigate the transcription of various other prosurvival and proliferative genes [75, 76].

In the absence of TCR signaling, Jak3 is absent and the proliferative response does not occur. However, activation of Lck and antiapoptotic signaling persists [75, 76]. It is also noteworthy that the IL2R signaling pathway, like most immune pathways, involves a wide breadth of redundancy and crosstalk between its different signaling cascades as well with those of other immune cell receptors, (such as the shared

involvement of Lck and Akt by the TCR). We use IL-2 during *in vitro* models of T and B cell activation to study lymphocyte proliferation and its inhibition by CPXV.

IIA6. In vitro T cell activation models

We mimic the MHC-dependent activation of murine T cells in the laboratory using plate-bound antibodies against CD3ε and the co-receptor, CD28 [79, 80]. Stimulation of the naive T cells induces a Th1 cytokine response, exemplified by TNFα production. We use intracellular TNFα staining as an indicator of T cell activation since induction of this cytokine occurs early in the T cell response**.** We also stain for IFNγ, though naive T cells, unlike mature cells, do not produce this cytokine upon activation [81], and the lack of IFNγ production in our studies provides further confirmation of the naive status of the murine T cell populations used.

T cell proliferation can be induced *in vitro* by culturing them in the presence of soluble anti-CD3 with exogenously supplied IL-2. We use this model to examine the proliferative response of naive T cells as well.

In addition to targeting antibodies against the TCR, there are many other mitogens used experimentally in the study of T cell activation pathways. These include phytohaemagglutinin (PHA, a lectin from red kidney beans), staphylococcal enterotoxin B (SEB), phorbol myristal acetate (PMA) and ionomycin. Additionally, genetically engineered mice with defined TCR specificities are available with which we can study the activation of T cells by APC presenting their specific antigenic peptide. One such model we use is the OT-I mouse line whose $CD8^+$ T cells bear a transgenic TCR specific for the SIINFEKL peptide (Ova 257-264) from ovalbumin.

When testing the responses of memory and effector T cells to TCR ligation, several other models are available. Memory T cells such as those taken from mice infected with a pathogen such as murine cytomegalovirus (MCMV), which causes a large T cell response, can be stimulated towards rapid TNFα and IFNγ responses by signaling through the TCR without costimulation by anti-CD28 [82, 83]. Another model used within the studies described was the isolation of memory T cells from LCMV-infected mice, which were then stimulated *ex vivo* with known LCMV immunodominant peptides [84, 85].

There are numerous readouts for T cell activation and functions. These include target cell killing, surface markers for differentiation states (such as CD11a, CD11b, CD44, CD62L etc.), calcium flux, blotting of signaling intermediaries and others. Most of the techniques used within this dissertation are based on flow cytometry analysis of cytokine production and cellular proliferation.

II B. B cell activation

II B1. BCR signaling

The B cell receptor (BCR) is made up of a membrane bound immunoglobulin (Ig) molecule associated with the Igα-Igβ heterodimer (CD79a and CD79b, disulfide linked). The physical mechanism of how the BCR signals is more controversial than it is for the TCR. The paradigm has been that aggregation of BCR complexes leads to B cell activation, though some hypothesize that the BCR actually exists as oligomeric complexes in the "off" state, and that antigen binding serves to dissociate these complexes and relieves them of inhibition [86-88].

Regardless of whether clustering or dissociation is the mechanism, when the specific antigen binds to the BCR **(Figure 2)**, the Src family kinase, Lyn, phosphorylates ITAMs in the cytoplasmic tail of the Igα-Igβ heterodimer. Another Src family kinase, Syk, can then bind to the ITAMS via its SH2 domain and become activated [89]. Syk then phosphorylates the Blnk adaptor protein, which can then serve as the scaffold for the signalosome assembly [90, 91]. Signalosome complex members recruited include Btk, Vav-1 and PLCγ2 [92].These proteins also possess pleckstrin homology domains that allow them to bind PIP3 in the inner leaf of the plasma membrane [93]. Btk phosphorylates PLCγ2 which is activated to cleave PIP3 into IP_3 and DAG [93]. IP_3 signals for the release of Ca^{2+} from the ER (by binding the IP_3 receptor). DAG binds to RasGRP and members of PKC family.

Past this, the signaling pathways branch out into multiple protein kinase cascades, (including the MAPK and Erk pathways), and GEFs (such as Ras and Raf) [93]. These pathways eventually lead to the translocation of various transcription factors into the nucleus, such as NFκB, AP-1 and NFAT, culminating in the activation, proliferation and differentiation of the B cell.

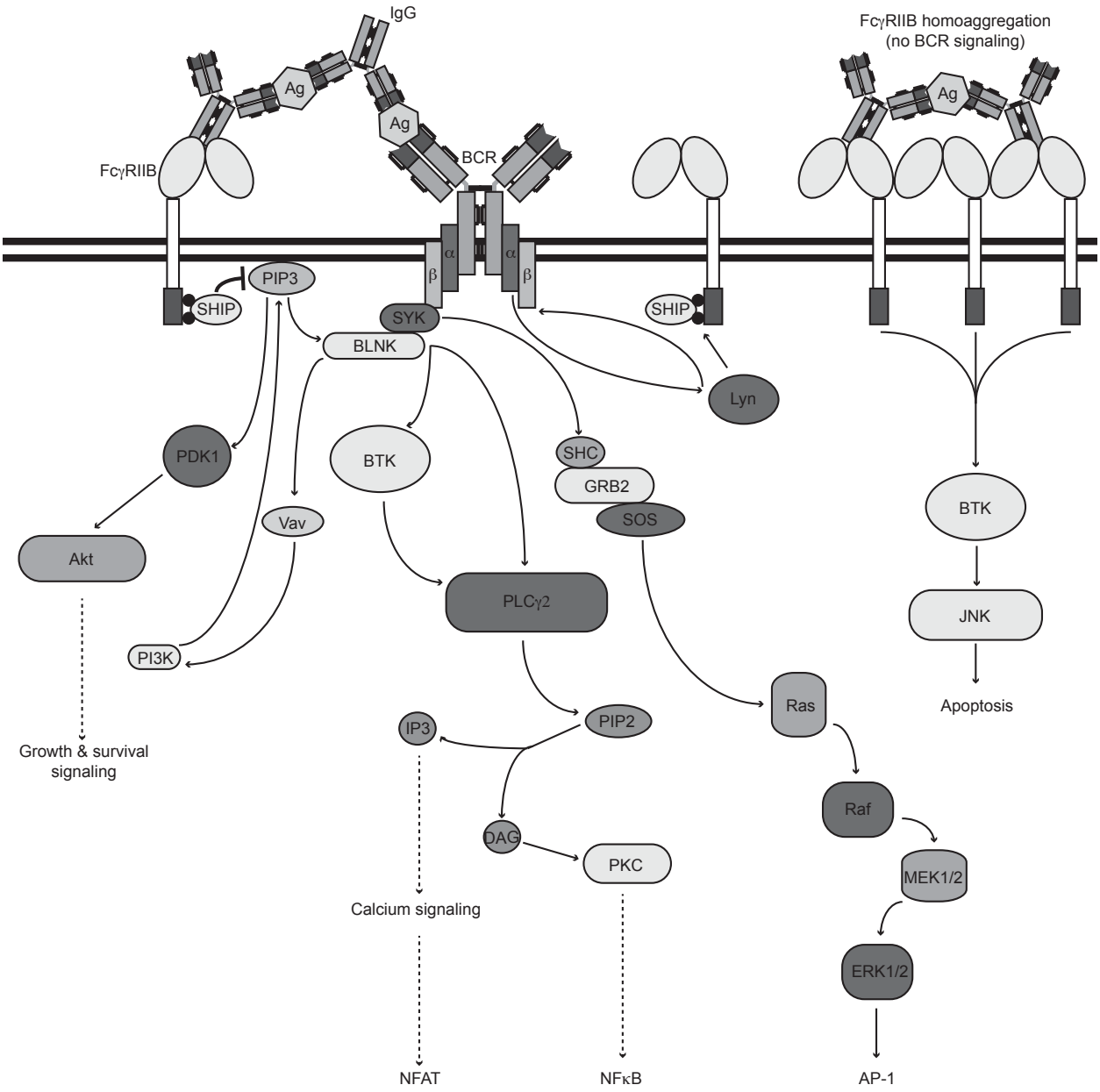


Figure 2 BCR and FcγRIIB signaling. A simplified schematic of major B cell receptor signaling pathways and their inhibition by FcγRIIB. FcγRIIB possesses ITIMs which are phosphorylated by Lyn, leading to the recruitment of SHIP and the depletion of the pool of PIP3, leading to diminished recruitment of pleckstrin-homology domain containing proteins (particularly Brk, PLCγ2 and Vav) and inhibiting signalosome formation. Lyn is the predominant Src family kinase in B cells and phosphorylates not only the ITAMs in the Igαβ heterodimer, but also key activating and inhibitory mediators such as Btk, Syk, FcγRIIB and CD22.

II B2. B cell costimulation

CD19 is a B cell and follicular DC transmembrane protein which plays an important role in B cell stimulation (as well as in non-antigen driven B cell development) [94]. It complexes with CD21, a complement fragment (C3d) receptor, and with the tetraspanin CD81. Together, CD19, CD21 and CD81 function as a coreceptor for the BCR, amplifying the activating signal [95]. When complexed with CD21, binding of complement fragment C3d provides a very strong coactivation signal to the B cell [96].

When not complexed with CD21, CD19 is phosphorylated by protein tyrosine kinases in its cytoplasmic domain forming SH2 motifs which function to directly and indirectly recruit a multitude of important BCR signaling mediators such as Lyn, Fyn, Btk, Vav, Akt and, importantly, PI3K. In this way CD19 acts as an adaptor and recruitment molecule for BCR signaling intermediaries, modulating B cell thresholds for activation [97, 98].

II B3. B cell inhibition: Lyn, SHIP & inhibitory FcγR.

As one might expect, there are numerous inhibitory signaling molecules that serve to dampen the B cell response and prevent the rise of autoimmunity. One well-studied player in B cell feedback inhibition is Lyn. Lyn, in addition to activating positive signaling molecules such as Btk, also phosphorylates the immunoreceptor tyrosine-based inhibitory motif (ITIM) of the FcγRIIB inhibitory Fc receptor when it is coligated along with the BCR [93, 99]. This allows for recruitment of the phosphoinositol phosphatase SHIP, which counters the conversion of PI(4,5)P2 to PIP3 by PI3K. It does

this by cleaving the phosphate at position 5 of PIP3, yielding PI(3,4)P2 (unlike PTEN, which removes the phosphate at position 3) [93] **(Figure 2)**. This leads to impaired recruitment of Btk and PLCγ as well as inhibiting the production of the secondary messengers IP_3 and DAG [99]. Lyn also phosphorylates SHP-1 phosphatase and CD22 (another inhibitory B cell receptor) which both function to negatively regulate of BCR signaling [99]. The balance between the positive and negative signals generated is crucial for proper B cell function, as demonstrated by the observation that autoimmune phenotypes are generated when Lyn is either over- or under-expressed in mice [100, 101].

II B4. In vitro B cell activation models

Ex vivo and *in vitro* there are several models with which one may study B cell activation and differentiation. Like those for T cells, the use of specific antibodies directed against the BCR is one popular method. Since B cells express inhibitory FcγIIRB, the species of antibody used must not cross-react with this receptor on the B cells in order to achieve the strongest activating signal without inhibition. One way around this limitation is to use antibodies that have been digested with pepsin to cleave away the Fc portion, leaving only $F(ab')_2$ fragments specific for the BCR (typically either anti-IgM or anti-IgD for naive B cells) [102-105]. Also like T cells, a survival/costimulatory signal is required for initial activation. In the absence of any other pro survival signal (such as that provided by complement fragment C3d, IL-4 or IL-2), immature B cells will undergo activation-induced cell death upon BCR stimulation [106]. Other mitogens such as lipopolysaccharide (LPS) (via TLR-4) or 8-merceptoguanosine

(a nucleoside derivative) can also act on B cells to induce proliferation [107]. Our laboratory's experimental readout for B cell activation in such assays is often measuring cell proliferation, although calcium flux, the detection of phosphorylated signal pathway intermediates, or the development of differentiation markers are also used [107].

II C. Antibodies & Fcγ Receptors

II C1. Antibodies

Antibodies (immunoglobulins) are Y-shaped glycoproteins produced by plasma cells which serve to neutralize invading pathogens and flag them for recognition by the immune system. They are comprised of a pair of heavy chains (which include a hinge region) bound to a pair of light chains **(Figure 3)**. The arms of the Y are known as the Fab domain and are specific for binding to a particular antigenic epitope. Also like the TCR, the genes encoding antibody specificity undergo V(D)J recombination (mediated by the RAG proteins) during B cell development [43]. After B cell activation and proliferation, these antigen-binding domains are further mutated within germinal centers in lymphoid tissues in order to increase their affinity for antigen in a process termed somatic hypermutation and affinity maturation. Affinity maturation requires help from $CD4^+$ helper T cells [43, 108]. The "stem" of the Y, made of the two heavy chains, is the Fc domain. The Fc (standing for "crystallizable fragment") has multiple forms based on the antibody isotype, (M, D, A, E, and G). Naive B cells initially express only membrane-bound IgD or IgM as their B cell receptor [43]. After activation and proliferation, they undergo class switch recombination wherein the Fc portion of the antibody is exchanged for that of a different isotype so that daughter cells of the initially activated

parent B cell express antibodies of the same specificity but of different isotypes [43, 108].

Antibodies serve a number of important immune functions when they bind to their cognate antigenic epitopes [43]. Firstly, by binding to a pathogen they may neutralize it by blocking important molecules on the pathogen surface which are needed for entering or infecting cells. Binding also serves to flag the pathogen for recognition by Fc receptor (FcR) bearing cells, such as macrophages, rendering them susceptible to phagocytosis in a process termed opsonization. Bound antibodies may serve as a nucleating seed for the recruitment of complement components on the surface of the pathogen. The accumulation of complement serves the purpose of opsonizing the pathogen for recognition by complement receptor-bearing phagocytes, and can form the complement membrane attack complex which may lyse and kill the pathogen directly [43]. Furthermore, since antibodies possess at least two antigen binding sites (more when they are multimerized, such as occurs with IgE dimers or IgM pentamers), they can serve to agglutinate multiple pathogens together into immune complexes which are readily recognized for phagocytosis. Antibodies transferred from mother to child both *in utero* and in the colostrum of breast milk after birth are important for neonatal immunity in the absence of a fully developed immune system [43]. Many of the functions for antibodies are mediated by the ability of their conserved Fc portions to bind to receptors on many different immune cells. The coupling of the antigen-specific Fab domains to a conserved Fc domain provides an important link between adaptive and innate cell functions.

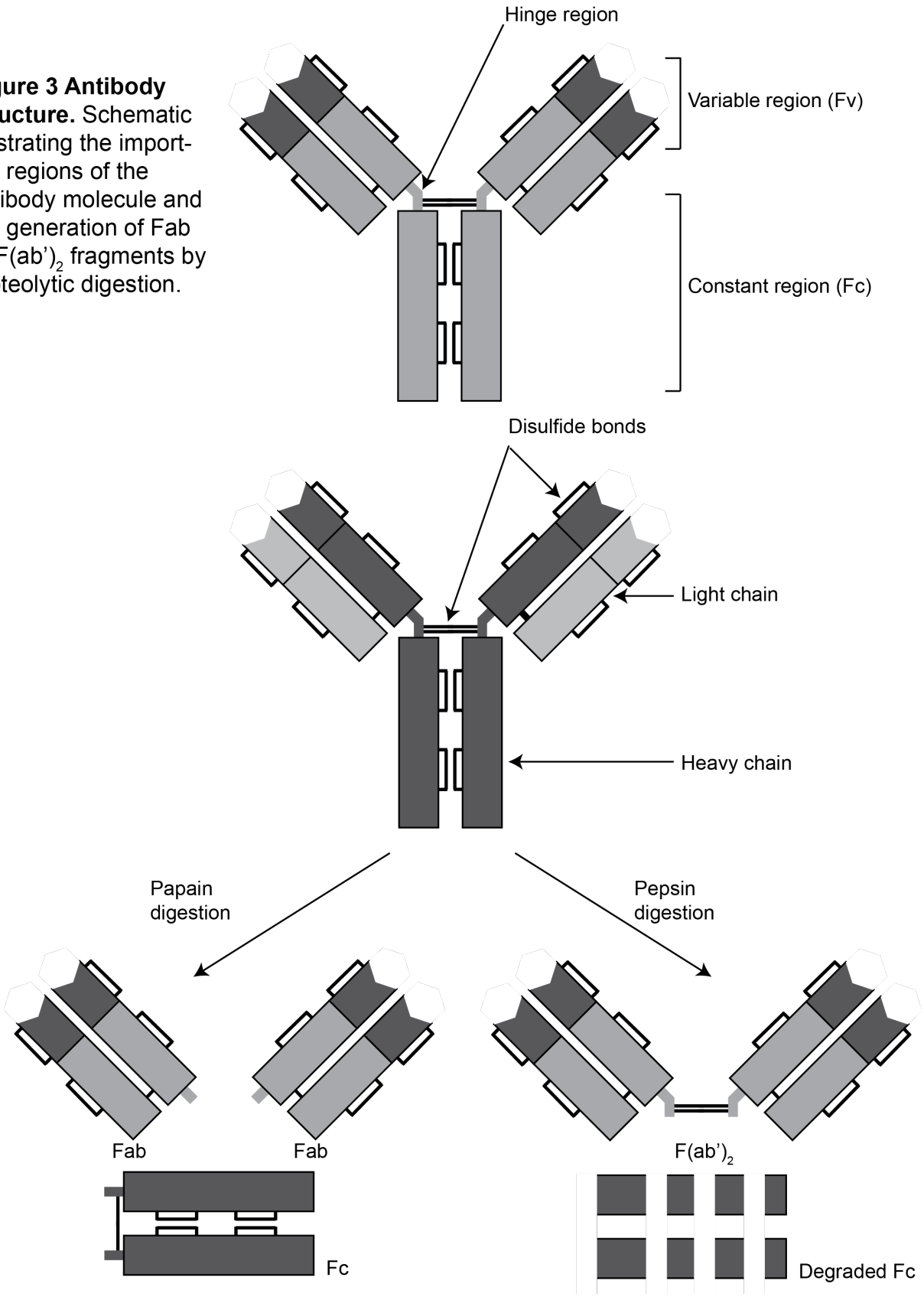


Figure 3 Antibody structure. Schematic illustrating the important regions of the antibody molecule and the generation of Fab or $F(ab')_2$ fragments by proteolytic digestion.

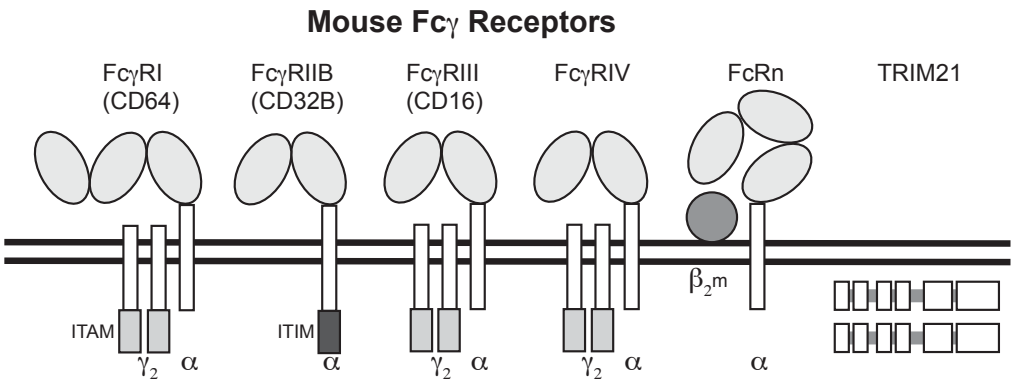
II C2. Fcγ receptors

Fc receptors are found on a wide range of immune cells, and serve to connect the humoral antibody response with other branches of the immune system. They are divided into different classes (γ, α and ε) depending on the antibody isotype Fc to which they bind. The Fcγ receptors **(Figure 4)** bind to IgG and are the relevant family of FcR for this work. In mice, there are six major known FcRs for IgG with different functions. Three of these (FcγRI, FcγRIII and FcγRIV) are associated with delivering activation signals when ligated (for macrophage phagocytosis and maturation, for example), and have varying affinities for the different subclasses of IgG (1, 2a, 2b and 3) [109, 110]. Their function stems from the presence of ITAMs in the cytoplasmic tails of associated γ chains which serve as binding sites for Src family tyrosine kinases involved in cellular activation pathways [109, 110]. FcRn (neonatal FcR) functions in the transfer of maternal IgG to the growing fetus as well as in recycling IgG in order to increase its effective serum half-life [109, 110]. TRIM21 is the only documented intracellular FcγR and functions both in cellular activation pathways as well as targeting bound antigen to the proteosome [109-111].

The only known inhibitory FcγR is FcγRIIB (CD32B). Instead of an ITAM, CD32B possesses an ITIM in its cytoplasmic tail which serves as a binding site for SHIP inositol-phosphatase [112-114]. SHIP counteracts the BCR activation signaling cascades mediated by tyrosine kinases **(Figure 2)**. There are two splice isoforms of FcγRIIB: FcγRIIB1 and B2 [115-117]. The B2 isoform is found on macrophages and dendritic cells and can mediate the endocytosis of bound immune complexes into these

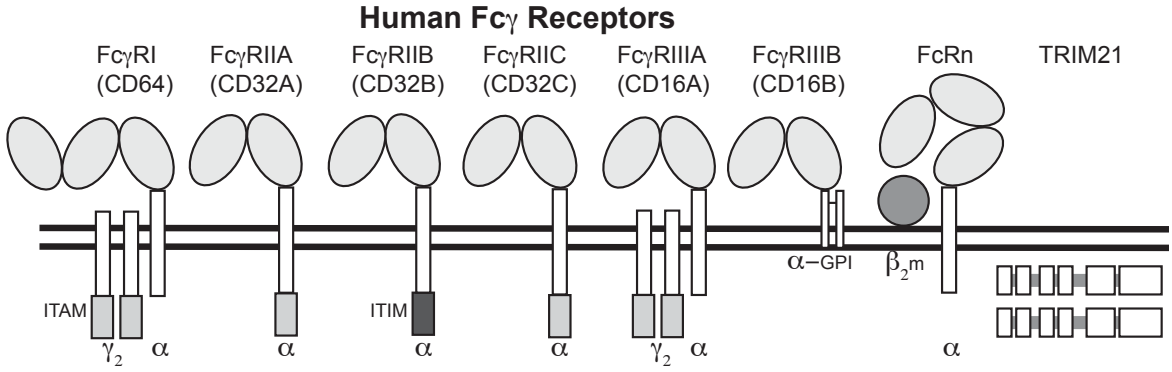
cells. The B1 isoform exists on B cells, and an in-frame 47 residue insertion in its cytoplasmic tail prevents it from mediating endocytosis [118-120]. FcγRIIB1 is the only known Fc receptor found on the surface of B cells (with the exception of some human splenic B cells which also express FcRn) [109, 121, 122] and plays a key role in both modulating the B cell response and in maintaining peripheral tolerance. It has been demonstrated that coligation of FcγRIIB with the BCR leads to a down modulation of the BCR signal [123-125]. In the absence of BCR ligation, FcγRIIB ligation and homoaggregation signals for B cell apoptosis via a distinct Btk - JNK pathway [126], and this trait is suspected to play an important role in maintaining B cell tolerance. This is supported by the observation that FcγRIIB KO mice develop autoantibodies and autoimmune pathology [127]. The B2 isoform found on dendritic cells has been shown to function in inhibiting dendritic cell maturation by immune complexes and subsequent cross presentation. Signaling via inhibitory FcγRIIB on DCs was demonstrated to limit the priming of naive T cells by these APC [112, 128].

As shown in **Figure 4**, there are more types of FcγR that exist in humans than in mice, making the assessment of their relative roles in immunity and disease more challenging.



Function	Activation	Inhibition	Activation	Activation	Recycling, neonatal transport, antigen uptake	Activation, proteosomal degradation
Subclass Binding	2a, 2b, little 3	1, 2a, 2b	1,2a, 2b	2a, 2b	1, 2a, 2b, 3	1, 2a, 2b, IgM
Affinity	High/Low	Low	Low	High	High pH<6.5	High
Expression	MN/Mø, DC	B,MN/Mø, PMN,DC, Basophil,Mast Eosin	NK,MN/Mø, PMN,DC, Basophil,Mast Eosin	MN/Mø, PMN	MN/Mø, PMN DC	B, T, NK, MN/Mø, PMN, DC, Basophil, Mast, Eosinophil, some endothelial

Figure 4 Distribution of mouse and human Fcγ receptors. "High" affinity indicates ability of receptor to bind monomeric IgG. Light grey boxes indicate activating ITAM domains. Adapted from Bruhns 2012 *Blood* and Bruhns & Jonsson 2015, *Blood* [109,110]



Function	Activation	Activation	Inhibition	Activation	Activation	Activation, Decoy	Recycling, neonatal transport, antigen uptake	Activation, proteosomal degradation
Subclass Binding	1, 3, 4	1, 2, 3, 4	1, 3, 4, little 2	1, 3, 4, little 2	1, 3, 4, little 2	1, 3, 4	1, 2, 3, 4	1, 2, 3, 4
Affinity	High	Low	Low	Low	High/Low	Low	High pH<6.5	Low
Expression	MN/Mø, DC, inducible on PMN&Mast	MN/Mø, PMN, DC, Basophils, Mast, Eosinophil Platelet	B,DC,Basophil some MN/Mø, PMN	NK, MN/Mø, PMN	NK,MN/Mø	PMN, some Basophils	MN/Mø PMN DC, Epith, Endoth, Splenic B	B, T, NK, MN/Mø, PMN, DC, Basophil, Mast, Eosinophil, some endothelial

III. Vaccinia and Immunity to Poxviruses

III A. Inoculation, Edward Jenner and the first vaccine

Before vaccination, there was inoculation. Inoculation involved infecting naive individuals with crushed smallpox pustules either intranasally or via scarification. This induced a form of the disease which was usually less severe than that acquired naturally, and upon recovery the inoculee was now immune. Inoculation had been in widespread use prior to the development of vaccination [129].

The first vaccine ever developed is credited to the English doctor, Edward Jenner in a story known to all immunologists [130]. He noted that milkmaids, known for their unblemished complexions, were spared the disfiguring scars caused by smallpox infection. He correctly hypothesized that this was due to contact with the pox-like pustules that sometimes occurred on the udders of the cows they milked. After a series of initial studies, in 1796 he took pus from a cowpox lesion on the hand of one such milkmaid. Jenner then scraped it into the skin of eight-year old James Phipps, the son of his gardener. The boy contracted a brief fever but no major illness. After Phipps had recovered, Jenner directly injected the boy with material taken from smallpox pustules and thus demonstrated that Phipps was now immune to the virus. Jenner's studies marked a new age for humanity in fighting infectious disease, and is a major landmark in the birth of the field of immunology.

III B. Vaccinia-induced immunity

Jenner's vaccine was passaged repeatedly and eventually made its way to the new world as societal resistance to the technique waned and vaccination became more commonplace. Though the specific origin of VACV is still murky, in one form or another it is still used to vaccinate against smallpox today. Although VACV was once considered to be an attenuated strain of a parental cowpox virus, genetic analysis suggests that it is actually more closely related to horsepox virus [131].

The efficacy of VACV in inducing immunity is so effective that the eradication of smallpox was able to be accomplished without having a complete understanding of how protection occurs. Since 1979, great strides have been taken and we now have a much better idea about the different roles of innate, cellular and humoral immunity in protection against poxviral infection and disease.

III C. Cross protection and the 2003 MPXV outbreak

Infection with one OPXV affords protection against other members of the genus and the discovery of vaccination by Jenner was dependent upon this fact. Poxviral protection lasts for potentially decades. A study of banked serum samples from over 300 people who received the smallpox vaccine demonstrated that anti-pox antibody titers are detectable for as long as 75 years after vaccination [132]. T cell immunity against poxviruses in vaccinated individuals was also found to last for decades, though it declines slowly over time [132]. A study of individuals exposed during the US monkeypox outbreak of 2003 provided further evidence for the long-lasting cross-protective immunity induced by vaccination. Individuals vaccinated up to 48 years previously were largely protected against infection or at least severe monkeypox illness

[133]. The immunity induced by VACV is therefore long term and may be considered to be effectively lifelong.

III D. MV and EV: antigens & epitopes

Most of what we know about poxviruses comes from studying the more abundant MV infectious form [134], as EV are difficult to isolate [17, 18]. It is thought that MV are largely responsible for spread between hosts and the EV for spread between cells within the host [18, 35, 135-137]. Therefore, it would appear that mechanisms combatting MV are critical for determining whether or not a host is protected against the virus, while targeting EV would be more important for limiting the severity of disease once infection has already occurred. In addition to playing different roles in infection, the different viral particles also display different antigenic epitopes to be recognized by the host.

III E. Adaptive immune responses to poxviral infection

In general, animal models have concluded that the humoral response is most important for protection against orthopoxvirus infection [138-141]. Most neutralizing antibodies are directed against the MV which is considered to be the form of the virus best suited for host-to-host spread [18, 35, 135-137].The cellular $CD8^+$ response appears to be more critical for limiting the virus spread within the host and limiting the severity of the resulting disease once infection has already occurred [138-141].

A study of antibodies generated during human VACV vaccination showed that while there are numerous MV proteins (including A27 and H3) targeted by virus-

neutralizing antibodies there was only one EV-displayed protein (B5) targeted for viral neutralization [142]. An optimal humoral response would be able to neutralize both MV and EV [143-147]. It has been demonstrated that a strong B cell response of mice to ectromelia virus (ECTV) or rhesus macaques to MPXV is crucial for poxvirus protection. Since $CD4^+$ T cells are needed for B cell help, antibody responses fail to occur in either mice or rhesus macaques lacking $CD4^+$ T cells [138, 140, 148].

$CD8^+$ T cells may be dispensable as long as solid humoral immunity exists [140], though in the absence of B cells, $CD8^+$ T cells are required to prevent mortality of infected mice [139]**.** They also appear to play a more important role in the case of newly acquired infections as T cells expand and become effectors faster than antibody responses [142, 149].

IV. Immune Evasion by CPXV

As mentioned earlier, CPXV possesses the largest genome and complement of immune-modulating proteins known among the *Orthopoxviridae*. Many (though by no means all) of these proteins have been identified and characterized. Interestingly, there seems to be no one immunomodulatory protein held in common by all of the OPXV [150], and each virus possesses its own repertoire of tactics for modifying the immune response of its specific host. CPXV subverts host immunity by targeting multiple intermediaries and pathways of both the innate and the adaptive immune system. As with research into poxviral life cycles, most studies of poxviral immune evasion have focused on VACV. However, orthologs of many VACV immunomodulatory proteins exist

in CPXV, and some of the VACV immune evasion mechanisms may be extrapolated to apply to CPXV as well.

IV A. Inhibition of host protein synthesis

OPXV inhibit host protein production such that more cellular resources can be devoted to poxviral genome replication and expression [6, 151, 152]. This effect seems to be exerted mainly at the level of host mRNA suppression. In VACV, D9 and D10 proteins work to decap host mRNA, leading to accelerated degradation [6, 153]. The hijacking of host translation initiation factors to viral replication factories also likely plays a role in limiting those cellular resources available to the host for most OPXV [6]. At least one protein associated with the Vaccinia virion has been shown to inhibit host protein translation by preventing the formation of the ribosomal 40s - Met tRNA ribosomal initiation complex [154].

IV B. Evading complement

Complement is an important branch of the innate immune system, consisting of several small proteins, mostly made in the liver, circulating as inactive precursors throughout the blood. Various triggers cause specific proteases to cleave these precursors into their active form, initiating one of three potential pathways that consist of amplifying cascades of further proteolytic activations. The primary functions of complement activation are chemotactic attraction of innate cells such as macrophages and neutrophils, opsonizing antigens for phagocytosis (particularly by C3b) and formation of the membrane attack complex, lysing foreign cells [43, 155, 156].

VACV MV have been shown to be susceptible to complement activation but EV are less so; this is the result of EV carrying host complement control proteins CD55 and CD59 within its outer envelope that render it resistant to complement activation [17, 157]. This is one way by which EV are particularly well suited to spread between cells within a host. This inhibition of complement activation is not absolute, however, and it has been demonstrated using C3 knockout mice that complement can play a significant role in enhancing the neutralization by specific VACV protein-directed antibodies by at least two means. For the anti-A33 antibodies, complement-mediated virolysis (lysis of the outer EV viral membrane), is required for the antibody to bind its target in the inner membrane and neutralize the virus. The anti-B5 antibody works with complement to neutralize virus by opsonization [157]. As such, control of complement is an important host evasion tactic used by these viruses.

CPXV possesses inflammation modulatory protein (IMP) which bears homology to the host C4b binding protein. The VACV ortholog of IMP (VCP), has been shown to bind and inhibit C3b and C4b in order to inhibit the classical and alternative pathways of complement activation [158-160]. Preventing complement activation is a mechanism by which the virus can avoid complement-mediated opsonization and antibody neutralization. IMP has been implicated in limiting host cell destruction during infection, presumably in order to preserve the integrity of host cells for further viral replication [161].

IV C. Avoiding TNFα & NFκB responses

IV C1. TNFα

TNFα and LTα (another member of the TNF superfamily) are potently proinflammatory antiviral cytokines released by several types of immune cells (including activated T cells, neutrophils, NK cells and others) during acute-phase infections [162]. These cytokines exert a wide range of effects on many cell types to control the spread of pathogens, and are critical mediators of immunity during viral infection

TNFα is mostly made by monocytes and macrophages, while LTα is mostly made by lymphocytes [163, 164]. In addition to the specific effects on immune cells, TNFα displays pleiotropic effects over most of the body's tissues, inducing defenses at the levels of individual cells, tissues, and the whole organism. It can induce the up regulation of reactive oxygen species (ROS) production in phagocytic cells or cause a cell to apoptose [43, 162]. At the level of tissues, it signals for inflammation, inducing further cytokine and chemokine production locally and an influx of immune cells [43, 162]. At the systemic level, it mediates the fever response as well as liver acute phase proteins and catabolic pathways [43, 162]. TNFα is capable of activating macrophages, inducing IL-2R expression in NK cells, and influencing the differentiation and maturation of T cells [162]. There are two known receptors for TNFα, TNFR1(p55) and TNFR2(p75). TNFR1 is fairly ubiquitously distributed on the body's tissues while TNFR2 is limited to immune cells [162, 165, 166]. Binding of TNFα to either of these receptors induces receptor trimerization and signals through three pathways characterized by extensive cross-talk. These are the NFκB activation pathway, activation of the MAPK pathway, or apoptosis via caspase-8 [162, 167]. The balance between these pathways in TNFα signaling is controlled by such factors as cell type and cytokine milieu, and this balance determines whether the cell's fate will be contributing towards inflammation or

undergoing apoptosis [162]. CPXV exerts numerous strategies to limit the TNFα response by targeting the cytokine as well as the NFκB or apoptotic signaling pathways induced.

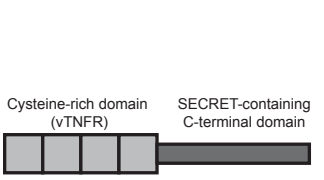
IV C2. vTNFRs and the Crms

CPXV encodes numerous factors to interfere with TNFα signaling. Chief among these are the Cytokine Response Modifier (Crm) proteins, a family of viral TNFα binding proteins (vTNFRs). All pathogenic poxviruses possess one or more members of this family but CPXV is the only one known to express versions of all the known Crm proteins **(Figure 5)**. These vTNFRs are characterized by sequence similarity to the extracellular portions of TNFR1 and TNFR2 and possess a cluster of cysteine-rich domains (CRDs) at their N terminus like cellular TNFRs [171, 172]. It seems plausible that the virus acquired versions of these genes from different hosts in its evolutionary history. CrmB, CrmC, CrmD, and CrmE are all vTNFR members of this family [10, 173-175]. CrmA is a viral serpin which will be discussed later in the context of avoiding apoptosis. A fifth member, vCD30, binds to CD153 and blocks its interaction with the cellular CD30 [176]. While Crms B, C, D, and E all bind to TNFα, CrmB and CrmD also bind to LTα. These vTNFR genes are also expressed at different times of infection [173, 175, 177, 178].

The presence of such an overlapping repertoire of vTNFRs emphasizes the importance of regulating this pathway for successful CPXV replication and dissemination. The different Crm vTNFRs display varied expression patterns as well as

different affinities for, and neutralizing capabilities on, the TNFα from different species, suggesting a role for these proteins in poxvirus species specificity [10, 168, 174].

CrmB and CrmD possess an additional C-terminal domain unrelated to the TNFR portion which has been termed the SECRET domain (for Smallpox Encoded Chemokine REcepTor). This domain has been shown to be able to bind and sequester a variety of CCL and CXCL chemokines [170]. CPXV 014 (also known as SCP-2), the focus of this dissertation, is a secreted member of this SECRET domain family. Close orthologs of CPXV 014 occur in all sequenced strains of CPXV (Brighton Red, GRI and GER-90) as well as ECTV (both Moscow and Naval strains) and horsepox (HSPV), though not in VACV, VARV or MPXV. However, the orthologous SECRET proteins SCP-1 and SCP-3 do occur in VACV and MPXV, though not in VARV [170].



	CPXV-BR	CPXV-GRI	CPXV-GER-90	ECTV-Naval	ECTV-Mos	MPXV-Zaire	VACV-WR	HSPV
CrmB	005 226	D2L I4R	CV3 CV206	EVN002P/ EVN205P (both truncated)	X	MV2 MV197	VACWR004 VACWR215	HSPV003 HSPV205
CrmC	191	A56R	CV173	EVN175P (truncated)	X	X	VACWR179 (truncated) (Sal19R)	HSPV175
CrmD	221	K2R	CV200	EVN006/ EVN201	EVM003	X	X	X
CrmE	Truncated	K3R	CV208	EVN005P/ EVN202P (both truncated)	X	X	X	X
SCP-1	224	204	CV204	X	X	MV184	VACWR206 (B23R)	HSPV225
SCP-2	014	012	CV012	EVN007	EVM008	X	X	HSPV009
SCP-3	207	189	CV188	EVN167	EVM162	MV172	VACWR189 (B7R)	HSPV210

Figure 5. Poxviral vTNFRs and SECRET domain proteins. Gene designations taken from viprbrc.org. Data based on vipbrc.org and BLAST analysis, as well as references [169-171]. "X" indicates no known ortholog present. All strains of CPXV used in this work were on the Brighton Red (BR) strain background.

IV C3. Inhibiting NFκB

In the NFκB TNFα pathway, binding of TNF to the receptor leads to the recruitment of TRAF and RIP by the TNFR-associated adaptor protein TRADD. TRAF then recruits IKK kinase complex where it is phosphorylated by RIP serine-threonine kinase. The phosphorylated IKK then phosphorylates IκBa, the protein that normally binds to NFκB and inhibits its translocation to the nucleus. The phosphorylated IκBa is then ubiquitinated and degraded, relieving the repression on the heterodimeric NFκB

transcription factor such that it can translocate to the nucleus and initiate the transcription of multiple pro-inflammatory genes.

CPXV possesses at least three known NFκB inhibitors which function by different mechanisms. All possess multiple ankyrin repeats, a motif known to facilitate protein-protein interactions. CPXV 077 appears to function as a bridging molecule between NFκB and the SCF E3 ubiquitin ligase complex, either targeting it for proteasomal degradation or otherwise inhibiting NFκB translocation to the nucleus [179, 180]. CPXV 006 binds to and inhibits the degradation of NFκB/p105 precursor protein, in this way interfering with the activation of NFκB precursors [181]. CPXV 041 is an ortholog of VACV K1L, which has been shown to inhibit the degradation of IκB and therefore translocation of NFκB to the nucleus [179].

IV C4. CrmA & Avoiding Apoptosis

Apoptosis is a normal innate response of a cell to viral infection and serves to limit the further subversion of its replicative machinery for the formation of more virus and viral release via cell lysis. CrmA is a discrete virally encoded serine protease inhibitor (serpin), among the first viral serpins discovered [182-185]. This Crm is not a vTNFR like those described above, but rather functions to prevent apoptosis of the host cell by multiple mechanisms. It has been shown to have specificity for granzyme B and inhibits the cytotoxic CTL and NK induction of apoptosis by this serine protease [186, 187]. It strongly targets the cysteine protease Caspase 1, therefore inhibiting the downstream processing of IL-1β. It also targets Caspase 8, and possibly Caspase 10, resulting in the inhibition of downstream apoptosis [185, 188, 189].

IV D. Interfering with interferons

The interferons (IFNs) are a group of immunomodulating cytokines first identified by their antiviral properties [190, 191]. They are divided into three categories based on receptor use and sequence homology. The type I IFNs include IFNα (itself a family of related proteins), IFNβ and others. There is only one type II IFN, IFNγ. IFN signaling promotes a Th1 immune response, and is a critical step in host antiviral defense. Effective control of VACV infection in mice has been shown to require both type I and type II IFNs [192]. The type III are the most recently identified class of IFNs and consist of the IFNλs [193]. Though IFNλ2 was demonstrated to inhibit VACV replication *in vivo* when expressed from recombinant VACV, this inhibition correlated with enhanced T cell responses, and IFNλ2 did not limit VACV replication in mouse fibroblasts *in vitro* [194].

IV D1. Type II IFN

IFNγ synthesis is restricted to only a few cell subsets: NK cells, $CD8^+$ and the T_h0 and T_h1 subsets of $CD4^+$ T cells. IFNγ is not induced directly by viral infection, but T cells produce IFNγ upon activation via the TCR and costimulation. Naive T cells will not make IFNγ initially, needing to progress towards an effector/memory phenotype first [81, 83]. NK cells are induced to produce IFNγ in response to IL-12 and TNFα produced by activated macrophages [195, 196]. This production is more rapid compared to that of T cells, and the secreted IFNγ binds to receptors on the macrophages, inducing them to produce more TNFα and IL-12, creating a positive amplification loop leading towards greater NK and macrophage activation [196]. The IFNγR is one of the most-studied

cytokine receptors and signals through a JAK 1/2 and STAT-1 pathway [195, 197]. Briefly, homodimeric IFNγ binds to the alpha chain of the IFNγR, inducing alpha chain dimerization and recruitment of the two beta chains. Transactivation by associated kinase allows the recruitment and activation of the JAKs, leading to the creation of STAT1 docking sites. The STAT1s are recruited and become phosphorylated, after which they dissociate from the receptor, homodimerize, become further phosphorylated on specific Ser residues then translocate to the nucleus where they instigate the transcription of IFNγ responsive genes [195].

IV D2. Type I IFNs

Unlike IFNγ, the type I IFNs are made by nearly all cell types after viral infection, particularly plasmacytoid dendritic cells [198]. Cytoplasmic double-stranded RNA, a hallmark of many viral replication cycles, activates the intracellular sensors RIG-1 and MDA-5 (or endosomal TLR-3) which possess RNA helicase activity. These activated dsRNA sensors signal for the activation of IRF3 and IRF7 which can then homo- or heterodimerize, migrate to the nucleus and induce IFNα and IFNβ transcription [196, 198]. Other stimuli such as TNFα, LPS, IL-1 and interferon itself can also signal for type I IFN production [196]. Secreted IFNα and IFNβ can then bind to cell surface IFNAR, which activates a JAK-STAT pathway resulting in transcription of several genes that help to inhibit viral replication and infectivity. This pathway exhibits some overlap with the JAK-STAT pathway utilized by IFNγ (involving STAT1 homodimers), but also involves a distinct parallel pathway utilizing STAT1-STAT2 heterodimer in association with p48, which forms a different transcription factor (the ISGF3 complex) which

migrates to the nucleus and interacts with distinct ISRE promoter elements [196]. Some of the downstream effects of this pathway include the upregulation of MHC class I expression and presentation, the inhibition of translation, degradation of viral RNA (via the phosphorylation of eIF-2 by PKR kinase), and the activation of APC and NK cells [43, 196, 198].

The IFNs orchestrate a number of antiviral and immunomodulatory activities. Both type I and type II IFN act to inhibit protein synthesis by the targeted cell via multiple mechanisms. Induction of 2'-5'-oligoadenylate synthetase by IFN and its subsequent activation by dsRNA leads to the activation of RNAseL which degrades ssRNA and therefore inhibits protein synthesis at the level of transcript stability [196, 199]. PKR is also induced by IFN and activated by dsRNA to phosphorylate and inactivate eIF-2, blocking protein synthesis at this step [196, 200]. Type I IFN are known to enhance the cytolytic activity of NK cells [196, 201, 202]. IFNγ is a major macrophage activating cytokine, reducing macrophage susceptibility to microbial infection and signaling for the inducible nitric oxide synthase and reactive oxygen intermediates they use to destroy pathogens [196, 203, 204].

Both type I and type II IFNs also promote antigen processing and presentation by enhancing MHC Class I and β2-microglobulin expression [196, 199]. IFNγ is capable of signaling for the upregulation of MHC Class I by itself; type I IFN cannot, though it enhances the ability of IFNγ to do so [196, 199, 204]. IFN signaling also increases the expression of TAP proteins needed for loading antigenic peptide onto MHC [196, 204]. IFNγ also modifies the proteasome subunit makeup to promote assembly of "immunoproteasomes" which are better able to generate MHC Class I - loadable

peptides [196, 204]. IFNγ is also crucial for promotion of a Th1 over a Th2 response, largely through it induction of macrophage-generated IL-12 [196].

IV D3. IFN evasion by CPXV

CPXV and many other poxviruses express a secreted IFNγ/β receptor (termed B18R in VACV) which can bind to infected cells and therefore competitively inhibit the antiviral actions of IFNγ & β upon them [205, 206]. IFNγ decoy receptors are also expressed [150, 207]. Furthermore, CPXV sequesters dsRNA via CPXV 069 (E3L is the VACV ortholog), which has been shown to inhibit IRF3 activation and IFNβ expression [150, 208-211]. CPXV 043 is a virally-encoded eIF-2α homolog which acts as a suicide substrate for PKR [212, 213]. Although the type I and type III IFNs utilize different receptors, their intracellular signaling pathways are nearly identical [214, 215] and VACV has been shown to limit the STAT phosphorylation and subsequent signal transduction of these pathways [216].

IV E. Interfering with IL-1β & IL-18

In addition to the aforementioned targeting of TNFα, IFN & NFκB signaling, CPXV and other orthopoxviruses interfere with other key immune cell cytokines.

IL-1β is another potently pro-inflammatory cytokine. It is expressed as an inactive precursor which is cleaved by cytosolic Caspase-1 in order to convert it into its active form [217]. IL-1β is involved in a large number of immune activities including lymphocyte proliferation, differentiation and apoptosis. CPXV targets this cytokine in multiple ways. As mentioned before, CrmA inhibits Caspase-1 and therefore processing of mature IL-

1β. The CPXV 209 protein (also possessed by VACV) is a secreted decoy receptor targeting IL-1β [218, 219].

IL-18 participates in the regulation of Th1 and Th2 responses, activates NK and $CD8^+$ T cells, and stimulates the production of a variety of other cytokines, including the all-important IFNγ. Like IL-1β, it must be cleaved by Caspase 1 into its active form, and as such is also inhibited by CrmA [184]. CPXV possesses a viral version of IL-18 binding protein, CPXV 024, a normal host antagonist and regulator of IL-18 activity which serves to sequester IL-18 away from its normal receptors and signaling actions [220, 221].

IV F. Disrupting chemokines & cell trafficking

All poxviruses studied thus far interfere with the chemokine signaling network in some fashion [150]. Several species possess chemokine-binding proteins which bind and sequester a wide variety of chemokines away from the host chemokine receptors. CPXV CBP-II (vCCI) is one such example, capable of binding to CC chemokines with high affinity. Interestingly, CBP-II from CPXV and other OPXV share no structural or sequence resemblance to the normal host G-protein coupled receptor (GPCR) ligands of the CC chemokines [82, 222-224]. They are capable of interacting with a large variety of CC chemokines with high affinity.

Also mentioned before are the so-called SECRET domain proteins first identified from VARV [170] **(Figure 5)**. Occurring either as the C terminal domain of CrmB or CrmD, or expressed and secreted as their own protein product (SCP-1, SCP-2 and SCP-3), these proteins have also been shown to bind with high affinity and

sequester a large, overlapping range of human and mouse CCL and CXCL chemokines with known roles in antiviral defense, particularly B, T and dendritic cell homing [170]. CPXV 014 (also known as SECRET-containing protein 2, SCP-2), the focus of this dissertation, belongs to this protein family, and close orthologs of it exist in ECTV and HSPV. Members of the SECRET domain family display little sequence similarity to other known viral chemokine binding proteins or to each other. However, they do possess two conserved Cys residues which are likely important for function [170]. CPXV is the only OPXV known to express versions of all the known SECRET domain proteins.

The sequestration of chemokines and disruption of effective immune cell chemotaxis has been shown to lead to a reduction in T cell priming, demonstrating how such tactics can benefit the virus [225].

IV G. Downregulating MHC Class I

One of the major ways by which CPXV avoids the CTL response is by downregulating MHC Class I on infected cells and therefore recognition by $CD8^+$ T cells. MHC Class I is expressed by all cells and is crucial for cell-mediated immunity. It displays peptides derived from proteins present in the cell within an antigen-binding groove for presentation to $CD8^+$ T cells and is a key means by which infected or transformed cells are targeted for destruction due to their display of aberrant peptide epitopes. It has been demonstrated by our laboratory and others that CPXV uses two genes to prevent MHC Class I presentation of antigens, CPXV 012 and CPXV 203 [226-228]. CPXV 012 acts by inhibiting the transporter associated with antigen processing (TAP), the protein responsible for loading peptides into the binding groove of MHC

Class I. CPXV 203 acts by binding to MHC Class I and retaining it in the endoplasmic reticulum via a KTEL retention sequence. In these ways, MHC Class I on the surface of CPXV-infected cells is downregulated, inhibiting recognition and killing by $CD8^+$ T cells.

IV H. Inhibiting NK cells

One of the drawbacks of evading CTL recognition by downregulating MHC Class I is that it opens up the potential for recognition by NK cells seeking out "missing self". NK cells are normally prevented from killing potential targets by the presence of MHC bearing normal cell epitopes alongside NK inhibitory ligands [43]. In the absence of MHC, and with the lack of NK-inhibiting signals or a presence of NK-activating signals, NK cells will target and kill aberrantly expressing and potentially virally infected cells. Like many viruses that downregulate MHC Class I, CPXV uses additional tactics to suppress NK cell activation and killing of infected cells.

NKG2D is one of the activating receptors for NK cells. It recognizes self proteins from the MHC Class I polypeptide-related sequence (MIC) and other families which become upregulated on the surface of transformed, infected or otherwise stressed cells. Ligation of this receptor in conjunction with low levels of surface MHC Class I can activate an NK cell to kill the target cell. CPXV (and MPXV) encodes an MHC Class I -like protein termed OMCP which binds with high affinity to both human and murine NKG2D, blocking its recognition of ligands such as MIC on the surface of infected cells, acting in a paracrine fashion as an antagonist of NK cell killing [229]. OMCP can also bind the poorly-defined, immunomodulatory receptor FCRL5 on the surface of innate B cells, monocytes and macrophages, demonstrating that like many other CPXV

immunoevasins, this protein has pleiotropic effects on different immune cell populations and pathways [230].

IV I. OPXV inhibition of T cells

A major impetus for the studies described in this work was the demonstration that MPXV is capable of preventing the response of human T cells to plate-bound anti-CD3 which cross-links and signals through the T cell receptor [231]. This inhibition was demonstrated to be MHC-independent and occurred *in trans*.

Further study revealed that this inhibition was the result of MPXV 197, a transmembrane protein belonging to the B22 family, and that orthologs of this protein found in CPXV (CPXV 219) and VARV (B22) are also capable of inhibiting the stimulation of human and rhesus macaque T cells by specific antigens *in vitro* [232]. MPXV 197 was also shown to suppress T cell responses during an *in vivo* rhesus macaque infection model [232]. However, a CPXV Δ12Δ203 mutant virus (lacking the proteins responsible for down-regulating MHC Class I) possessing intact CPXV 219 showed a restoration of murine poxvirus-specific $CD8^+$ T cell activation, indicating that the inhibition exerted by CPXV 219 is species-specific and ineffective against mouse $CD8^+$ T cells [232]. We wondered if CPXV possessed other MHC-independent T cell inhibiting activities, which is how we became involved in the project detailed within.

We began this project by examining the effects of CPXV infection on the polyclonal activation of naive murine T cells *in vitro*. These experiments led us to initially identify CPXV 014 as a naive T cell inhibitor. However, further examination indicated that this T cell inhibition by CPXV 014 was limited to the antibody-driven *in vitro* naive T

cell activation model used. As I describe in the following body of work, we realized along the way was that in order to properly explain our observations and determine the real role of CPXV 014 in viral pathogenesis, we needed to look beyond T cells and examine their relationship with other cells, both *in vitro* and *in vivo*. This has since led us away from T cells and into the fine details of antibody-driven activation models, B cell inhibition and Fc receptor functions.

Chapter 2: Materials and Methods

I. Mice:

BALB/cByJ and C57BL/6 mice were purchased from The Jackson Laboratory and housed under SPF conditions. Splenocytes from the sole CD32B KO (Jackson labs stock# 002848 B6;129SFcgr2B^{tm1Ttk}/J) mouse were generously provided by the laboratory of David Parker, OHSU. All animal experiments were reviewed and approved by the OHSU institutional animal care and use committee (IACUC).

II. Cells and Media:

II A. Cells

A20 (BALB/c B cell lymphoma line) (ATCC®-TIB208™)

MC57 (C57BL/6 fibrosarcoma) (ATCC®-CRL2295™)

DC2.4 (C57BL/6 dendritic cell line) [233]

BSC-40 (Cercopithecus aethiops, kidney) (ATCC®-CRL2761™)

Vero (Cercopithecus aethiops, kidney) (ATCC®-CCL81™)

CHO cells (ATCC®-CCL61™)

293F Cells (Life Technologies #R790-070)

II B. Media

"T Cell Proliferation media" was Hi-Glucose DMEM (Gibco #11995-065) supplemented with 55μM B-mercaptoethanol (BME), 10% FBS, 100 μM penicillin/streptomycin, 100 μM non-essential amino acids (NEAA, Corning #25-025-CI).

"Stimulation media" was RPMI + 5% FBS + 100 μM Pen/Strep+ 2 mM L-Gln + 10 mM HEPES

(Cellgro #25-060-CI).

"B Cell" proliferation media was DMEM + 4.5 g/L glucose + 2 mM L-Gln + 50 μM BME + 100 μM NEAA + 10% FBS + 100 μM Pen/Strep + 10 mM HEPES + 1 mM sodium pyruvate

"DC2.4 Media": RPMI 1640 + 2 mM L-Gln + 100 μM Pen/Strep + 10% FBS + 100 μM NEAA + 50 μM BME

BSC40 and Vero cells were maintained in MEM + 10% FBS +2 mM L-Gln + 100 μM Pen/Strep

Gibco Freestyle 293 expression media, cat#12338-018 (for CPXV014-His expression)

Thermo Scientific SFM4CHO Utility media, cat#SH3051602 (for CPXV014-Fc expression)

A20 cells (ATCC® TIB-208), were maintained in RPMI + 10% FBS + 2 mM L-Gln + 100 μM Pen/Strep.

III. Viruses:

III A. Viruses

CPXV_BR (ATCC®-VR302™) (Accession #NC_003663)

VACV_WR (ATCC®-TVR1354™) (Accession #NC_006998)

VACV-EGFP – based on VACV_WR, EGFP under a poxviral early/late promoter is inserted into the TK gene. A gift from Jon Yewdell NIH).

Deletion mutants CPXV A518, A694, A529 A530, A531 and Δ015 CPXV were all gifts from the laboratory of David Pickup, Duke University. All strains of CPXV used during these studies were based on the Brighton Red (BR) strain, which was isolated from a lesion on the hand of a milkmaid in England in 1937 [234, 235].

III B. Purification

Viruses were purified using standard poxviral protocols [236]. Infected BSC-40 cell lysates were

precleared and layered onto a 36% sucrose cushion. The virus was isolated by centrifugation at 40,000 *g* for 80 min. The virus was then additionally purified by centrifugation (22,500 *g*, 40 min) through a 25%–40% continuous sucrose gradient before being resuspended in 1 mM Tris-HCl (pH 8.0), and titered. Standard plaque assay titrations were performed on BSC-40 cells [236].

IV. ∆14 CPXV:

IV A. pCR2.1_∆014CPXV: This plasmid was constructed by splicing the first and last 150 bp terminal segments of CPXV 014 to the 5' and 3' termini of a pT7 E/L EGFP_GPT cassette using splice overlap extension PCR technique (SOE-PCR) [237]. The 3' and 5' 150 bp termini of CPXV 014 were cloned from wild type (WT) CPXV (Brighton Red) genomic DNA using primer pairs "d14Int#1" + "d14Int#2" and "d14Int#5" + "d14Int#6" respectively (See **Table 1**). The EGFP and GPT cassette (under the control of the poxviral EL and 7.5K promoters, respectively) was cloned from the previously published pT7 E/L EGFP_GPT plasmid (a gift from Grant McFadden) [238] using primer pair "d14Int#3" + "d14Int#4." The EGFP-GPT cassette, the 3' and 5' terminal 014 segments were then fused to each other by SOE-PCR using the "d14Int#1" and "d14Int#6" primer pair. This construct was then cloned into the pCR 2.1 TOPO-TA vector (Invitrogen) and propagated in OneShot® Top 10 chemically competent *E. coli* (ThermoFisher #404010) using the manufacturer's protocol.

IV B. Construction of Recombinant ∆014 CPXV (Figure 16): Two µg of the pCR2.1_∆CPXV014 plasmid was transfected into 1 x 10^6 BSC-40 cells using Life Technologies Lipofectamine 2000 according to manufacturer's protocol. Transfected cells were infected 3 hours later with WT CPXV (Brighton Red) at an MOI=1 and were cultured under GPT selection in media using 250 µg/ml xanthine (Sigma #X0626), 15 µg/ml hypoxanthine (Sigma #H9377) and 32 µg/ml mycophenolic acid (sigma #M5255). The resulting EGFP-positive virus was isolated using 3 successive rounds of plaque purification under the same selection. RT-PCR analysis of the surrounding open reading frames (ORFs) 012, 013 & 015 was performed to ensure neighboring genes were not disrupted. Isolation of viral genomic DNA was performed by infecting BSC-40 cells with virus at an MOI of 5, then lysing the cells and purifying the DNA using a Qiagen DNeasy Blood and Tissue Kit (Cat # 69506) according to the manufacturer's instructions. The presence of the insert in the ∆14 CPXV virus was first checked by PCR (using Thermo DreamTaq polymerase #EP0701). The PCR reaction consisted of 30 cycles with an annealing temperature of 55°C and a 3-minute extension step at 72°C) using internal primer pair "d14#1" + "d14#6" and external primer pair "L152F" + "R188R." Isolation of Sequencing of both WT CPXV and ∆014 CPXV was conducted by the ONPRC sequencing core facility using an Illumina MiSeq. Libraries were constructed by the OHSU core facilities. Sequence analysis and alignment with reference CPXV_BR genome was carried out by Daniel Malouli, Früh laboratory, using Geneious v8.1.4.

IV C. Screening of ∆14 CPXV by RTPCR: RNA was isolated from ∆14 CPXV or WT CPXV-infected (MOI=5) BSC-40 cells using a Qiagen RNAeasy kit according to the manufacturer's instructions. Genomic DNA was digested for 15 minutes at 37°C followed by addition of 25 mM

EDTA (1:10, 2.5 mM final concentration) and incubation for 5 minutes at 65°C. dNTPs and random hexamers were added, the sample was split into 2 tubes for either mock or reverse transcriptase inclusion. cDNA was generated using reverse transcriptase (or mock) and cycling in a PCR machine: 25°C for 10 min, 50°C for 30 min then 85°C for 5 min. We then used the generated cDNA to screen the expression of multiple surrounding genes via PCR: vDNA Pol (using primers “MVA056L-67F” and “MVA056L-312R”), CPXV 012 (primers “12A” and “12B”), CPXV 013(“13A” and “13B”), & CPXV 015 (“15A” and “15B”). PCR cycler settings were 94°C melting, 55°C annealing, 72°C extending, 30 cycles, and Thermo DreamTaq polymerase (Thermo cat# EP0711). The resulting PCR products were run on a 1% agarose gel and stained with ethidium bromide for visualization. Results confirmed that the viral polymerase and other surrounding genes were undisrupted and expressed normally in the ∆14 virus relative to WT virus.

V. qRT PCR for CPXV 014 expression kinetics:

BSC-40 cells were infected with WT CPXV at an MOI of 3 in 6-well plates. After 30 minutes, the cell supernatant was discarded and replaced with fresh media +/- 40 µg/ml arabinose C. At 1, 2, 4, 6, 8 and 10 hours post-infection the supernatant was discarded and the cells were scraped and harvested into RLT + B-mercaptoethanol for loading into a QiaShredder column, centrifugation and storage at -80°C until RNA isolation. RNA was isolated from WT CPXV-infected (MOI=5) or uninfected BSC-40 cells using a Qiagen RNAeasy kit according to the manufacturer’s instructions. Genomic DNA was digested for 15 minutes at 37°C followed by addition of 25 mM EDTA (1:10, 2.5 mM final concentration) and incubation for 5 minutes at 65°C. dNTPs and random hexamers were added, the sample was split into 2 tubes for either

mock or reverse transcriptase inclusion. cDNA was generated using reverse transcriptase (or mock) and cycling in a PCR machine: 25°C for 10 min, 50°C for 30 min then 85°C for 5 min. qPCR was performed on an Applied Biosciences StepONE PCR cycler using SYBR Green and the primers “14qPCR-F”, “14qPCR-R”, “CPXV021-F” and “CPXV021-R”, “CPXV066-F” and “CPXV066-R” as recorded in **Table 1**. GAPDH was used as the reference gene for normalization. Cycles to threshold were calculated using the equations:

$Ct_{goi} - Ct_{norm(GAPDH)} = \Delta Ct$ sample.

ΔCt sample $- Ct_{calibration(Ct\ baseline\ at\ T=1)} = \Delta\Delta Ct$.

“Relative Expression” $= 2^{(-\Delta\Delta Ct)}$

VI. Recombinant proteins:

VI A. pcDNA3.1_CPXV014Fc

CPXV014 was cloned from genomic WT CPXV (Brighton Red) DNA using the primers “EcoRI-CPV14F” and “Bste2-Thr-CPV14R” (**Table 1**). These primers introduced the Kozak sequence and an EcoRI digestion site at the 3’ end as well as thrombin and BSTE II cleavage sites were introduced at the 5’ end. This fragment was then cloned into pRK5-Fc (A gift from the laboratory of Dr. Scott Wong, OHSU) directly upstream from the human IgG1Fc tag. The resulting CPXV14-Fc fragment was subsequently excised using EcoRI and HindIII and inserted into the pcDNA3.1 expression vector under the control of a CMV promoter **(Figure 7)**. The plasmid was propagated in OneShot® Top 10 chemically competent *E. coli* (ThermoFisher #404010) using the manufacturer’s protocol.

VI B. Generation of CPXV 014-Fc

The pcDNA3.1_CPXV14Fc plasmid was transfected into CHO cells using Life Technologies Lipofectamine 2000 (Thermo Fisher cat# 11668010), which were raised under selection. Transfected clones were then single cell sorted on a BD LSRII flow cytometer and grown up in 96 well plates. The highest-expressing clones were identified using a dot-blot of the supernatants harvested from wells confluently colonized with the individual stable transfectants, then probed with anti-human Fc antibody. The highest-expressing transfectant clone was then cultured in Mediatech CELLGRO flasks (10-126-4 BD Biosciences No.:353137), in the presence of tissue-culture safe protease inhibitor (Sigma #P1860) for one month. Supernatants were harvested twice weekly and frozen at -80°C. CPXV 14-Fc was then purified from the supernatants via FPLC affinity chromatography on an Amershand Phamacia Biotech ÅKTA FPCL UPC900 P920 using GE Healthcare HiTrap™ Protein G HP 1 ml columns (FisherSci #45-000-053) according to device manufacturer protocols. Following elution at low pH, the recombinant protein was dialyzed twice (Fisherbrand Dialysis Tubing Nominal MWCO 12,000-14,000 kDa) at 4°C. Dialysis was conducted for 12 hours in 5L PBS, then again for 6 hours in 5L fresh PBS. The dialyzed protein was concentrated using Amicon Ultra Ultracel – 50K concentration columns (Cat #UFC8-050-08). Protein concentration was determined using both NanoSpec and Bradford assays. The protein was flash-frozen in liquid nitrogen and stored at -80°C until use.

VI C. pFM1-2R_SCP2 and expression of His-tagged constructs.

The plasmids, pFM1-2R_SCP-2_CPXV_BR, pFM1-2R_ECTV-CrmD, pFM1-2R_ECTV-CrmD_CTD3Lys, pFM1-2R_ECTV-CrmD_CTD3Ala, pFM1-2R_EVM008, pFM1-

2R_CPXV221CTD-His were a gift from the Fremont laboratory**.** The pFM1-2R_SCP-2_CPXV_BR plasmid encoding CPXV014-His was expressed in HEK293F cells and purified from the culture supernatants according to published protocols [239]. Briefly, 200 ml shaker cell cultures of 293F cells at 1 x 10^6 cells/ml are transiently transfected with 200 µg of the pFN1-2R plasmid using linear polyethylene (Polysciences Inc #23966). Supernatants are harvested on days three and six following transfection and stored at -80°C until purification by passing it over a column packed with 4 ml of Qiagen NiNTA Superflow resin (Cat#30410). Following elution from the Ni-NTA resin the recombinant protein was dialyzed twice into PBS as described for CPXV014-Fc above, then concentrated using Amicon Ultra-15, Ultracel 10K columns. The protein was flash frozen with liquid nitrogen and stored at -80°C until use. This CPXV 014-His (SCP-2) construct is the same as the WT gene except for the inclusion of a C-terminal His tag and substitution of the native secretion signal with a high efficiency secretion signal taken from baculovirus [239]. The pFM1-2R_ECTV-CrmD, pFM1-2R_ECTV-CrmD_CTD3Lys, pFM1-2R_ECTV-CrmD_CTD3Ala, pFM1-2_CPXV221CTD-His and pFM1-2R_EVM008 plasmids also include the baculovirus secretion signal, the C-terminal His tag, and were also expressed using transient transfection of the same 293F cell line. Their respective recombinant proteins were purified identically.

VI D. PNGase F digestion

Purified recombinant CPXV 014-His or CPXV 014-Fc was treated by PNGase F (Thermo # P0704S) according to manufacturer's directions overnight at 37°C, then subjected to heat-inactivation of the enzyme followed by electrophoresis on a 12% acrylamide gel.

VII. *Ex vivo* T cell activation assays

VII A. *Ex vivo* Naïve T cell ICS Activation Assays

(Figure 8) Spleens were harvested from naive female 6-10 week old BALB/cByJ mice and single cell suspensions were generated by gently forcing spleens through a 70 μm cell strainer. Red blood cells were lysed using 2 ml ACK lysis buffer (Lonza #10-548E) for 2 min at room temperature. The remaining splenocytes were washed and resuspended in stimulation media at a concentration of 2 x 10^7 cells/ml. The cells were plated into round-bottom 96 well plates for pre-incubation in the presence of infected cells/virus free supernatant or recombinant protein at 37°C, 5% CO2 for 4 hours. The cell mixtures were then transferred to flat-bottom 96-well plates which had been coated overnight with 10 μg/ml both anti-CD3ϵ and anti-CD28 antibodies. Brefeldin A (Sigma #B6542) was added to a final concentration of 4 μg/ml. The plates were centrifuged for 2 min at 1200RPM and then incubated for 6 hrs at 37°C, 5%CO_2. Following this stimulation, the cells were washed 3x in FACS buffer (PBS + 3% FBS) prior to surface staining overnight at 4°C with the following antibodies (see **Table 2**): anti-mouse CD3ε-PerCP_5.5 (1:100 dilution), anti-mouse CD4-Pacific Blue (1:100), anti-mouse CD8-APC (1:50), and LIVE/DEAD Fixable Aqua Dead cell stain (1:100). After surface staining, cells were washed three times with FACS buffer, then fixed/permeabilized with BD Cytofix/Cytoperm (BD Biosciences #554722) for 30 min at 4°C, followed by two washes with BD PermWash (BD Biosciences #554723). Intracellular cytokine staining was then carried out for 1 hr at 4°C with the following antibodies: anti-mouse IFNγ-Alexa 700 (1:100), and anti-mouse TNFα-APC_Cy7 (1:100). Following intracellular cytokine staining, samples were washed 3x with BD PermWash and analyzed using a BD LSRII flow cytometer. Data was analyzed using TreeStar FlowJo

software v 10.1. Gating was performed according to the schema detailed in **Figure 6**.

*VII A1. Using Infected A20 cell*s:

A20 cells were infected overnight with indicated virus at MOI=5, washed three times with RPMI +5% FBS + Pen/Strep + L-Gln + HEPES ("Stimulation media") to remove free virus, and resuspended in same at a density of 1 x 10^7 cells/ml. Spleens were harvested from naive female 6-10 week old BALB/c mice. Single cell suspensions were generated by gently forcing spleens through a 70 µm cell strainer. Red blood cells were lysed using 2ml AKC lysis buffer (Lonza #10-548E), washed and resuspended in stimulation media at 2 x 10^7 cells/ml. Splenocytes were mixed with infected A20 cells in round-bottom 96 well plates at a ratio of 2:1 and coincubated at 37°C, 5% CO2 for 4 hours. The cell mixtures were then transferred to flat-bottom 96-well plates which had been coated overnight with 5 µg/ml both anti-CD3 and anti-CD28 antibodies. (See **Table 2**) Brefeldin A (Sigma #B6542) was added to a final concentration of 4 µg/ml. The plates were centrifuged for 2 min at 1200RPM and incubated for 6 hrs at 37°C, 5% CO_2. Following this stimulation, the cells were washed three times in FACS buffer (PBS + 3% FBS) prior to surface staining overnight at 4°C with the following antibodies (see **Table 2**): anti-mouse CD3ε-PerCP_5.5 (1:100 dilution), anti-mouse CD4-Pacific Blue (1:100), anti-mouse CD8-APC (1:50), and LIVE/DEAD Fixable Aqua Dead cell stain (1:100). After surface staining, cells were washed three times with FACS buffer, then fixed/permeabilized with BD Cytofix/Cytoperm (BD Biosciences #554722) for 30 min at 4°C, followed by two washes with BD PermWash (BD Biosciences #554723). Intracellular cytokine staining was then carried out for 1 hr at 4°C with the following antibodies: anti-mouse IFNγ-Alexa 700, and anti-mouse TNFα-APC_Cy7. Following intracellular cytokine staining, samples were washed three times with BD

PermWash and analyzed using a BD LSRII flow cytometer. A20 or MC57 cells were monitored for infection rates by fixing and permeabilizing them (as described above), staining with a biotin-conjugated polyclonal rabbit anti-VACV antibody (ViroStat #8107, see **Table 2**) followed by Streptavidin-PE_Cy7, and analysis on a BD LSRII flow cytometer. EGFP expression was also monitored for relevant mutant viruses.

VII A2. Using Virus-Free Supernatants:

For some assays, cells were co-incubated with virus-free supernatants taken from infected MC57 cells rather than with the infected cells themselves. These supernatants were generated by infecting MC57 cells grown to confluency in six-well plates with an MOI of 2 of the indicated virus. The cells were initially infected for 1 hour at 37°C using 250 µl per well of stimulation media, then 250 more µl of media was added and the cells were incubated at 37°C, 5% CO_2 overnight. The following morning, the supernatants were harvested and then centrifuged at 1500 rpm for 5 min at 4°C to remove cell debris. The resulting supernatants were centrifuged at 13,500 rpm for 90 min to remove any poxvirus particles. These virus-free supernatants were harvested and frozen at -80°C until use. Infection levels of the MC57 cells were monitored for consistency between different viruses by fixation, permeabilization and intracellular staining with polyclonal rabbit anti-VACV antibody, followed by analysis by flow cytometry as described for the A20 cells above. Samples of supernatant were used to treat BSC-40 cells in order to confirm the absence of virus by plaque assay. When this conditioned media was used in our T cell assays, we added it at a ratio of 1:1 with existing media for 4 hours prior to stimulation.

VII A3. Using Recombinant Protein

For assays using recombinant proteins, the indicated concentration of protein in stimulation media was added to the splenocytes for 4 hours prior to stimulation and allowed to remain in the culture throughout stimulation.

VII A4. Using PMA/Ionomycin Stimulation

Spleens from naive female 6-10 week old BALB/c mice were harvested and processed as above. Splenocytes were resuspended in stimulation media at 2 x 10^7 cells per ml and coincubated with either virus-free supernatants (generated as described above) in a 1:1 ratio, or with 5 µg of either CPXV 014-His or human IgG for 4 hours at 37°C. The cells were then treated with 50 ng/ml PMA (FisherSci #BP685-5), 1 µg/ml ionomycin (FisherSci #BP2527-1) and 4 µg/ml Brefeldin A (Sigma #B6542) for 6 hrs at 37°C, 5% CO_2. Following stimulation, the cells were washed, then stained for surface markers, followed by fixation, permeabilization and intracellular cytokine staining as described for the naive T cell assays above.

VII B. OT-I and SIINFEKL T cell activation assay

Splenocytes from age matched naive female age-matched BALB/cByJ mice were isolated and red blood cells lysed as described above. Splenocytes were resuspended in stimulation media at 2 x 10^7 cells per ml in 96 well plates (100 µl/well) and incubated in the presence of the 5 µg/ml either CPXV 014-His or hIgG1 for four hours prior to stimulation with the indicated concentration of SIINFEKL peptide (Sigma #S7951, Batch #054M4731V, FW: 963.13 g/mol reconstituted in H_2O at 1mg/ml) and 4 µg/ml Brefeldin A (Sigma #B6542) for 6 hours at 37°C, 5%CO_2. Following this stimulation cells were subjected to the same surface staining, ICS and flow cytometry analysis as described above. This assay was performed by David Edwards.

VII C. Memory T cell activation assays

VII C1. MCMV memory T cell assay using anti-CD3

Splenocytes from 5 age and sex-matched C6F1 (C57BL/6 x BALB/c) mice infected intraperitoneally with 5 x 10^5 pfu MCMV 21 days previously were generously donated by the laboratory of Dan Streblow, OHSU. Red blood cells were lysed using ACK lysis buffer (Lonza #10-548E) and the cells were stored at -80°C in 10%DMSO 90%FBS until used. A flat-bottomed 96 well plate was coated overnight with 5 µg/ml anti-CD3. Splenocytes were thawed, washed three times with "Stimulation media," and plated at 1 x 10^6 cells/well in the presence of the indicated concentrations of recombinant proteins or media only for two hours. The anti-CD3 coated plate was washed three times with PBS, the splenocytes were transferred to it, and Brefeldin A (Sigma #B6542) was added for the six-hour stimulation at 37°C 5% CO_2 in a total volume of 200 µl/well. Following this stimulation, the cells were washed three times in FACS buffer (PBS + 3% FBS) prior to surface staining overnight at 4°C with the following antibodies (see **Table 2**): anti-mouse CD3ε-PerCP_5.5 (1:100 dilution), anti-mouse CD4-Pacific Blue (1:100), anti-mouse CD8-PE-Cy7 (1:50), anti-CD44-PE_CF594 (1:100) and LIVE/DEAD Fixable Aqua Dead cell stain (1:100). After surface staining, cells were washed three times with FACS buffer, then fixed/permeabilized with BD Cytofix/Cytoperm (BD Biosciences #554722) for 30 min at 4°C, followed by two washes with BD PermWash (BD Biosciences #554723). Intracellular cytokine staining was then carried out for 1 hr at 4°C with the following antibodies: anti-mouse IFNγ-Alexa 700 (1:100), and anti-mouse TNFα-APC_Cy7 (1:100). Following intracellular cytokine staining, samples were washed three times with BD PermWash and

analyzed using a BD LSRII flow cytometer. Data was analyzed using FlowJo v10.1.

VII C2. LCMV memory T cell assay using peptide stimulation

Whole splenocyte populations were obtained from C57BL/6 mice infected with LCMV (Armstrong strain) at least 60 days previously. Memory T cells were stimulated by the indicated concentrations of LCMV antigenic peptide (GP33 or NP396) for five hours in the presence of Brefeldin A and 5 µg/ml CPXV 014-His (226 nM) or ECTV CrmD CTD 3Lys-His (215 nM) negative control protein. Intracellular staining for IFNγ was performed as previously described. Data generated by the laboratory of Dr. Jeff Nolz, OHSU

VII D. T Cell proliferation assays

VII D1. Anti-CD3/IL-2 proliferation T cell assay

Spleens were harvested from naive female 6-10 week old BALB/c mice. Spleens were gently pressed through a 70 µm cell strainer to generate single-cell suspensions, washed using “T Cell Proliferation media” (Hi-Glucose DMEM + 10% FBS + 55 µM B-mercaptoethanol + NEAA + P/S) and resuspended in 37°C PBS at 5 x 10^6 cells/ml. The cells were CFSE-loaded by adding 2 µM CFSE (BD Biosciences #565082, dissolved at 5mM in DMSO for storage at -20°C and was diluted to a working solution of 2 µM in 37°C PBS immediately before using) in a 1:1 ratio (1 µm final concentration) and incubated at 37°C for 10 min. Ice-cold FBS was then added to the CFSE cell mix in a 1:1 ratio, incubated at room temperature for 5 min, then a 5x volume of proliferation media was added. The cells were centrifuged at 1500rpm, washed 2 additional times with proliferation media, and resuspended at 5 x 10^6 cells/ml in the same. At this point, all

cells received 100 U of recombinant mouse IL-2 (R&D Systems #402-ML/CF) to provide minimal survival signals during the proliferation assay. The cells were plated in 96 well plated at 2.5 x 10^5 cells/well in 200 µl of media. CPXV014-His or control proteins were added in the indicated quantities. Soluble anti-mouse CD3 (see **Table 2**) was added at a concentration of 1 µg/ml and rmIL-2 was added at 1 µg/ml. The splenocytes were then incubated in the dark for three days at 37°C 5%CO_2. They were washed three times with FACS buffer, stained for life/death (LIVE/DEAD Fixable Aqua Dead cell stain, 1:100) and surface markers CD3ε-PerCP_5.5 (1:100 dilution), CD4-Pacific Blue (1:100), and CD8-APC (1:50), then analyzed on a BD LSRII cytometer as described above. Data was analyzed using TreeStar FlowJo software v9.8.

VII D2. PHA proliferation T cell assay

These assays were performed exactly as the anti-CD3 plus IL-2 proliferation assays described above except that instead of stimulation with anti-CD3 and IL-2 the cells were treated with 10 µg/ml phytohaemagglutinin (Thermo Scientific Remel™ PHA Reagent Grade Cat#R30852701).

VII E. T cell enrichment

For some T cell activation assays, the Milteny Biotek Pan-T cell isolation kit II (#NC9048934) was used to enrich murine splenic T cells to >98% $CD3^+$ via negative selection using the manufacturer's instructions. "LD" columns (#NC9775202) and the MidiMACS separation system were used for the magnetic separation steps. The mouse $CD8^+$ T Cell Isolation Kit 2 (#NC9062466) was utilized for some experiments using the same protocol.

VIII. B Cell proliferation assay

Spleens were harvested from naive female BALB/cByJ aged 6-10 weeks. Single cell suspensions were generated by gently forcing spleens through a 70 μm cell strainer. The resulting cell suspensions were washed with “B Cell Proliferation Media”, counted and resuspended at 5 x 10^6 cells/ml. The total splenocyte populations were then loaded with 1 μM CFSE (BD Biosciences #565082, dissolved at 5 mM in DMSO for storage at -20°C and was diluted to a working solution of 2 μM in 37°C PBS immediately before using), as described above for the T cell proliferation assays, before resuspension at the same cell concentration in B cell proliferation media. A flat bottomed 96 well plate was coated with 50 μg/ml anti-IgM $F(ab')_2$ overnight at 4°C then washed twice with PBS. The cell suspensions were plated at a concentration of 1.25 x 10^6 cells/ml in the presence of 1 nM IL-2 (R&D Systems #402-ML/CF). Recombinant CPXV 014-His or control proteins were added in the concentrations indicated and left for the duration of the stimulation. The plate was spun at 1200 rpm for 2 minutes to bring the cells into contact with the $F(ab')_2$-coated plate surface, and they were cultured for three days at 37°C 5% CO_2. After this incubation, they were washed three times with FACS buffer (PBS + 3% FBS), then stained with the following antibodies (see **Table 2**): LIVE/DEAD Fixable Aqua Dead cell stain (1:100), CD3ε-PerCP_5.5 (1:100 dilution), CD45R/B220-PE (1:125), CD19-PE-Cyanine7 (1:100). Cells were then analyzed on a BD LSRII cytometer as described above. Cells were gated according to the strategy: Time → Lymphocytes → Singlets → Live cells → $CD3^-$ → $CD19^+B220R^+$. Data was analyzed using TreeStar FlowJo software (v9.8).

IX. Statistical Analyses

All statistical analyses were performed using GraphPad Prism 6.0. Unless otherwise noted, all

P values were calculated using an unpaired, two-tailed Student's t test.

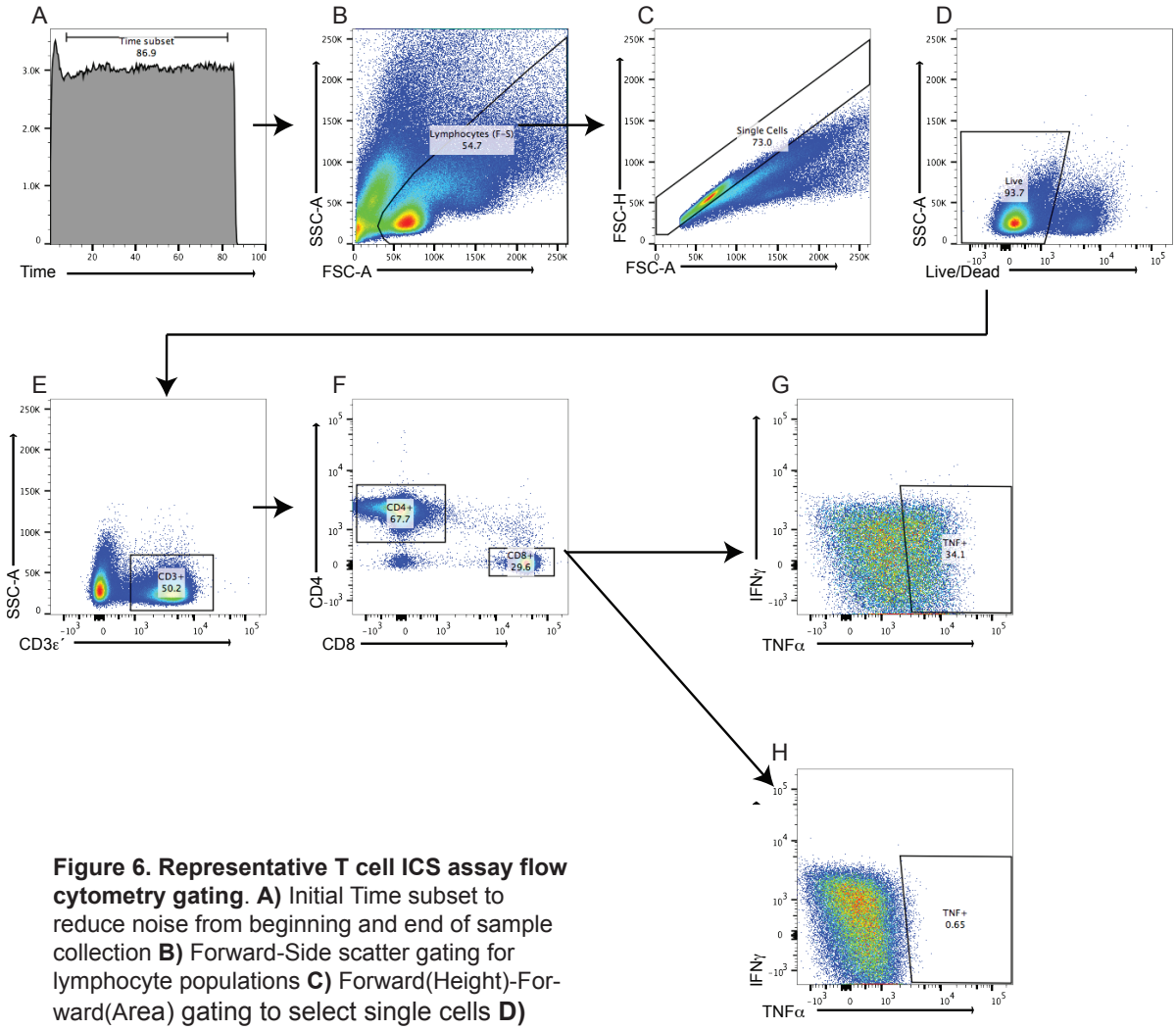
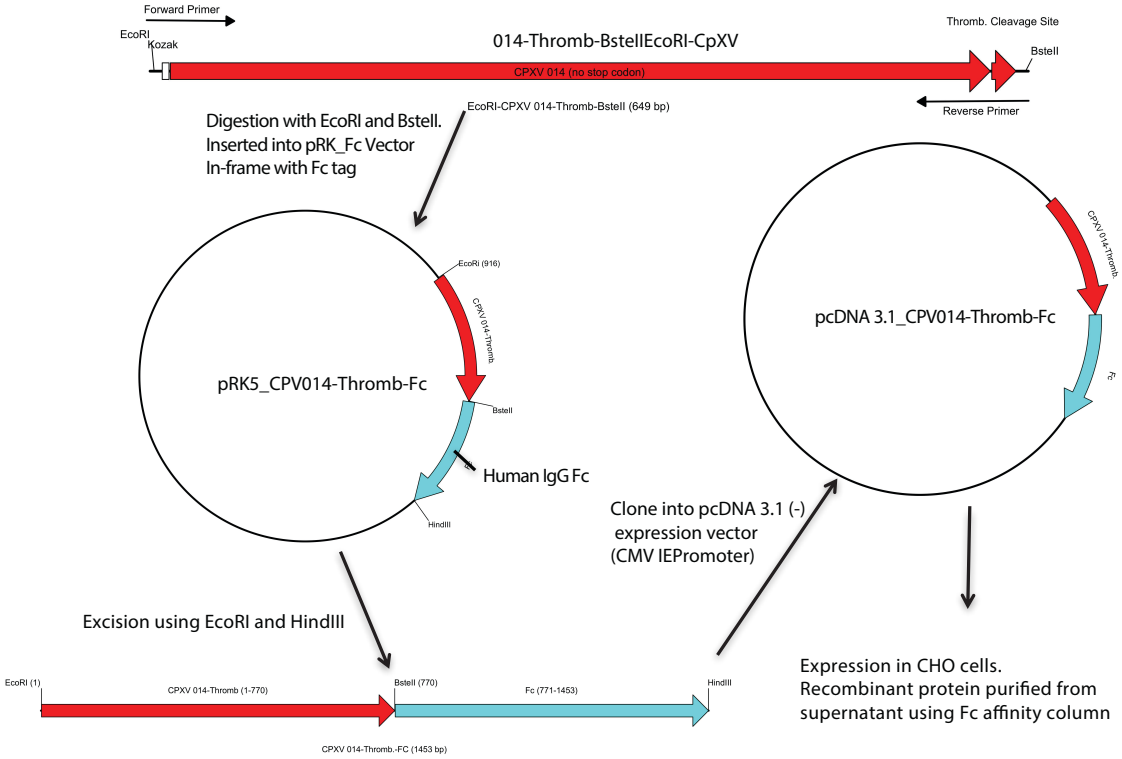


Figure 6. Representative T cell ICS assay flow cytometry gating. **A)** Initial Time subset to reduce noise from beginning and end of sample collection **B)** Forward-Side scatter gating for lymphocyte populations **C)** Forward(Height)-Forward(Area) gating to select single cells **D)** Gate for live cells **E)** gate for CD3+ cells **F)** gating on CD4+ and CD8+ T cell populations. **G)& H)** Gating for IFNγ/TNFα ICS. CD8+ population shown. **G)** Upper plot is with typical anti-CD3 + anti-CD28 stimulation; **H)** lower plot is with unstimulated cells.

Figure 7 Cloning strategy for construction of recombinant CPXV 014-Fc. CPXV014 was cloned from genomic WT CPXV (Brighton Red) DNA using the primers EcoRI-CPV14F and Bste2-Thr-CPV14R (**Table 1**). These primers introduced the Kozak sequence and an EcoRI digestion site at the 3' end as well as thrombin and BSTE II cleavage sites were introduced at the 5' end. This fragment was then cloned into pRK5-Fc (A gift from the laboratory of Dr. Scott Wong) in frame and directly upstream from the human IgG1Fc tag. The resulting CPXV14-Fc fragment was subsequently excised using EcoRI and HindIII and inserted into the pcDNA3.1 expression vector under the control of a CMV promoter.



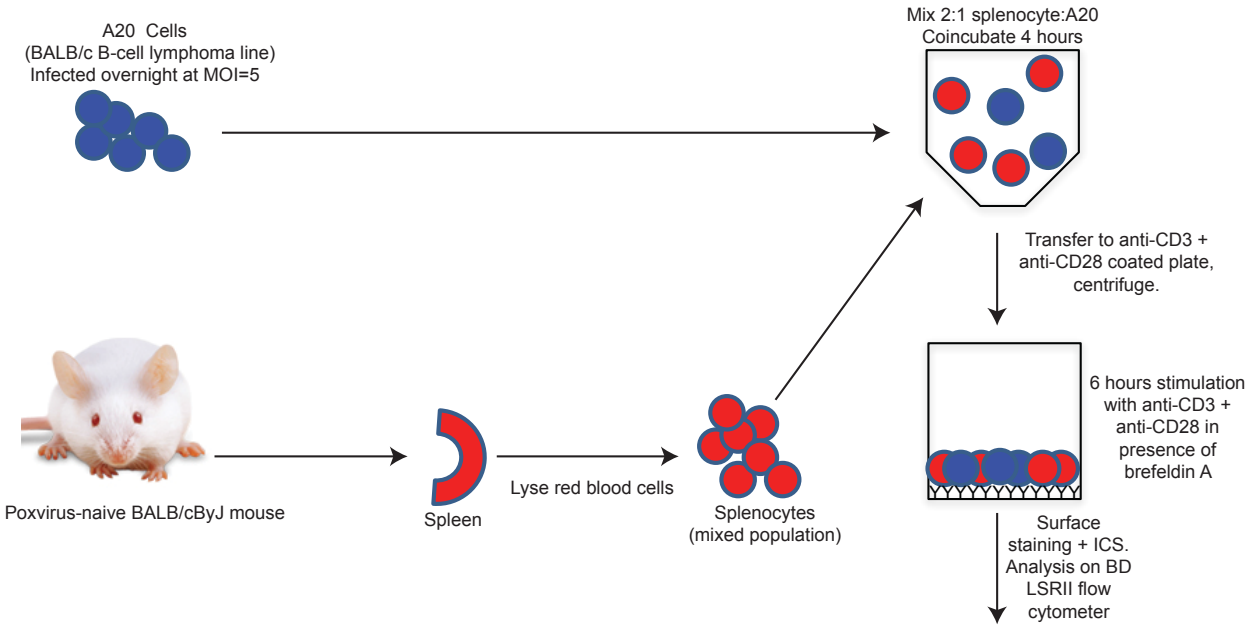


Figure 8. Naive T cell ICS activation assay. As described in Methods.

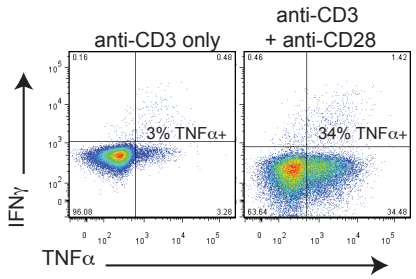


Table 1. Primer Sequences

Plasmid/Primer Name	Primer Sequence (5' - 3')
pCR2.1_∆014CPXV	
d14Int#1	AACACAATACTAATATTCGCATCATTATTTGTTGCATC
d14Int#2	GCTATTTTTAAATCCATATGACTAGTAGATCCTCTAGAA CAAGATGATACGTTAACTGGATATAGTTCAAAATCTATA G
d14Int#3	CTATAGATTTTGAACTATATCCAGTTAACGTATCATCTT GTTCTAGAGGATCTACTAGTCATATGGATTTAAAAATAG C
d14Int#4	GATCTGATAGACAGTTGCCTATTAAAGTGAAATTTTCTC TAGAGCGGATCCGCAGGTTTGC
d14Int#5	GCAAACCTGCGGATCCGCTCTAGAGAAAATTTCACTTT AATAGGCAACTGTCTATCAGATC
d14Int#6	AGCGCACATATTTGGAGGTATACCGTTAATATC
L-152-F	AGAAGCTGTACGAGCATAGTAACTTTTTATCAGACG
R-188-R	ACAATCATGTGGACCGGATAAACCACGA
pcDNA3,1CPXV14Fc	
EcoRI-CPV14F	ATGAATTCGCCGCCATGATAAACATAAACATAAACACA ATACTAATATTCGCATCATTATTTGTTGC
Bste2-Thr-CPV14R	CAGGTGACCTGAGATCCTCTTGGCACCAGATTAATGGT TCTGGTAGCGCACATATTTGGAGGTAT
qRT PCR for CPXV 014 expression profile	
14qPCR F	TGAATTGACAATTACTGTTAATAGTACAGATTGTGATCC
14qPCR R	GTGTAAAATTACCGATTAATACAGGTTCCGTTTCAG
CPXV021 F	CAAACGCTACAACAGATATTCCAGCTATCAG
CPXV021 R	TGATGTTGTAGTGTCTGGTTTTTCTGAACGTTG
CPXV066 F	GAAGGAAGATATCTGGTTCTAAAAGCCGTTAAAG
CPXV066 R	CACGTCCGTTCTCATAAACGGGACTT
RTPCR screening of ∆014 CPXV	
MVAA056L-67F	AGATGTCGAAA TGGCGAGAC
MVA056L-312R	CATGGTGGCATTTTGTATGC
CPXV012A	TCCCCGCGGATGTTTATCATGCGTGAATC
CPXV012B	TCCCCCCGGGTCAGATGATGCTGTCCAATT
CPXV013A	ATGGAGGAGTACGTGAATATAATTCTGGG

CPXV013B	TTATTTTCTAACGAATGTAACGAATTGGCGTTC
CPXV015A	ATGAAGATGAATATTATCTTTTTATCCGCTATCGTAACC
CPXV015B	TCATGATGAGTATTTATGATAACAAAGTTTTGTATTATC CGG
CrmA (CPXV 207) Screening primers	
CPX207-F	ATGGATATCTTCAGGGAAATCGCATCTTC
CPX207-R	TTAATTAGTTGTTGGAGAGCAATATCTACCAAC
CPX207INT-F	CTTCACTGAGGGGAAAATTAATCCACTATTGGAT
CPX207INT-R	CCATTTCCGTTGGAGATACGTAAAAGGG
Cloning Plasmids	
pRK5-Fc	Gift from Scott Wong, based on BD Pharmigen pRK5 plasmid (Cat#556104)
pcDNA3.1(+)	Thermo Fisher V79020
pcR 2.1 TOPO TA	Thermo fisher K450001

Table 2. Antibodies and cell stains

Supplier	Host	Antibody/Stain	Clone	Cat #
AVES Labs Inc	Chicken	Chicken anti-CPXV014C-terminal domain		
AVES Labs Inc	Goat	Goat-anti-chicken-IgY - HRP	polyclonal	H-1004
BD Bioscience		Streptavidin_PE_Texas Red		551487
BD Bioscience	Rat	Anti mouse TNFα-APC_Cy7	MP6-XT22	560658
BD Bioscience	Rat	Anti-mouse CD28	37.51	553294
BD Bioscience	Rat	Anti-mouse CD3ε	145-2C11	553057
BD Bioscience	Rat	Anti-mouse CD4-FITC	RM4-5	561835
BD Bioscience	Rat	Anti-mouse CD4-Pacific Blue (Rat)	RM4-5	558107
BD Bioscience	Rat	Anti-mouse CD4-PE	RM4-5	553047
BD Bioscience	Rat	Anti-mouse CD44-PE_CF594	IM7	562464
BD Bioscience	Rat	Anti-Mouse CD45R/B220-PE	RA3-6B2	553090
BD Bioscience	Rat	Anti-mouse IFNγ- FITC	XMg1.2	554411
BD Bioscience	Rat	Anti-mouse IFNγ-Alexa 700	XMg1.2	557998
BD Bioscience	Rat	Anti-mouse TNFα-PE	MP6-XT22	554419
BD Bioscience	Rat	Anti-mouseCD16/CD32 (“FC Block”), not functional grade (for nonspecific background reduction)	2.4G2 (IgG1)	553142
Bethyl labs	Goat	Anti-human IgG-Fc	polyclonal	A80-

				104P
BioX	Human	hIgG	polyclonal	BE0092
eBioscience		Streptavidin-PE_Cy7		25-4317-82
eBioscience	Goat	Anti-mouse IgM, μ chain specific, $F(ab')_2$ fragment	polyclonal	16-5092-85
eBioscience	Rat	Anti-Mouse CD19-PE-Cyanine7	1D3	25-0193-81
eBioscience	Rat	Anti-mouse CD3ε-PerCP_5.5	145-2c11	45-0031-82
eBioscience	Rat	Anti-mouse CD8-APC (Rat)	53-6.7	17-0081-83
eBioscience	Rat	Anti-mouse CD8-PE_Cy7	53-6.7	25-0081
eBioscience	Rat	Anti-mouseCD16/CD32 (“FC Block”), functional grade	93 (IgG2a)	16-0161-82
eBioscience	Rat	IgG2a κ Isotype Control Functional Grade Purified	eBR2a	16-4321-81
Jackson Immunoresearch	Goat	Anti-mouse IgM,μ chain specific	polyclonal	115-005-020
Life Technologies		LIVE/DEAD® Fixable Aqua Dead Cell Stain		L34957
Sigma	Mouse	Murine IgG	polyclonal	I8765-10MG
ViroStat	Rabbit	Rabbit Antibody to Vaccinia virus – Biotin conjugated	polyclonal	8107
ViroStat	Rabbit	Rabbit Antibody to Vaccinia virus – FITC conjugated	polyclonal	8103

Chapter Three: Identification of CPXV 014

I. Initial Discovery

This project began by testing whether CPXV-infected cells could inhibit an *ex vivo* activation assay for naive murine splenocytes. Whole populations of splenocytes were isolated from poxvirus-naive BALB/cByJ mice and coincubated with infected A20 cells (a syngeneic B cell line) for four hours prior to six hours of T cell stimulation in the presence of brefeldin A using plate-bound anti-CD3 and anti-CD28 **(Figure 8)**. The cells were then stained for surface markers and intracellular cytokines. Activation of naive T cells was detected using flow cytometry to examine the TNFα response. We discovered that A20 cells infected with CPXV, but not VACV, were able to inhibit the TNFα response to the agonistic antibodies **(Figure 9)**. This inhibition was much more pronounced with $CD8^+$ T cells than with $CD4^+$ T cells.

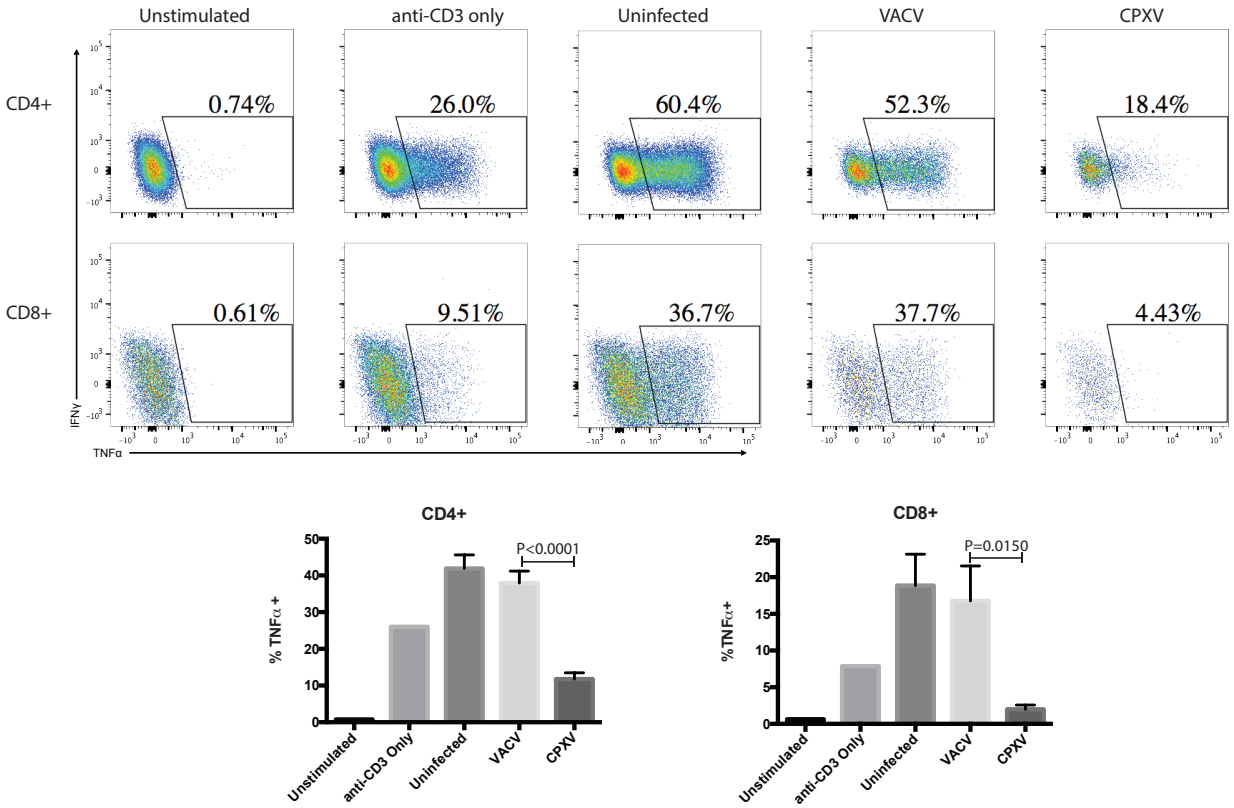


Figure 9 Cowpox inhibits naive T cell activation by anti-CD3 and anti-CD28 antibodies.
The *ex vivo* naive T cell ICS activation assay using infected A20 cells was performed as described in Methods. Briefly, A20 target cells were infected at an MOI of 5 for 16 hours, then washed 3 times to remove residual virus. Splenocytes from naive, female, Specific Pathogen Free (SPF) , age-matched Balb/cByJ mice were mixed with infected A20 cells at a 2:1 ratio (splenocyte:A20) for 4 hours before being stimulated with plate bound anti-CD3 and anti-CD28 for 6 hours in the presence of Brefeldin A (BFA). Next, cells were surface stained and subjected to Intracellular Cytokine Staining (ICS). Cells were gated according to the strategy shown in **Figure 6**. Error bars indicate the mean ± Standard Error of Mean (SEM), comprising of 5 mice per group. Data from one representative experiment shown. P values calculated by two-tailed unpaired Student's T test.

We monitored direct T cell infection using a polyclonal anti-VACV antibody which cross reacts with CPXV. What infection occurred was at such low levels (<1%) that direct infection of T cells seemed unlikely to account for the levels of inhibition observed (data not shown). This inhibition still occurred to a lesser extent, in cells treated with ST-246 **(Figure 10)**, an anti-poxviral drug which prevents viral egress from infected cells by targeting CPXV 061, a membrane protein required for EV formation and ortholog of VACV F13L [240-242]. These results supported the hypothesis that the inhibition of naive T cell activation seen in this assay was not due to direct infection (even unproductive) of the T cells by CPXV, and was instead due to some factor expressed by

the infected cells. The next questions were what gene or genes are responsible and how does this inhibition occur?

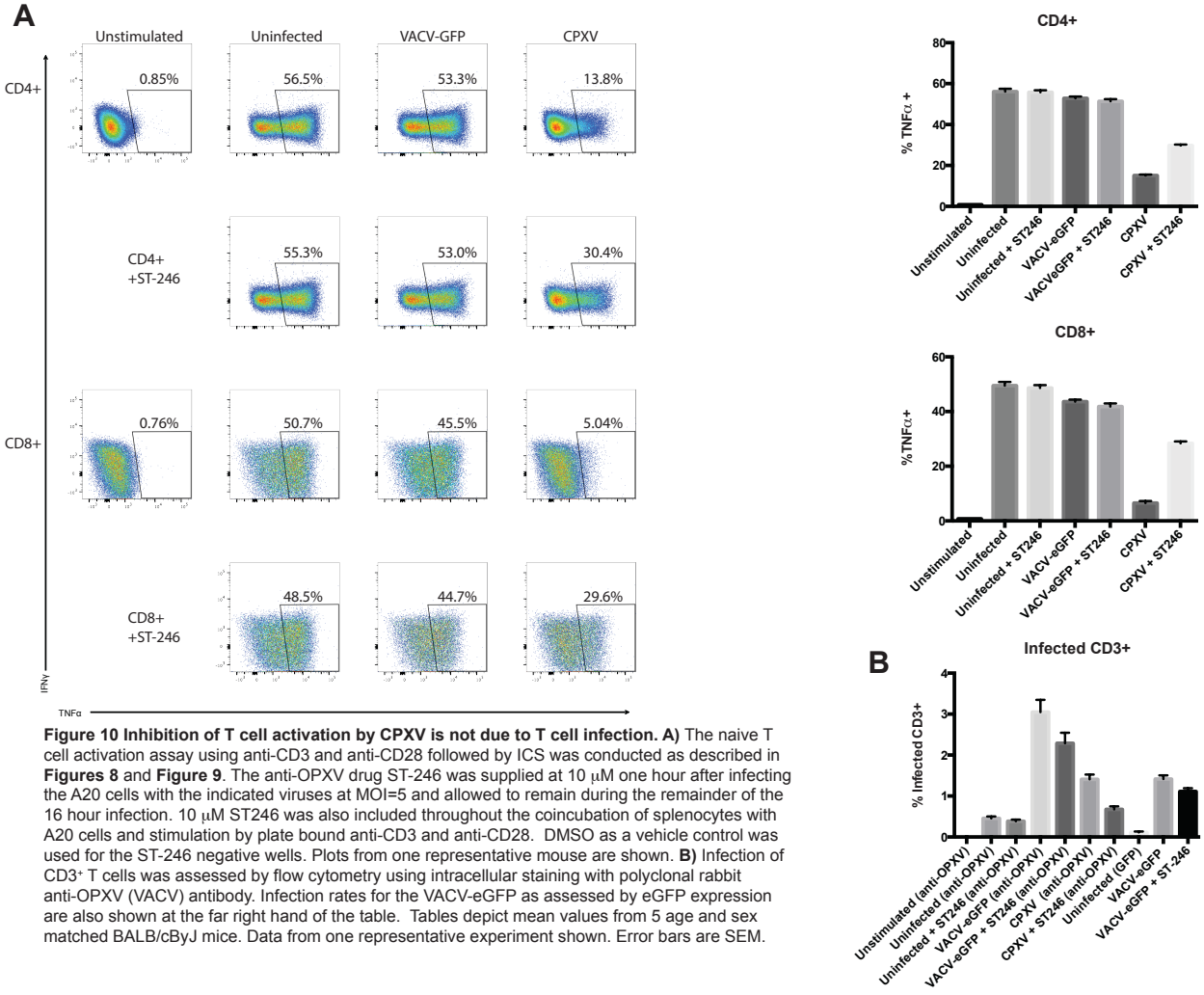


Figure 10 Inhibition of T cell activation by CPXV is not due to T cell infection. A) The naive T cell activation assay using anti-CD3 and anti-CD28 followed by ICS was conducted as described in **Figures 8** and **Figure 9**. The anti-OPXV drug ST-246 was supplied at 10 μM one hour after infecting the A20 cells with the indicated viruses at MOI=5 and allowed to remain during the remainder of the 16 hour infection. 10 μM ST246 was also included throughout the coincubation of splenocytes with A20 cells and stimulation by plate bound anti-CD3 and anti-CD28. DMSO as a vehicle control was used for the ST-246 negative wells. Plots from one representative mouse are shown. **B)** Infection of $CD3^+$ T cells was assessed by flow cytometry using intracellular staining with polyclonal rabbit anti-OPXV (VACV) antibody. Infection rates for the VACV-eGFP as assessed by eGFP expression are also shown at the far right hand of the table. Tables depict mean values from 5 age and sex matched BALB/cByJ mice. Data from one representative experiment shown. Error bars are SEM.

II. Deletion Mutant Mapping

In order to determine which gene or genes expressed by CPXV could be responsible for the observed phenotype, we conducted a series of loss-of-function T cell assays (utilizing plate-bound anti-CD3 and anti-CD28 to stimulate and ICS for TNFα as a readout of activation). To this end, we obtained a series of CPXV mutants with overlapping deletions from the Pickup laboratory at Duke University **(Figure 11a)**. Since

the variable immune modulating genes of the poxviruses occur towards the flanks of the large linear genomes, we began with two mutants possessing large deletions in either flank. As shown in **Figure 11b**, deletion of the left-hand flank of the genome, but not the right hand flank, restored the T cell TNFα response in our assay. Next, we examined if any of three overlapping deletion mutants within this flank would also restore T cell activation in our assay. As shown in **Figure 11c**, when the region encompassing ORFs 11 through 16 was deleted, the mutant virus again was no longer able to inhibit naive T cell stimulation.

Since VACV possesses orthologs to CPXV 011 and 013, and does not inhibit naive T cell activation by plate-bound antibodies, these two ORFs were considered unlikely to be responsible for the observed phenotype. CPXV 016 was present in one of the other mutants that still inhibited T cells (CPXV A531), so that gene could not be responsible. CPXV 012, as described earlier, has already been characterized by ours and other laboratories as an inhibitor of MHC Class I loading by TAP [226, 228]. That left CPXV 014 and 015 as the potential candidate genes for inhibition in our assay. CPXV 015 has previously been characterized as a secreted viral homolog of CD30 [176]. Sequence analysis indicated CPXV 014 also possesses a putative secretion signal sequence. Our next step was to determine if this poxviral inhibitor of naive T cells was also secreted by infected cells.

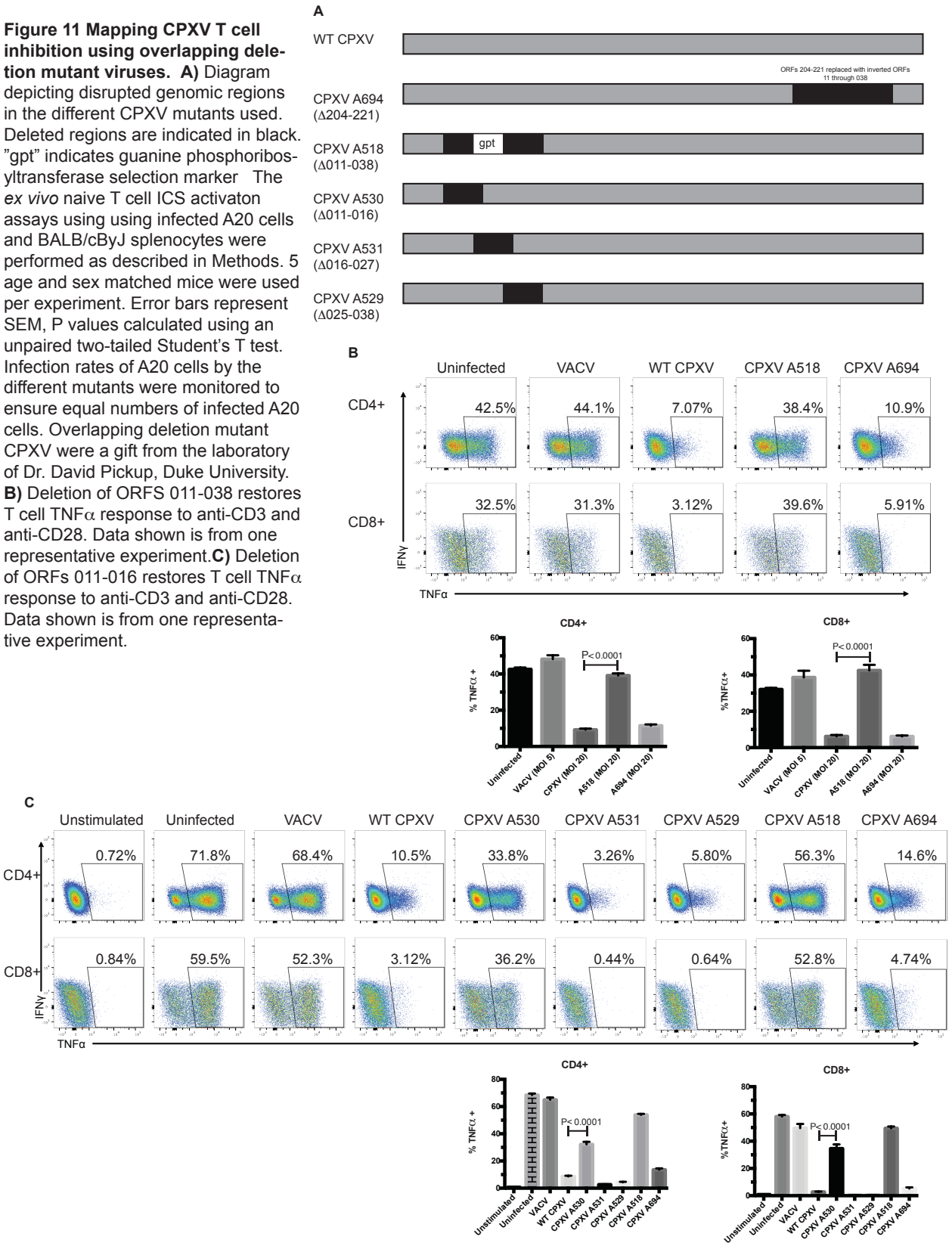


Figure 11 Mapping CPXV T cell inhibition using overlapping deletion mutant viruses. A) Diagram depicting disrupted genomic regions in the different CPXV mutants used. Deleted regions are indicated in black. ”gpt” indicates guanine phosphoribosyltransferase selection marker The *ex vivo* naive T cell ICS activaton assays using using infected A20 cells and BALB/cByJ splenocytes were performed as described in Methods. 5 age and sex matched mice were used per experiment. Error bars represent SEM, P values calculated using an unpaired two-tailed Student’s T test. Infection rates of A20 cells by the different mutants were monitored to ensure equal numbers of infected A20 cells. Overlapping deletion mutant CPXV were a gift from the laboratory of Dr. David Pickup, Duke University. **B)** Deletion of ORFS 011-038 restores T cell TNFα response to anti-CD3 and anti-CD28. Data shown is from one representative experiment.**C)** Deletion of ORFs 011-016 restores T cell TNFα response to anti-CD3 and anti-CD28. Data shown is from one representative experiment.

III. A Soluble Factor

We performed a variant of our plate-bound antibody and ICS naive T cell assay in which the splenocytes were incubated with virus-free supernatants taken from infected cells instead of coincubating them with infected cells directly. The supernatants were generated by infecting MC57 fibroblasts overnight with wild-type CPXV or VACV at a multiplicity of infection (MOI) of 5. The supernatants were then subjected to ultra-high speed centrifugation in order to pellet out any virus particles or cell debris, and they were demonstrated to be virus-free by overlaying them on susceptible fibroblasts and looking for plaque formation (data not shown). The splenocytes were coincubated with these supernatants for four hours prior to a six-hour stimulation with plate-bound anti-CD3 and anti-CD28.

As shown in **Figure 12**, the virus-free supernatants from CPXV-, but not VACV-infected cells were capable of inhibiting T cell activation in our assay. These results confirmed that the viral inhibitor under study is secreted from the infected cells.

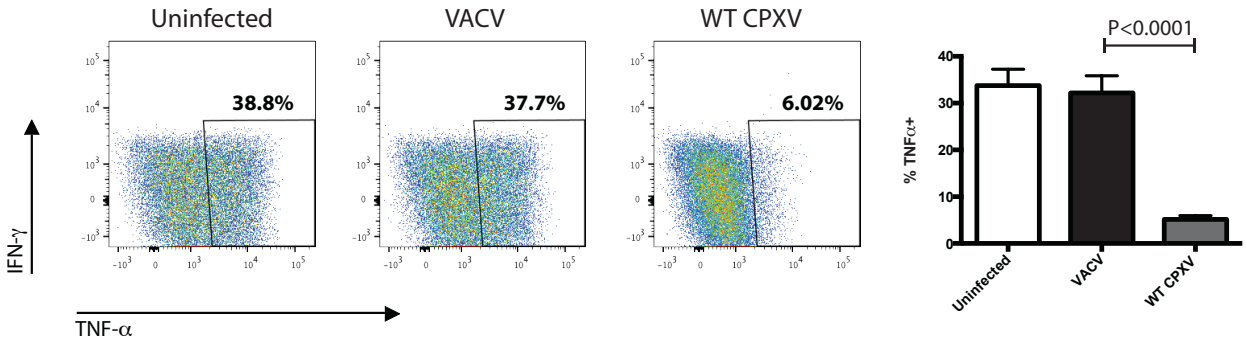
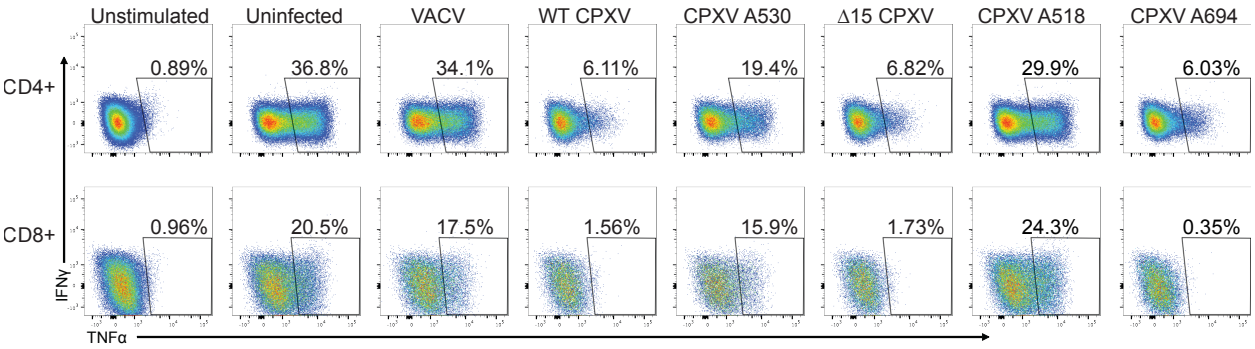


Figure 12 CPXV encodes a secreted, soluble T cell inhibitor. The *ex vivo* naive T cell ICS activation assay using virus-free supernatants was performed as described in Methods. Briefly, MC57 fibroblastswere infected (MOI=5) for 16 hours with the indicated viruses. Supernatants were harvested and virus was removed by ultracentrifugation (18,000 rpm, 90 m, 4C). SPF BALB/cByJ (6 weeks old, age & sex matched) mouse splenocytes were pre-treated for 4 hrs with a 1:1 ratio of supernatants to media prior to stimulation with plate bound anti-CD3 and anti-CD28 in the presence of BFA for 6 hrs. Cells were surface stained followed by ICS. Data was collected on a BD LSR2 and then analyzed with FloJo software v10.1 (TreeStar). Values depicted represent the total percentage of TNFα+ gated on Live $CD3^+$ $CD8^+$ lymphocyte single cells following background subtraction. Graph depicts the mean responses for the pooled data of 3 individual experiments each with 5 mice. Plots depicted are representative results from one mouse. Error bars are SEM, P value calculated using unpaired two-tailed Student's T test.

IV. vCD30 (CPXV 15) vs. CPXV 014

We obtained a disruption mutant of CPXV 015 (vCD30) from the Pickup lab and tested if disabling this gene would lead to a restoration of T cell stimulation in our assay. As in the initial mapping experiments, we incubated naive murine splenocytes with A20 cells which had been infected with WT CPXV or the mutant virus before stimulating them with plate-bound anti-CD3 and anti-CD28. As shown in **Figure 13**, inactivation of CPXV 015 did not reverse the phenotype of inhibition demonstrated by wild-type CPXV. This left CPXV 014 as the most likely remaining candidate, and we focused on determining if this gene was necessary and sufficient for the inhibition observed.



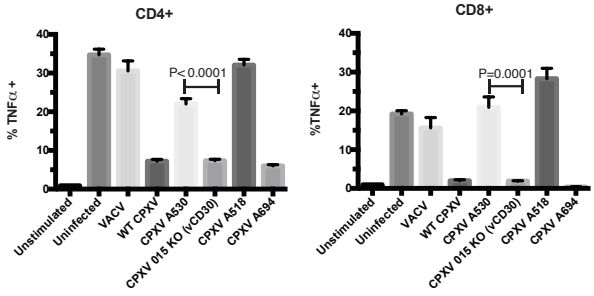


Figure 13 Deletion of CPXV 015 (vCD30) does not restore naive T cell activation by anti-CD3 + anti-CD28. The *ex vivo* naive T cell ICS activation assay using infected A20 cells was conducted and analyzed as before. Tables indicate mean TNFα+ T cells from 5 age and sex matched BALB/c-ByJ mice in one representative experiment. Error bars indicate SEM, P values calculated using unpaired two-tailed Student's T test.

V. CPXV 014 and Relations

V A. SECRET domain proteins

CPXV 014 consists of 202 amino acid residues and has a molecular weight of 22.5 KDa. It possesses a secretion signal and is predicted to be heavily modified by N-linked glycosylation. The above mentioned results strongly implicated CPXV 014 as the responsible CPXV gene for the inhibition seen in our T cell assay. A review of the literature indicated that CPXV 014 (also known as SCP-2), belongs to the poxviral family of chemokine-scavenging SECRET domain-containing proteins [170], which are themselves part of a larger, more recently defined pox viral immune evasion (PIE) domain protein family [243].

The first SECRET domain protein identified was in the CTD of VARV CrmB, but several more in CPXV, ECTV, MPXV and VACV were quickly identified, either independently expressed or as the CTD of CrmB or CrmD **(Figure 5)**. These were found to be broad-spectrum chemokine binding proteins. CPXV 014 (SCP-2), CPXV 224 (SCP-1) and their ECTV orthologs were shown by surface plasmon resonance to bind CCL25, CCL27, CCL28, CXCL11, CXCL12b, CXCL13 and CXCL14, despite bearing no sequence similarity to known host or viral chemokine receptors. Indeed, they bear little sequence similarity to each other, indicating the likely existence of a specific chemokine-binding fold in this domain. However, they do share two conserved cysteine residues, likely critical to their function [170]. The bound chemokines are known to be involved in T, B and dendritic cell chemotaxis and recruitment, particularly to the skin and mucosa [170, 244-246].

V B. PIE Domain proteins

The PIE domain proteins, including the SECRET domain family, share a conserved β-sandwich fold structure (despite little sequence similarity) [243]. The ancestral function of these proteins is thought to be binding chemokines, and many family members retain this function [243]. However, it appears as though several PIE domain proteins have evolved other, more specialized functions and members have been demonstrated to bind other non-chemokine ligands such as GM-CSF, MHC class I, and various glycosylaminoglycans [243]. None of them are deemed to be essential to poxviral replication and all possess signal peptides for their secretion [243]. This family includes the CrmB and CrmD vTNFRs as well as CPXV 203 (mentioned earlier in the

context of MHC Class I ER retention), which gives a small glimpse into the variety of ligands bound by this family and the versatility of the PIE domain structure [243].

V C. Orthologs and alignments

We identified CPXV 014 using the Brighton Red (BR) strain of CPXV, but it exists with a high (>95%) degree of sequence identity in the two other most commonly used laboratory strains, Gri-90 and Ger-91 **(Figure 14a)**. Similar orthologs of CPXV 014 exist in ECTV, as well as raccoonpox and horsepox (**Figure 14b)** though not in MPXV, VACV or VARV. As mentioned, it has been shown to bind a variety of CCL and CXCL chemokines by virtue of its SECRET domain structure. Additionally, unpublished data generated by our collaborators in the laboratory of Daved Fremont indicated that it binds murine FcγR. However, it has never been reported to have any activity upon T lymphocytes. Since naive T cells are not believed to express any FcR [248], and chemokine sequestration seems unlikely to play a role in inhibiting T cell activation during *in vitro* assays (since cell migration and chemotaxis are not expected to have an effect within the limited confines of a 96-well plate well), we hypothesized that our T cell assay data represented a new and undescribed immunomodulatory activity of this protein.

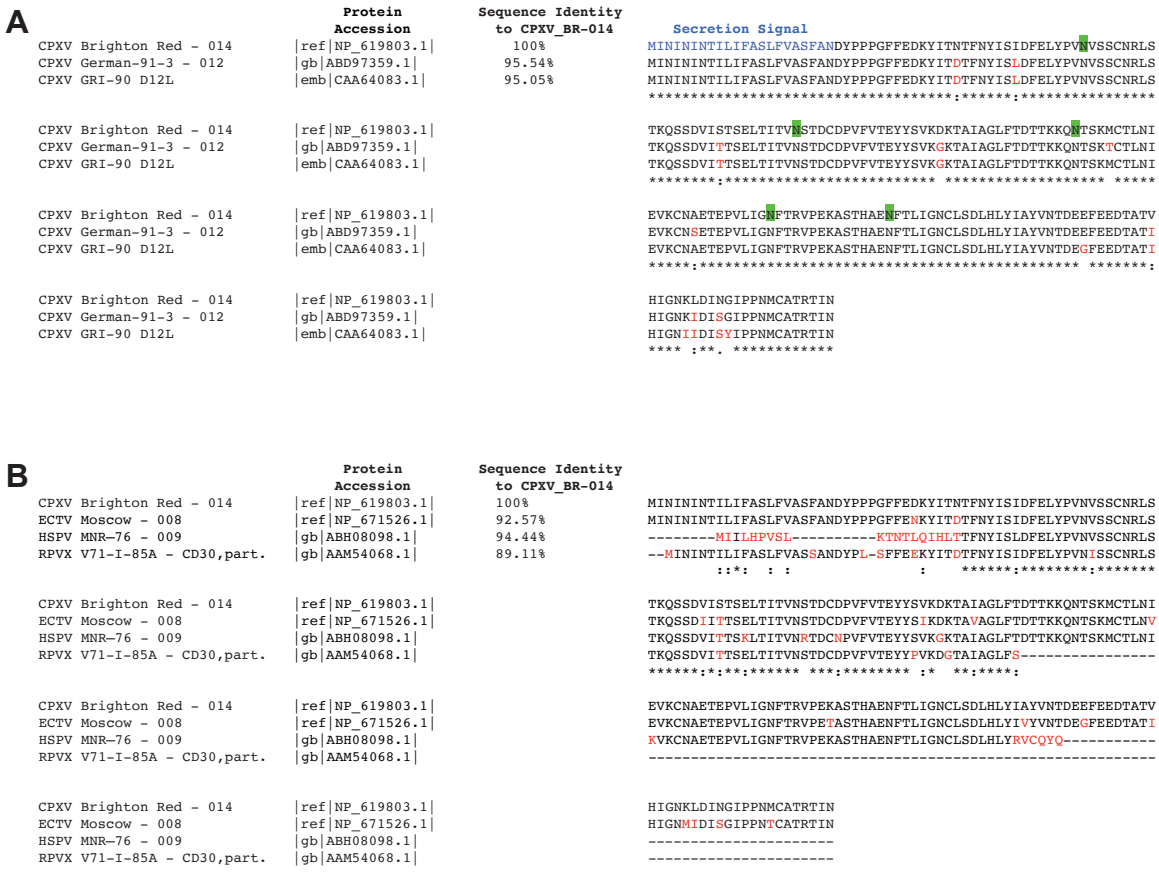


Figure 14 Alignment of CPXV 014. A) Alignment of CPXV 014 protein (SCP-2) between different lab strains of CPXV. Secretion signal is indicated in blue. Predicted N-linked glycosylation sites are highlighted in green. **B)** Alignment of CPXV014 protein with closest OPXV orthologs. Differences from CPXV 014 (BR) sequence are indicated in red. Glycosylation predictions from GlycoEP (www.imtech.res.in). Sequence identity calculated using CLUSTAL O(1.2.1). [247]

VI. Expression of CPXV 014

We wished to know the kinetics of CPXV 014 expression so we conducted a qPCR analysis of CPXV 014 mRNA relative to prototypically early or late CPXV genes. Early genes are those expressed before viral DNA replication, while late genes require viral genomic replication. As shown in **Figure 15**, CPXV 014 follows a late gene expression profile like that of a model late gene CPXV 066, but not like the early gene CPXV 021 [181]. Additionally, its expression was inhibited by late-gene inhibitor arabinose C (a pyrimidine nucleoside analog inhibiting poxviral replication) [249] just as CPXV 066 was. This indicates that CPXV 014 expression requires viral genomic

replication like other late genes.

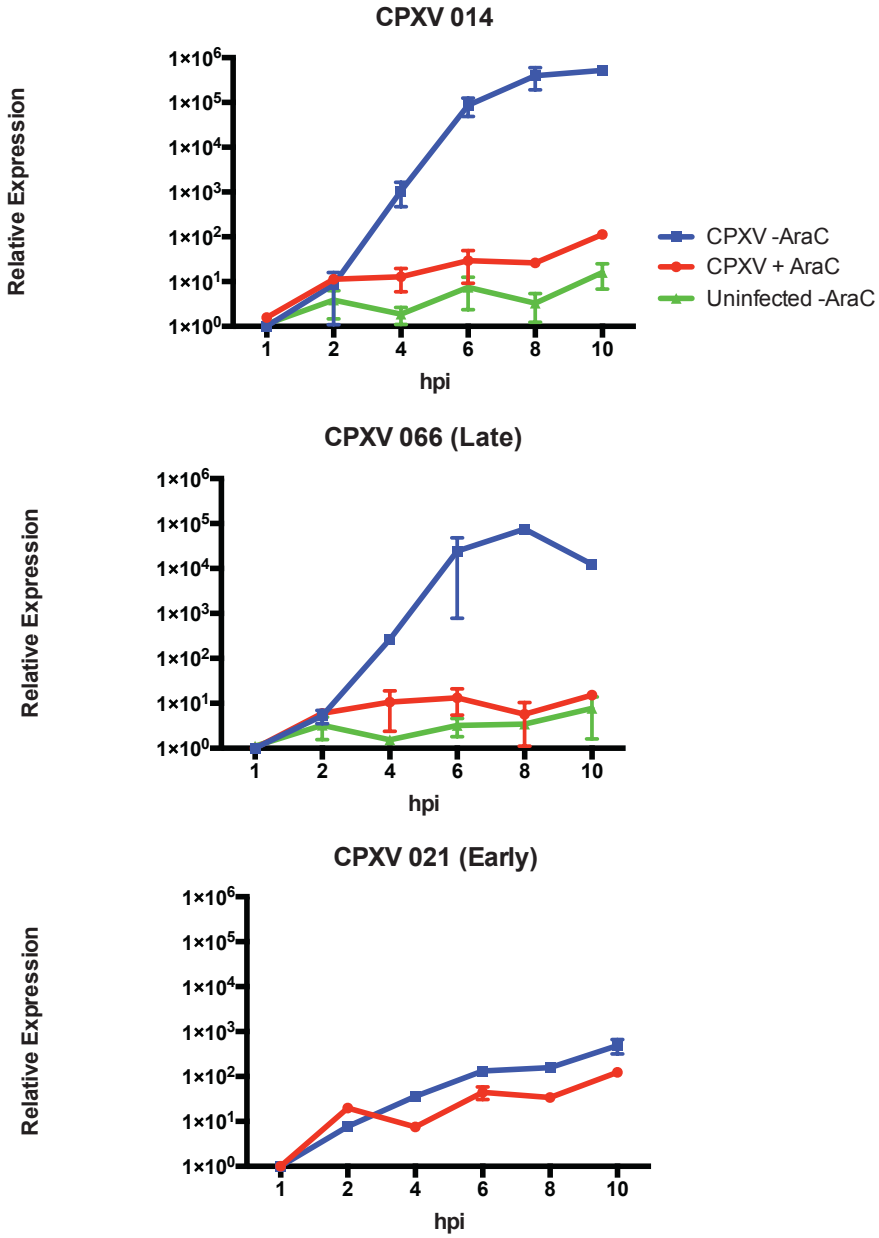


Figure 15 CPXV 014 Exhibits typical late gene expression profile. BSC-40 cells were infected at MOI=3 in the presence or absence of the late-gene inhibitor arabinose-c (40 µg/ml). RNA was isolated at the indicated timepoints. qPCR was used to quantitate the amount of CPXV 014, CPXV 066 (a prototypical late gene) and CPXV 021 (a prototypical early gene). Expression levels are portrayed as relative to GAPDH housekeeping gene expression. Data was collected using an Applied Biosciences StepOne cycler and software.

Chapter Four: CPXV 014 inhibits an unexpected cofactor of *in vitro* T cell activation

I. Making ∆14 CPXV

I A. ∆14 CPXV Construction

In order to determine if CPXV 014 was sufficient for the inhibition of naive murine T cells *in vitro*, we generated a mutant CPXV wherein ORF 014 was disrupted by replacing the middle (306 bp) coding section with a EGFP/GPT cassette under pox viral promoters **(Figure 16)**. The full description for the generation of this virus is detailed in the methods section, and only a brief outline is provided here.

The 150 nucleotide 5' and 3' termini of the 606bp CPXV 014 gene were fused to the 5' & 3' ends of a previously constructed EL_EGFP_7.5K_GPT cassette using splice-overlap extension PCR. The resulting cassette was cloned into the pCR2.1_TOPO-TA vector using manufacturer's instructions). The resulting pCR2.1_∆014CPXV plasmid was transfected into BSC-40 cells which had been infected with WT CPXV (BR) allowing it to recombine with the WT genome. The resulting viruses were cultured in GPT selection media and screened for GFP expression. GFP positive plaques were purified and screened for the insert via PCR. Expression of surrounding genes was verified by RT-PCR to ensure that expression was not altered **(Figure 17)**. In addition, the final clone of the ∆14 CPXV virus was then subjected to genomic sequencing in order to confirm the replacement of CPXV 014 and the integrity of any other ORFs.

l B. Repairing CPXV 207

When the initial ∆14 CPXV virus was sequenced, it was revealed that an additional genetic lesion had occurred in CPXV 207 (CrmA), another immune modulating gene. In order to repair the virus such that CPXV 014 was the only disrupted gene, we took advantage of the natural ability of CPXV to recombine with itself during infection. Vero cells were infected with both the ∆14-∆207 virus and the WT CPXV using an MOI of 2. After 24 hours, the resulting virus was harvested and subjected to plaque purification under the same selective conditions described above. 24 GFP^+ plaques were isolated, screened for the EGFP/GPT insertion in CPXV 014 and intact CrmA, and finally sequenced until we identified a clone in which CPXV 207 was repaired by recombination with WT CPXV but in which CPXV 014 remained disrupted. This repaired ∆14 CPXV is the isolate used in the studies shown here unless otherwise indicated.

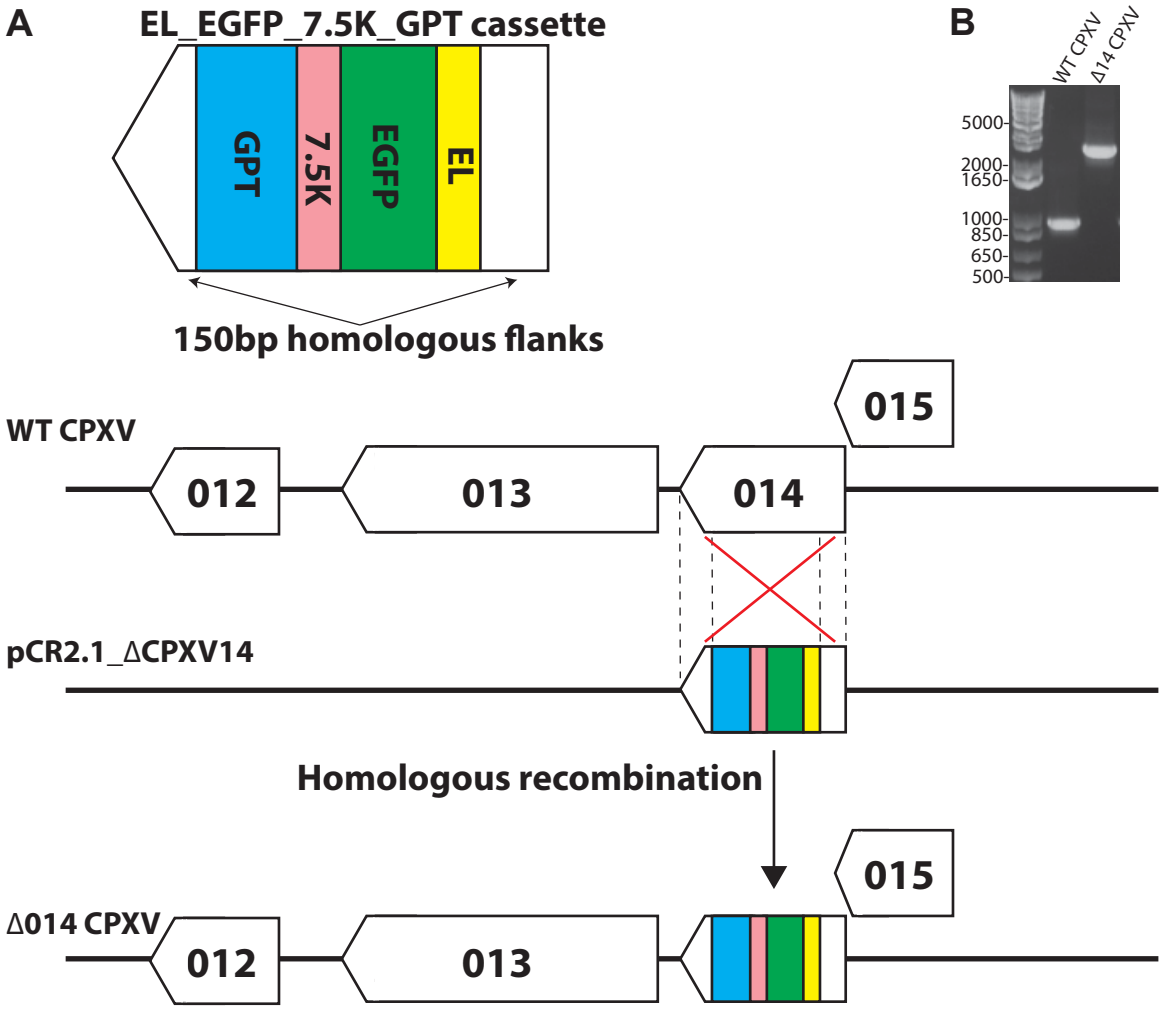
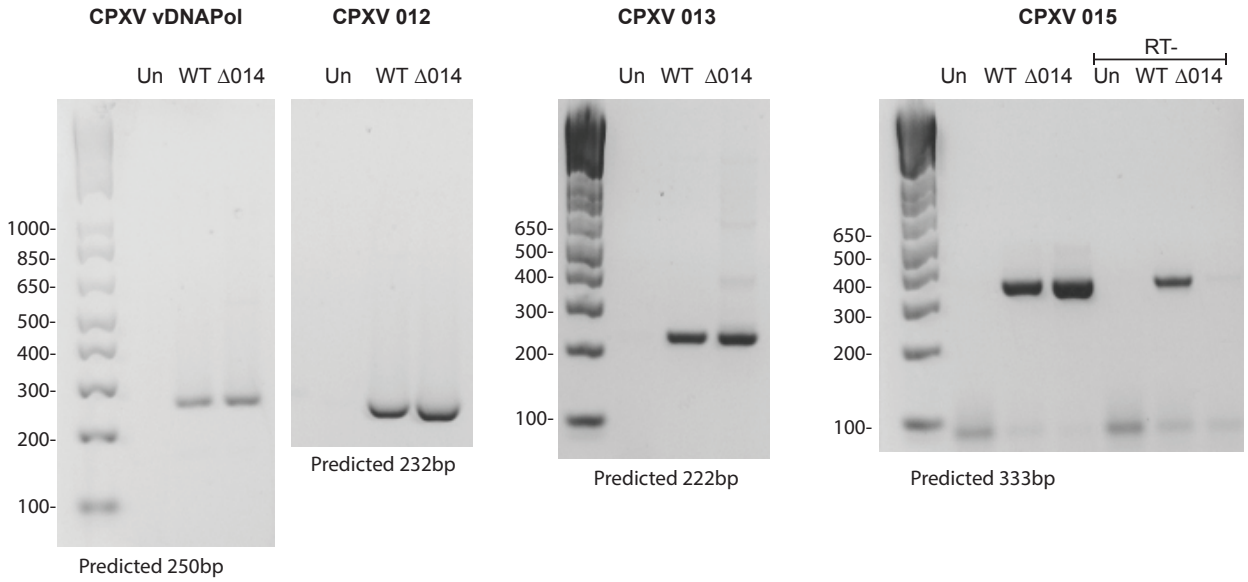


Figure 16 Generation of Δ14 CPXV by homologous recombination. **A)** Schematic showing insert and pCR2.1_Δ14 recombination with WT CPXV as described in Methods. **B)** PCR confirmation of Δ14 CPXV cassette. PCR analysis was conducted on genomic DNA from both WT and Δ14 CPXV virus using primer pair L-152-F and R-188-R (**Table 1**). Predicted band sizes were 941bp for WT CPXV and 2536bp for Δ14 CPXV containing the EL_EGFP_7.5K_GPT. cassette.

Figure 17 Genes surrounding the disrupted CPXV 014 gene in CPXV Δ014 maintain normal expression patterns and are not disrupted. RNA was isolated from BSC-40 cells infected overnight at an MOI of 5 by WT or Δ14 CPXV using a Qiagen RNAeasy kit according to the manufacturers instructions. Genomic DNA was digested, dNTPs and random hexamers were added, and the samples were split into 2 tubes for either mock or reverse transcriptase inclusion. cDNA was generated using reverse transcriptase according to published procedures. We then used the generated cDNA to screen the expression of multiple surrounding genes via PCR : vDNA Polymerase (using primers "MVA056L-67F" and "MVA056L-312R"), CPXV 012 (primers "12A" and "12B"), CPXV 013 ("13A" and "13B"), and CPXV 015 ("15A" and "15B") (See **Table 1**). The resulting PCR products were run on a 1% agarose gel and stained with ethidium bromide for visualization. Un=Uninfected cell. RT- = negative control with no reverse transcriptase.



II. Deleting CPXV 014 Restores T Cell Activation

II A. *In vitro* Δ14 CPXV T cell assays

Once we had a confirmed CPXV mutant virus with disrupted ORF 14, Δ14 CPXV, we tested this virus in our plate-bound anti-CD3 anti-CD28 naive T cell activation assay. Uninfected or VACV-infected A20 cells were included as negative controls. As seen in **Figure 18**, disruption of CPXV 014 resulted in a restoration of the naive T cell TNFα response relative to WT CPXV. From these results, we concluded that CPXV 014 was necessary for the inhibition of naive T cell activation by plate bound antibodies.

In order to ensure that this phenotype was not specific to BALB/cByJ mice, this assay was also performed with splenocytes isolated from C57BL/6 mice in which the

virus demonstrated the same reversal of T cell inhibition **(Figure 19)**.

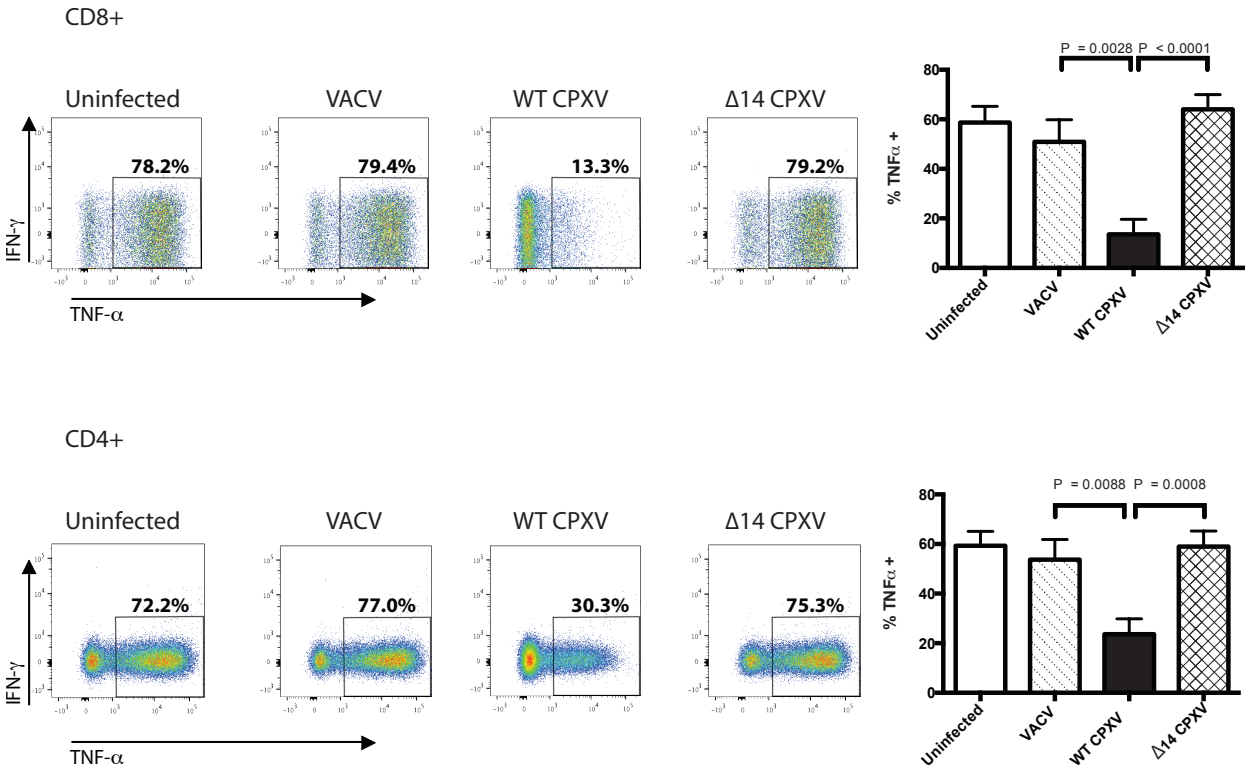


Figure 18 Inactivation of CPXV 014 restores anti-CD3/anti-CD28-mediated naive T cell stimulation. *Ex vivo* naive T cell ICS activation assay using infected A20 cells was performed as described in Methods. Briefly, A20 target cells were infected at an Multiplicity of Infection (MOI) of 5 for 16 hrs, then washed 3 times to remove residual virus. Splenocytes from naive, SPF BALB/c mice, age and sex matched, were mixed with infected A20 cells at a 2:1 ratio (splenocyte:A20) for a 4 hr preincubation and then stimulated with plate bound anti-CD3 and anti−CD28 for 6 hrs in the presence of BFA. Next, cells were surface stained and then subjected to ICS. Cells were gated according to the strategy outlined in **Figure 6**. Data pooled from 2 independent experiments each with 5 mice per group. Error bars represent SEM, P values were calculated using an unpaired two-tailed students T test.

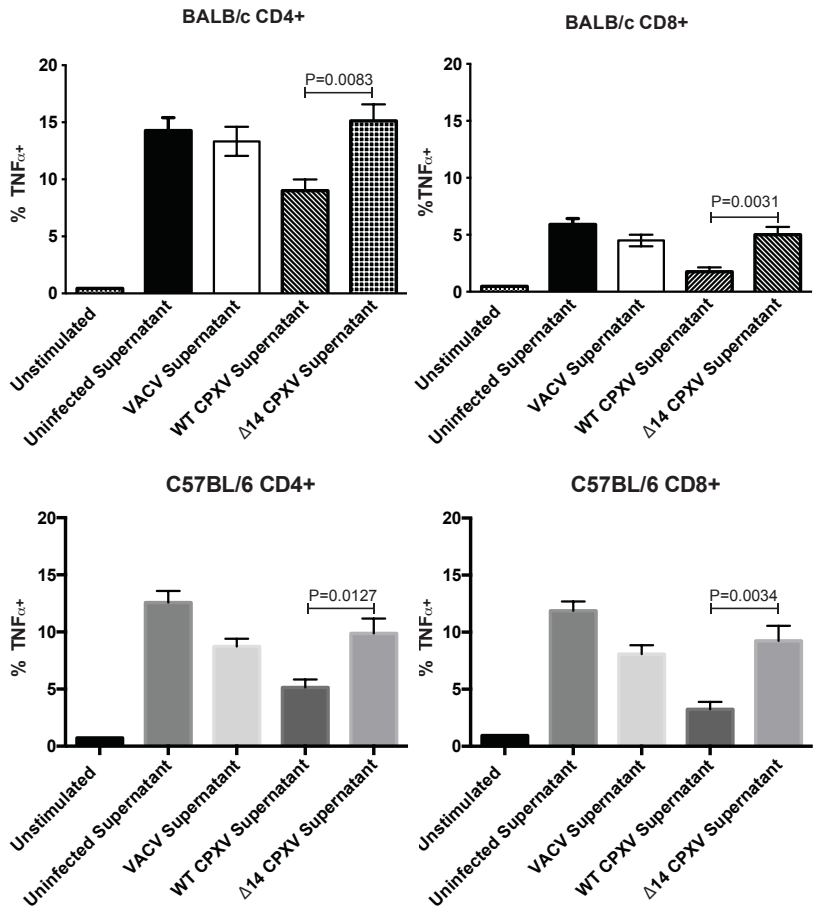


Figure 19 CPXV 014 is required for inhibition of naive T cell activation by plate-bound anti-CD3 and anti-CD28 in both BALB/cByJ and C57BL/6 mice. The *ex vivo* naive T cell ICS activation assay using virus-free supernatants was performed as described in the methods section for two groups of five age- and sex-matched mice, either BALB/cByJ or C57BL/6. Gating was performed according to the strategy outlined in **Figure 6**. Error bars depict SEM, P values calculated using unpaired two-tailed Student's T test.

II B. *In vivo* Δ14 CPXV T cell responses

In addition to testing the Δ14 CPXV *ex vivo* in mouse splenocytes, we also studied the effect of the gene deletion would have on CPXV virulence during an *in vivo* mouse infection model. David Edwards infected age matched female C57BL/6J mice with WT or Δ14 CPXV in two models of infection: intraperitoneally (**Figure 20a**) or intranasally (**Figure 20b** and **20c**). The intraperitoneal route generates a systemic infection and viral replication in the spleen and ovaries was determined by qPCR for genomic copies and plaque assay respectively. The intranasal route is intended to mimic the natural route of infection of VARV, and less CPXV is required for lethality compared to intraperitoneal inoculation. The virulence of the different CPXV is

measured in this experiment by weight loss (**Figure 20b**) and mouse survival (**Figure 20c**). As shown in **Figure 20**, in both models the Δ14 virus exhibited significantly lower virulence. We observed that Δ14 CPXV replicated to lower titers than WT virus, and most of the mice given Δ14 CPXV survived the infection compared to 100% lethality with the WT CPXV at the dose administered.

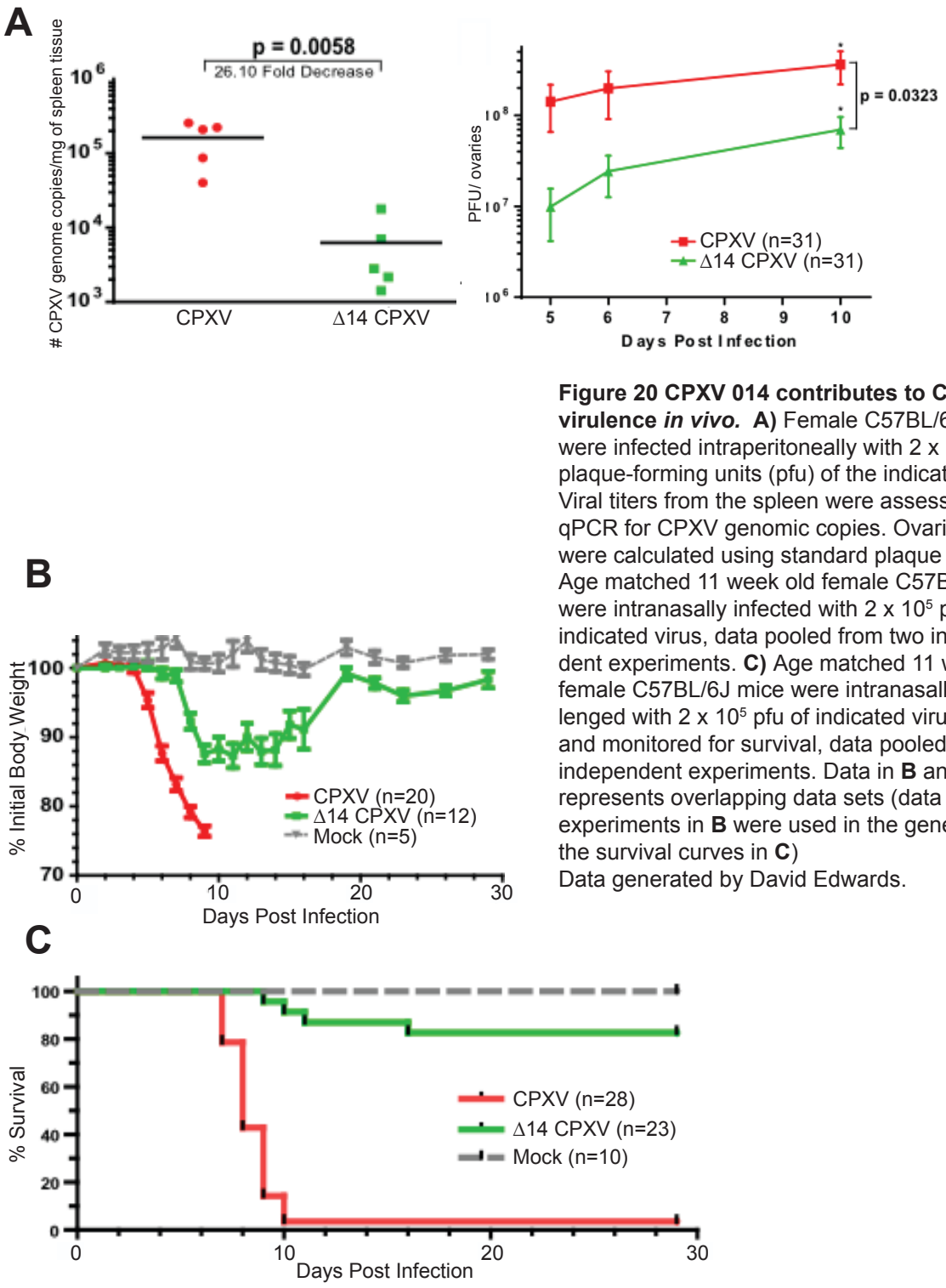
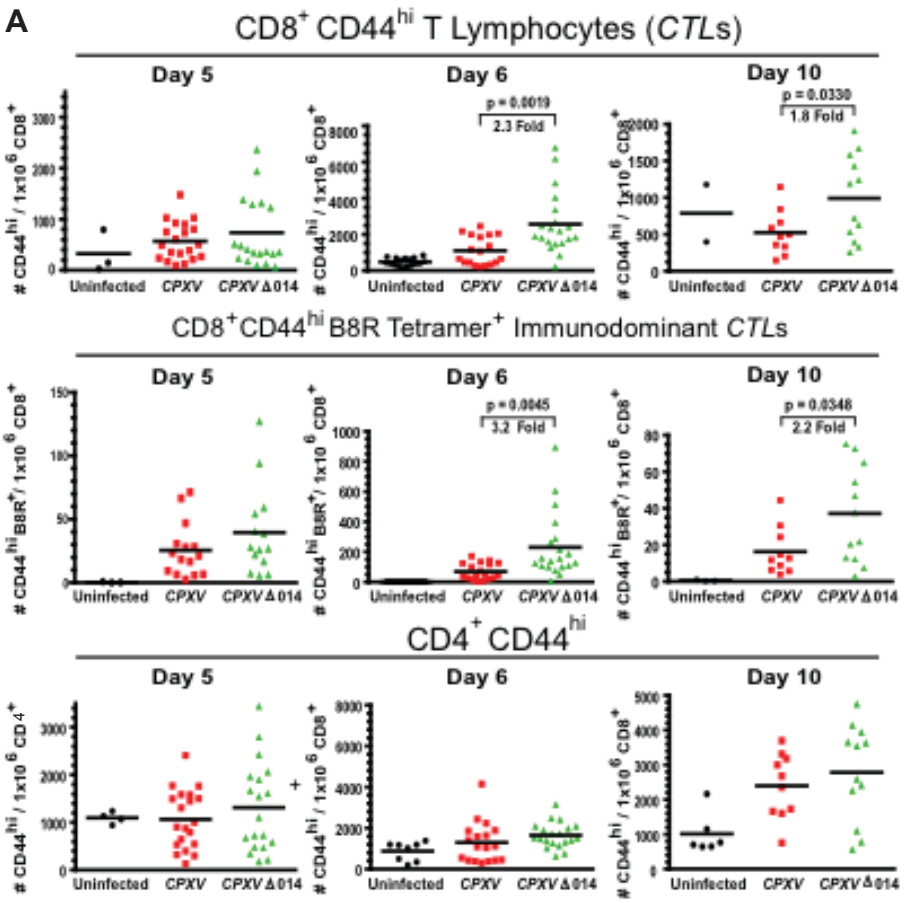


Figure 20 CPXV 014 contributes to CPXV virulence *in vivo*. **A)** Female C57BL/6J mice were infected intraperitoneally with 2 x 10^6 plaque-forming units (pfu) of the indicated virus. Viral titers from the spleen were assessed by qPCR for CPXV genomic copies. Ovarian titers were calculated using standard plaque assay. **B)** Age matched 11 week old female C57BL/6J mice were intranasally infected with 2 x 10^5 pfu of indicated virus, data pooled from two independent experiments. **C)** Age matched 11 week old female C57BL/6J mice were intranasally challenged with 2 x 10^5 pfu of indicated virus and monitored for survival, data pooled from 3 independent experiments. Data in **B** and **C** represents overlapping data sets (data from the experiments in **B** were used in the generation of the survival curves in **C**)
Data generated by David Edwards.

Since CPXV 014 was identified using T cell activation assays, we wished to determine if there were any differences in T cell priming between WT and Δ14 virus *in*

vivo. Mice were given a systemic WT CPXV or Δ014 CPXV infection by injecting 2 x 10^6 pfu intraperitoneally and splenocytes were harvested at the indicated time points. The splenocytes were then stained for surface markers including CD44 (a marker of effector-memory T cells) and with the B8R tetramer (which stains $CD8^+$ TCRs specific for the immunodominant B8R CPXV epitope) to assess the extent of T cell priming. As seen in **Figure 21a (top row)**, deletion of ORF 014 from the virus results in a higher overall number of ($CD8^+CD44^{High}$) memory CTLs at six and ten days post-infection relative to mice infected with the WT virus. Tetramer staining for TCRs directed against the immunodominant B8R CPXV epitope **(Figure 21a, middle row)** further indicated that Δ14 CPXV elicits a greater number of CPXV-specific memory CTLs at six and ten days post infection than its WT counterpart. This difference was largest at day six post-infection, with the Δ14 CPXV eliciting roughly three times as many specific CPXV-memory $CD8^+$ T cells. In **Figure 21b** this data is quantified alongside viral titers taken from the ovaries of these mice, and shows that Δ14 virus (lower panel) replicates to significantly lower levels than the WT virus (upper panel). These *in vivo* data further suggested that CPXV 014 acts to inhibit naive $CD8^+$ T cell activation and priming. This defect in T cell priming was only significant for $CD8^+$ populations, but not for $CD4^+$ populations, as shown in **Figure 21a (bottom row),** which shows the overall number of $CD44^{High}$ $CD4^+$ T cells detectable in the spleen. Overall, these data indicate that CPXV 014 acts to inhibit $CD8^+$ T cell priming in this model of systemic CPXV mouse infection.



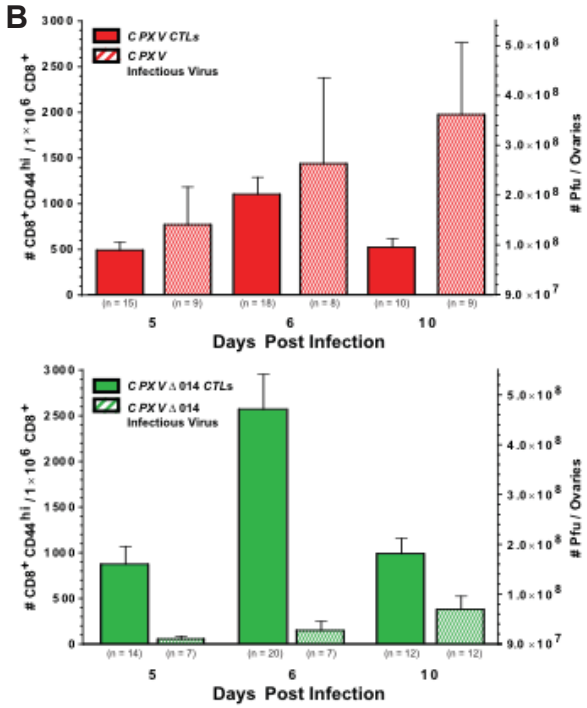


Figure 21 CPXV 014 Inhibits Acute Phase CD8⁺ Priming. A) CPXV014 signifantly inhibits day 6 CTL priming of the general memory pool. Female C57BL/6J mice were infected with 2 x 10^6 pfu (i.p) of the indicated virus. Splenocytes were harvested on the indicated days and surface stained. Cells were gated on lymphocyte size (FSC vs SSC) -> Live -> $CD3^+$ -> $CD8^+$. Either $CD44^{high}$ gated or cross-reactive VACV specific (CPXV cross-reactive) B8R $Tetramer^+$ gated lymphocytes were quantified. Graphs depicts the mean ± SEM. Data pooled from three or more seperate independent experiments. **B)Deletion of CPXV 014 enhances CD8⁺ expansion** Graphed points depict mean values, error bars are SEM. Data generated by David Edwards.

Our collaborators in the laboratory of Jeff Nolz (OHSU) tested the Δ14 CPXV in an ear scarification model to determine if Δ014 CPXV also exhibited greater T cell priming in a different infection setting. Briefly, 1 x 10^5 pfu of either Δ014, Δ203 or WT CPXV were inoculated into one ear of C57BL/6 mice by scarification in order to cause a localized infection. Δ203 CPXV no longer expresses the MHC Class I ER retention protein encoded by that ORF. The Δ203 virus was included in the experiment because it works as a positive control for the restoration of $CD8^+$ T cell priming since a greater number of CTLs will be activated by CPXV infection in the absence of this MHC Class I down-regulating protein. Furthermore, we wished to assess the relative contributions of CPXV 014 and CPXV 203 to T cell priming in an *in vivo* infection model. Ten days later, the mice were euthanized and the ears were harvested to assay for viral titers in the site of inoculation. As shown in **Figure 22c**, there was no significant difference between the different viruses in their local replication within the ear, indicating that there were no significant replicative defects in the Δ014 and Δ203 viruses. T cells from the draining lymph node (DLN) were harvested and stimulated with one of the two immunodominant CPXV antigenic epitopes, B8R or A47 and were subsequently stained for CD44 and IFNγ expression. Effector memory $CD44^+CD8^+$ T cells that are specific for these CPXV epitopes respond to stimulation with these peptides by producing IFNγ. As seen in **Figures 22a and 22b**, splenic T cells taken from mice infected with the Δ14 virus showed a greater frequency and number of T cells responsive to stimulation with specific peptide than those taken from WT-infected mice. The restoration of T cell priming exhibited by the Δ014 virus was less than that shown by Δ203 CPXV, indicating that CPXV 014 contributes less to the inhibition of T cell priming *in vivo* relative to CPXV

203. The frequency of CPXV-specific DLN memory T cells was also assessed by staining for CD44 and with B8R tetramer (specific for TCRs binding the B8R immunodominant epitope). As shown in **Figure 22d**, the ∆014 CPXV infection resulted in a greater frequency of B8R-specific memory T cells than the WT virus. Again, this increased T cell priming was more pronounced in the mice infected with the ∆203 CPXV. Together these data indicate that while CPXV 014 does contribute to evasion of $CD8^+$ T cell priming *in vivo*, the down-regulation of surface MHC Class I by the CPXV 203 plays a larger role. Collectively, these data further indicate that CPXV 014 contributes to limiting T cell priming during infection. Finally, $CD8^+$ T cells were surface stained to assess the generation of memory and effector cells. CD127 is the α chain of the IL-7 receptor, which signals for survival and proliferation in T cells, and is a marker for precursor memory T cells [251]. Killer cell Lectin-like Receptor G1 (KLRG1) is a coinhibitory receptor expressed by antigen-experienced T cells with decreased proliferative capacity, and is commonly used as a surface marker for short-lived effector T cells (SLEC) [252, 253]. As shown in **Figure 22e**, infection with ∆014 CPXV resulted in a greater frequency of $CD127^-KLRG1^+$ SLEC and a reduced frequency of $CD127^+KLRG1^-$ memory precursor cells than WT CPXV. The increase in the proportion of SLEC in mice infected with ∆014 CPXV relative to those infected with WT CPXV is potentially the result of increased T cell priming and initial expansion of these cells in the absence of CPXV 014. However, infection with the ∆203 CPXV led to the opposite trend, with an increased proportion of precursor memory T cells and decreased SLEC relative to WT CPXV infection. Both of these results indicate that CPXV 014 and 203 affect the priming of $CD8^+$ T cells and the generation of memory *in vivo,* but further

functional studies of these different $CD8^+$ T cell populations would be required to make more substantive conclusions about the relative impacts on T cell differentiation of CPXV 014 and 203.

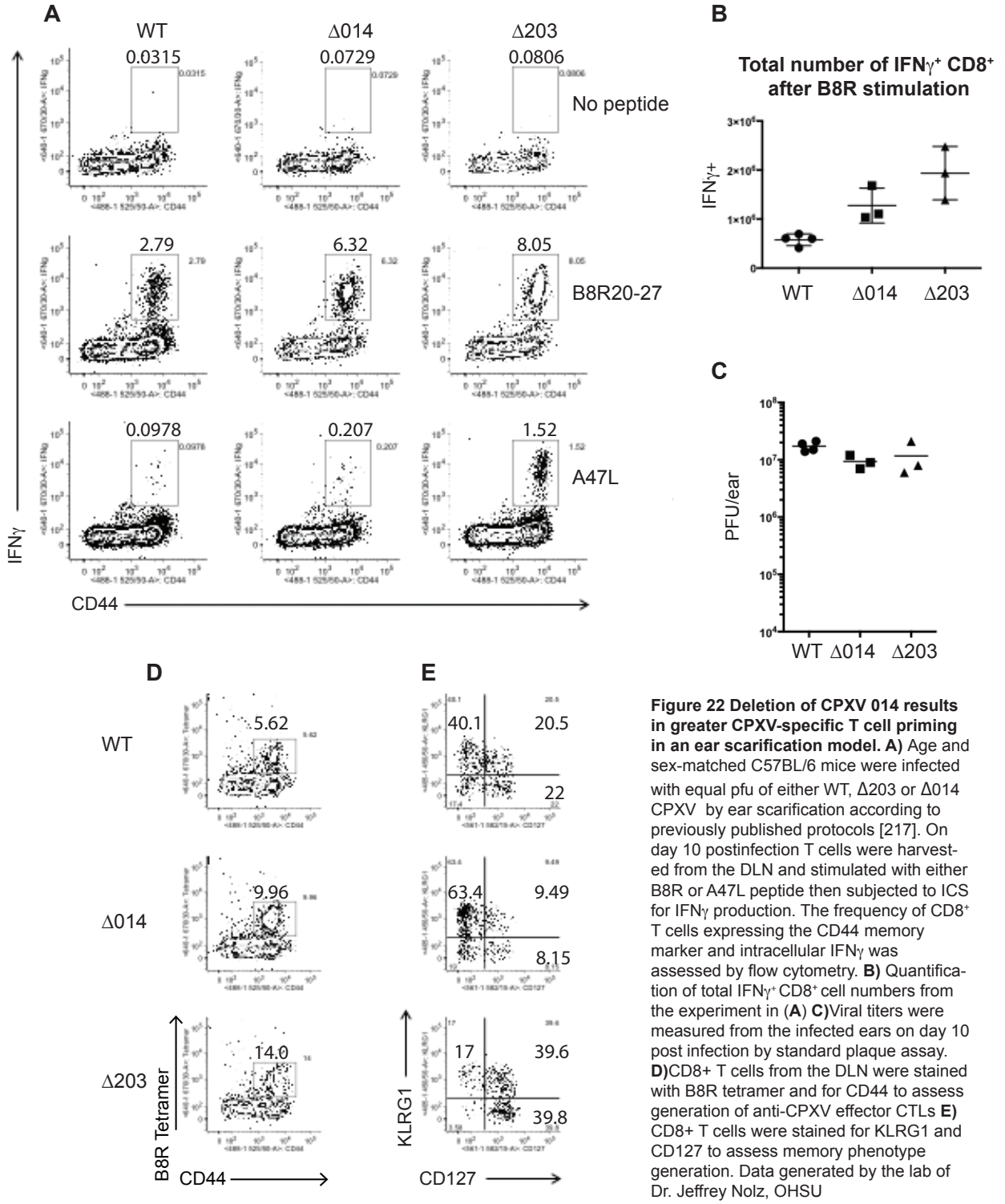


Figure 22 Deletion of CPXV 014 results in greater CPXV-specific T cell priming in an ear scarification model. A) Age and sex-matched C57BL/6 mice were infected with equal pfu of either WT, Δ203 or Δ014 CPXV by ear scarification according to previously published protocols [217]. On day 10 postinfection T cells were harvested from the DLN and stimulated with either B8R or A47L peptide then subjected to ICS for IFNγ production. The frequency of $CD8^+$ T cells expressing the CD44 memory marker and intracellular IFNγ was assessed by flow cytometry. **B)** Quantification of total $IFN\gamma^+ CD8^+$ cell numbers from the experiment in (**A**) **C)**Viral titers were measured from the infected ears on day 10 post infection by standard plaque assay. **D)**CD8+ T cells from the DLN were stained with B8R tetramer and for CD44 to assess generation of anti-CPXV effector CTLs **E)** CD8+ T cells were stained for KLRG1 and CD127 to assess memory phenotype generation. Data generated by the lab of Dr. Jeffrey Nolz, OHSU

III. Recombinant CPXV 014

III A. CPXV 014-Fc construction and purification

In order to determine if CPXV 014 is sufficient to cause the observed T cell inhibition we decided to produce a recombinant, exogenously expressed version of the protein. The first construct we made was CPXV 014 with a thrombin-cleavable human Fc tag at its C terminus (the construction is detailed in the Materials and Methods section as well as outlined in **Figure 7**). The expression plasmid was stably transfected into Chinese Hamster Ovary (CHO) cells using Lipofectamine 2000. These cells were grown in the presence of ampicillin to select for expression of the recombinant protein and sorted into single cell clones. The single cell stable transfectants were screened via a dot-blot assay for the highest expressing clone (using anti-human Fc antibody to detect the tagged protein in supernatants harvested from the stably transfected cells), which was isolated and cultured (data not shown). This stable transfectant cell line was cultured in the presence of protease inhibitors in MediaTech CellGro flasks for up to one month at a time before the supernatant was harvested. The recombinant protein was purified from the supernatant via FPLC affinity chromatography to protein G, followed by two rounds of dialysis in PBS and concentration using molecular weight cut-off (MWCO) columns (also called desalting columns, these centrifugal filter devices retain macromolecules greater than a specific molecular weight while allowing for the passage of solute and smaller molecules, allowing for the concentration of protein suspensions).

The expressed protein retained its predicted size (49 KDa, unglycosylated) and N-linked glycosylations (increasing the molecular weight by roughly 23 KDa) as confirmed by Western blots of the recombinant protein with or without PNGase treatment, which cleaves N-linked glycosylations (**Figure 23**).

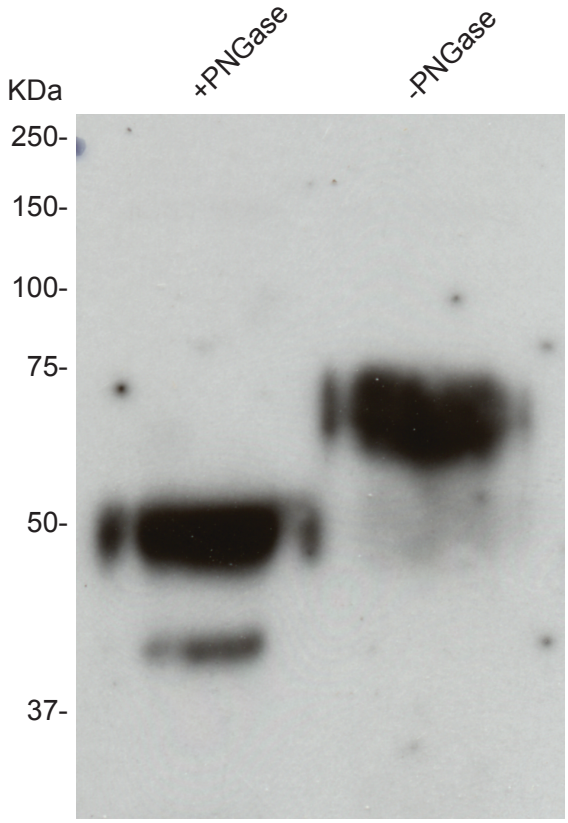


Figure 23 Recombinant CPXV 014-Fc is expressed by stably transfected CHO cells and is modified by N-linked glycosylation. Purified CPXV 014-Fc was treated or left untreated with PNGase overnight at 37°C then run on a 10% agarose SDS gel before blotting with anti-human Fc (1:10,000). Predicted size of CPXV 014-Fc without glycosylation is 49KDa.

III B. CPXV 014-Fc Inhibits T cell activation by plate-bound antibodies

Prior to protein purification we tested if the supernatants taken from the transfected cells could inhibit T cells as shown with virus-free supernatants taken from infected cells. We mixed the indicated supernatants in a 1:1 ratio with cell media for four hours before stimulating the T cells for six hours with anti-CD3 and anti-CD28. We observed an inhibition of T cell activation, however, the inhibition was not a strong as

that observed using virus-free supernatants taken from CPXV-infected cells **(Figure 24**). Supernatant taken from untransfected CHO cells, as well as virus-free supernatants from VACV- or ∆011-016 CPXV-infected MC57 cells were used as negative controls.

We then tested the fully purified recombinant protein in T cell assays by coincubating the splenocytes with the protein for four hours prior to stimulation with plate bound antibodies for six hours. No viruses or A20 cells were included in these assays. As shown in **Figure 25**, the recombinant protein was able to inhibit naive T cell activation in this assay. **Figure 25a** shows the TNFα responses of the $CD4^+$ and $CD8^+$ T cells from one representative mouse, and these are quantified in **25b** and **25c**. Virus-free supernatants (generated as described earlier) from WT CPXV-infected MC57 cells were used as a positive control for naive T cell inhibition. Human IgG1 was used as a negative control protein for the hIgG1 Fc-tagged CPXV 014. As observed with our previous assays, the inhibition of the TNFα response of naive T cells to plate-bound antibodies was more evident in the $CD8^+$ T cells (**Figure 25c)** than the $CD4^+$ T cells **(Figure 25b)**. From these data we concluded that CPXV 014-Fc was sufficient for the observed inhibitory phenotype in the absence of other viral products or infection.

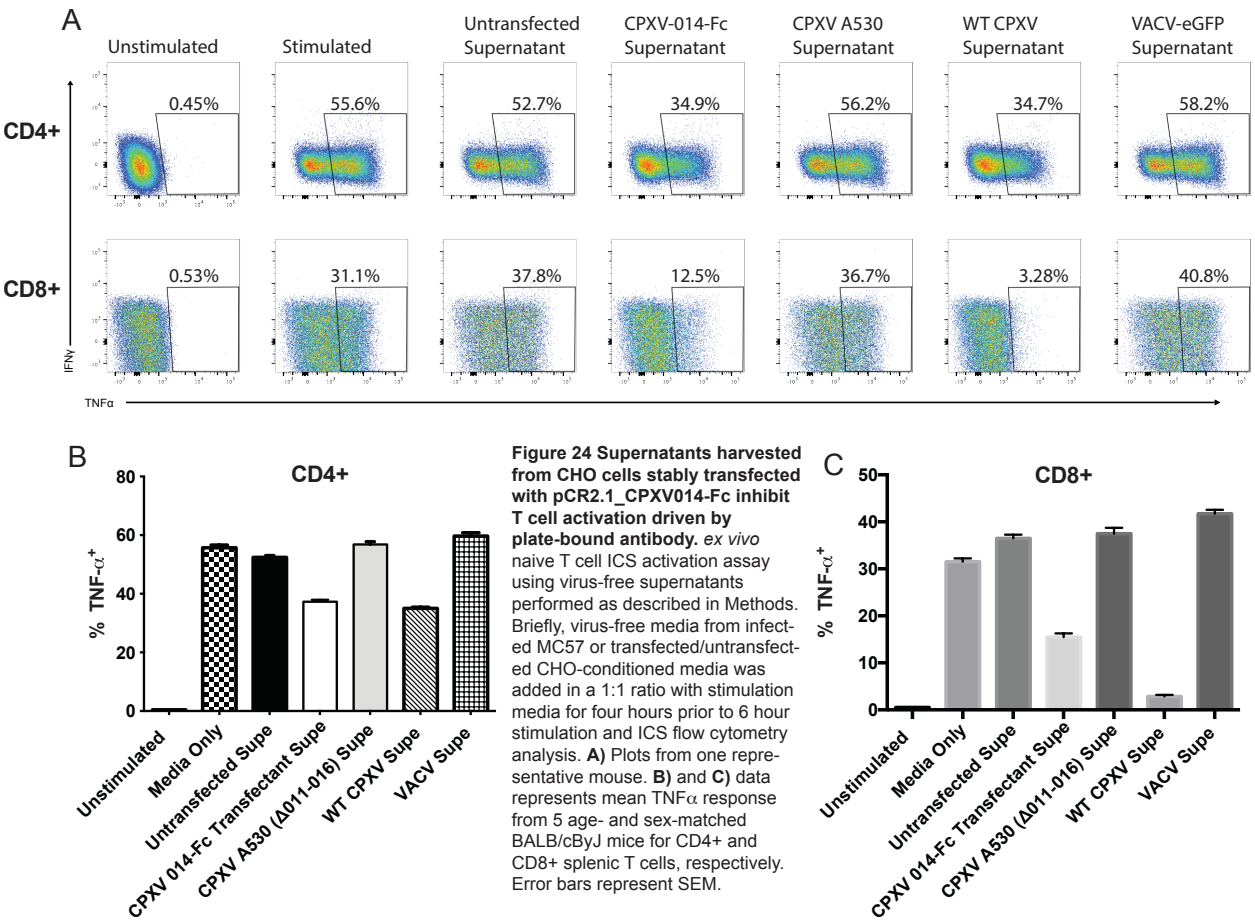


Figure 24 Supernatants harvested from CHO cells stably transfected with pCR2.1_CPXV014-Fc inhibit T cell activation driven by plate-bound antibody. *ex vivo* naive T cell ICS activation assay using virus-free supernatants performed as described in Methods. Briefly, virus-free media from infected MC57 or transfected/untransfected CHO-conditioned media was added in a 1:1 ratio with stimulation media for four hours prior to 6 hour stimulation and ICS flow cytometry analysis. **A)** Plots from one representative mouse. **B)** and **C)** data represents mean TNFα response from 5 age- and sex-matched BALB/cByJ mice for CD4+ and CD8+ splenic T cells, respectively. Error bars represent SEM.

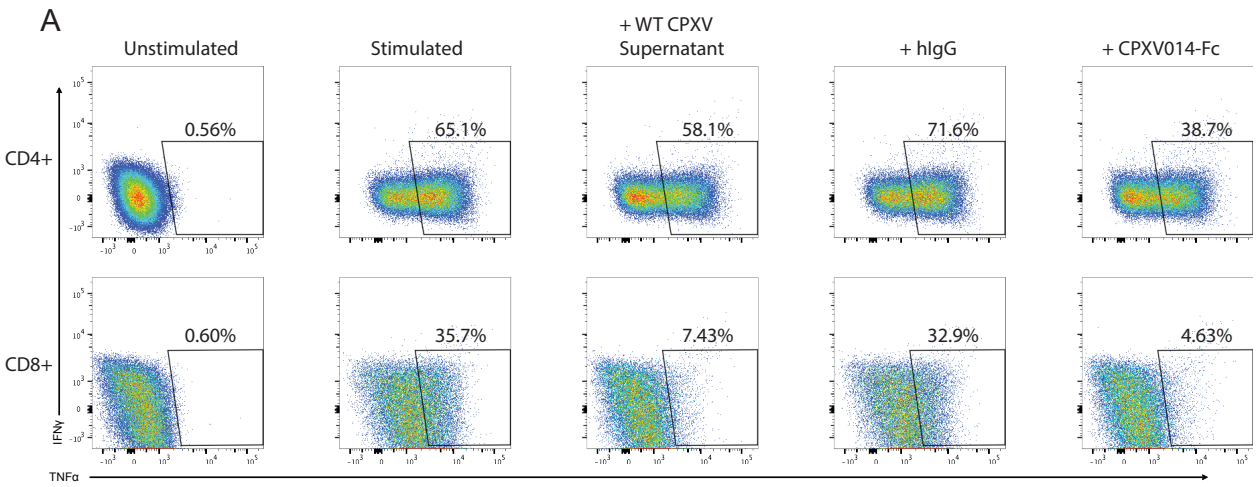
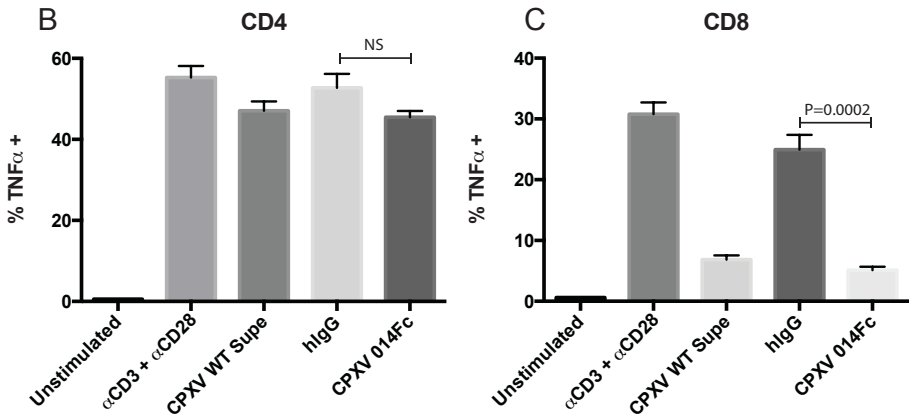


Figure 25 Recombinant CPXV 014-Fc inhibits naive T cell activation by plate bound antibodies. Splenocytes from 5 age-and sex matched BALB/cByJ mice were treated 1:1 with virus-free WT CPXV-infected MC57 supernatant or 5 μg/ml of the recombinant protein or hIgG control for 4 hours prior to stimulation and flow cytometry as previously described. **A)** Plots show responses from one representative mouse. **B)** and **C)** Tables show mean TNFα responses from $CD4^+$ and $CD8^+$ T cells respectively. Error bars are SEM. P values calculated using unpaired two-tailed Student's T test.



III C. CPXV 014-His

While the CPXV 014-Fc recombinant protein was found to be effective in inhibiting T cell activation in the assays we conducted, this construct had a few problems. Firstly, the Fc tag on the protein is almost the same size as the protein itself, and steric hindrance seemed to prevent recognition of the protein by a chicken-anti-CPXV014 CTD antibody (**Table 2**) we use for Western blots of infected cell lysates. Secondly, we had recently learned from our collaborators in the laboratory of Daved Fremont (Washington U., St Louis) that CPXV 014 is able to bind FcR, and that the presence of a human Fc tag on the protein could potentially confound the effects of that

interaction. Thirdly, the expression levels reached using the stably transfected CHO cells were low, making it difficult to obtain enough recombinant protein to perform the experiments.

These issues prompted us to collaborate with the Fremont lab (Washington University, St. Louis), who have substantial experience in structural biology and expression of recombinant proteins. They provided us with an alternative CPXV 014 construct and expression system. Briefly, this construct, which is on a pFM1.2 background, replaces the native poxviral secretion signal with one taken from baculovirus (which exhibits optimal activity in HEK293F cells), and adds a His tag on the C terminus instead of an Fc tag. This plasmid is transiently transfected into HEK293F cells that subsequently produce large amounts of recombinant protein. We purified protein from the supernatants three and six days post-transfection using affinity chromatography to NiNTA resin [239]. This plasmid and technique allowed us to purify milligram quantities of the CPXV recombinant protein using a fraction of the time and resources required for generating and purifying the Fc-tagged version. Therefore, we decided to only use this protein generation system as soon as it became available to us. As shown by the Western blot in **Figure 26**, when treated with PNGase, the CPXV 014-His ran at its predicted molecular weight of 22.07 KDa. In the absence of PNGase treatment CPXV 014-His ran between roughly 35 and 50 KDa, indicating that this construct is subject to varying degrees of N-linked glycosylation like the virus-expressed and Fc-tagged proteins.

We tested purified CPXV 014-His in our naive T cell activation assay as we did for the Fc-tagged protein. We found that recombinant His-tagged CPXV 014 was fully

capable of inhibiting naive T cell activation in this assay confirming our observations with CPXV 014-Fc. As shown in **Figure 27**, CPXV 014-His, but not an inactive His-tagged orthologous protein from ECTV (described in section VII of this chapter), was able to inhibit naive T cell activation by plate bound antibodies when supplied in picomolar quantities. As previously observed, the inhibition by CPXV 014 was more pronounced in $CD8^+$ than $CD4^+$ T cells. Interestingly, there did not appear to be a typical dose-response curve to this inhibition. Inhibition of naive T cell activation by CPXV 014 seemed to an all-or-nothing phenomenon with no apparent intermediate effects.

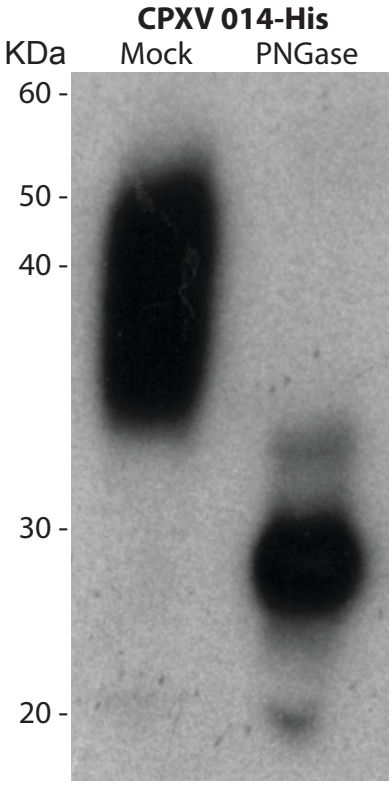


Figure 26 Recombinant CPXV 014-His is modified by N-linked glycosylation. Purified recombinant CPXV 014-His (Predicted MW = 22.07 KDa) was treated by PNGase overnight at 37°C, then subjected to electrophoresis on a 12% acrylamide gel. Western blotting was performed using AVES chicken anti-CPXV014 CTD (1:750 dilution) followed by goat-anti-chicken IgY-HRP (1:5000 dilution).

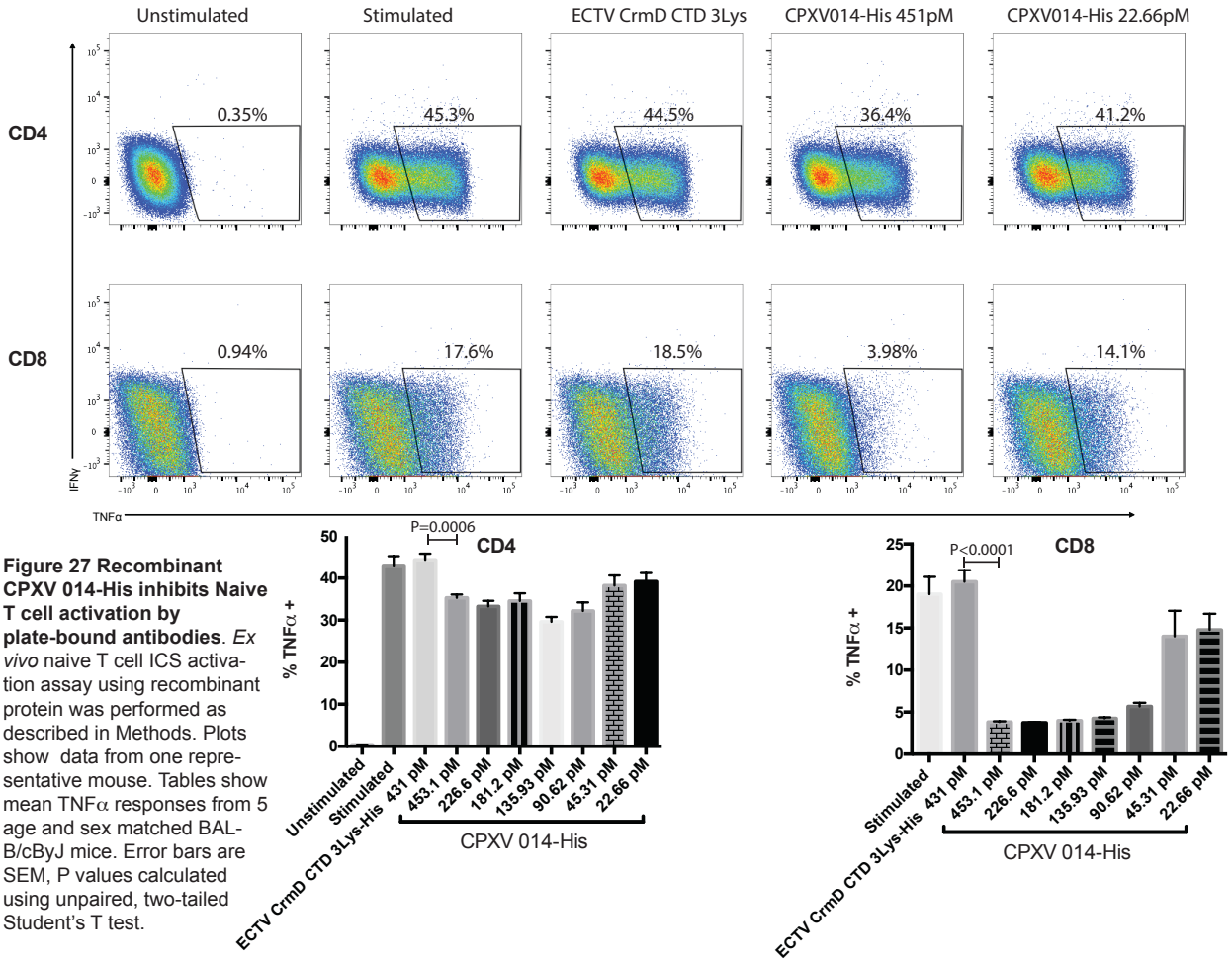


Figure 27 Recombinant CPXV 014-His inhibits Naive T cell activation by plate-bound antibodies. *Ex vivo* naive T cell ICS activation assay using recombinant protein was performed as described in Methods. Plots show data from one representative mouse. Tables show mean TNFα responses from 5 age and sex matched BALB/cByJ mice. Error bars are SEM, P values calculated using unpaired, two-tailed Student's T test.

IV. CPXV 014 Inhibits T Cell Proliferation Driven by Anti-CD3 and IL-2

The previous paragraphs describe the restoration of T cell activation by a CPXV 014 deletion virus and the inhibition of T cell activation by the recombinant protein. Our next experiments were focused on whether CPXV 014 inhibits T cells in other activation models.

We wanted to see if, in addition to inhibiting the TNFα response, CPXV 014 was capable of suppressing the proliferative response of CPXV-naive T cells to TCR ligation. To assess this, we loaded murine BALB/cByJ splenocytes with carboxyfluorescein succinimidyl ester (CSFE). CFSE is a fluorescent cell dye that is highly cell-permeable and rapidly taken into the cytoplasm by cells. Once taken up, intracellular esterases

cleave its two acetate groups which renders it less membrane-permeable, so that the rate of CFSE uptake exceeds that of its diffusion out of the cell [254]. Inside the cell, CFSE binds covalently via its succinimidyl moiety to amine groups (particularly lysine) on intracellular proteins and is retained for long periods of time [254]. As loaded cells divide, their CFSE fluorescence decreases in a predictable fashion, and the discrete fluorescence peaks from different generations of divided cells can be visualized using flow cytometry. CFSE- loaded splenocytes were stimulated with soluble anti-CD3 and recombinant murine IL-2 for 3 days in the presence of CPXV 014-His or an inactive mutant version of an orthologous protein (described in section VII of this chapter). The cells were subsequently stained for surface markers and assayed via flow cytometry for proliferation. Analysis of the data was conducted using the FlowJo 9.8 proliferation platform. “Fraction divided” refers to the percentage of cells in the final culture that have divided at least one time during the incubation period. “Precursor frequency” refers to the percentage of cells from the initial culture that divided at least one time during the incubation period. “Expansion index” refers to the fold expansion of the culture. As seen in **Figure 28**, CPXV 014, but not the nonactive orthologous control protein, was able to significantly inhibit $CD4^+$ T cell proliferation in this model as judged by all three of the proliferation metrics described above. $CD8^+$ T cell proliferation was also inhibited, though to a lesser extent, by CPXV 014-His. However, this was only statistically significant for the precursor frequency and not for the fraction divided or the overall expansion of the culture during incubation. This contrasts with the plate-bound anti-CD3/anti-CD28 and ICS assays in which inhibition of the TNFα response were more pronounced for $CD8^+$ T cells than $CD4^+$ T cells.

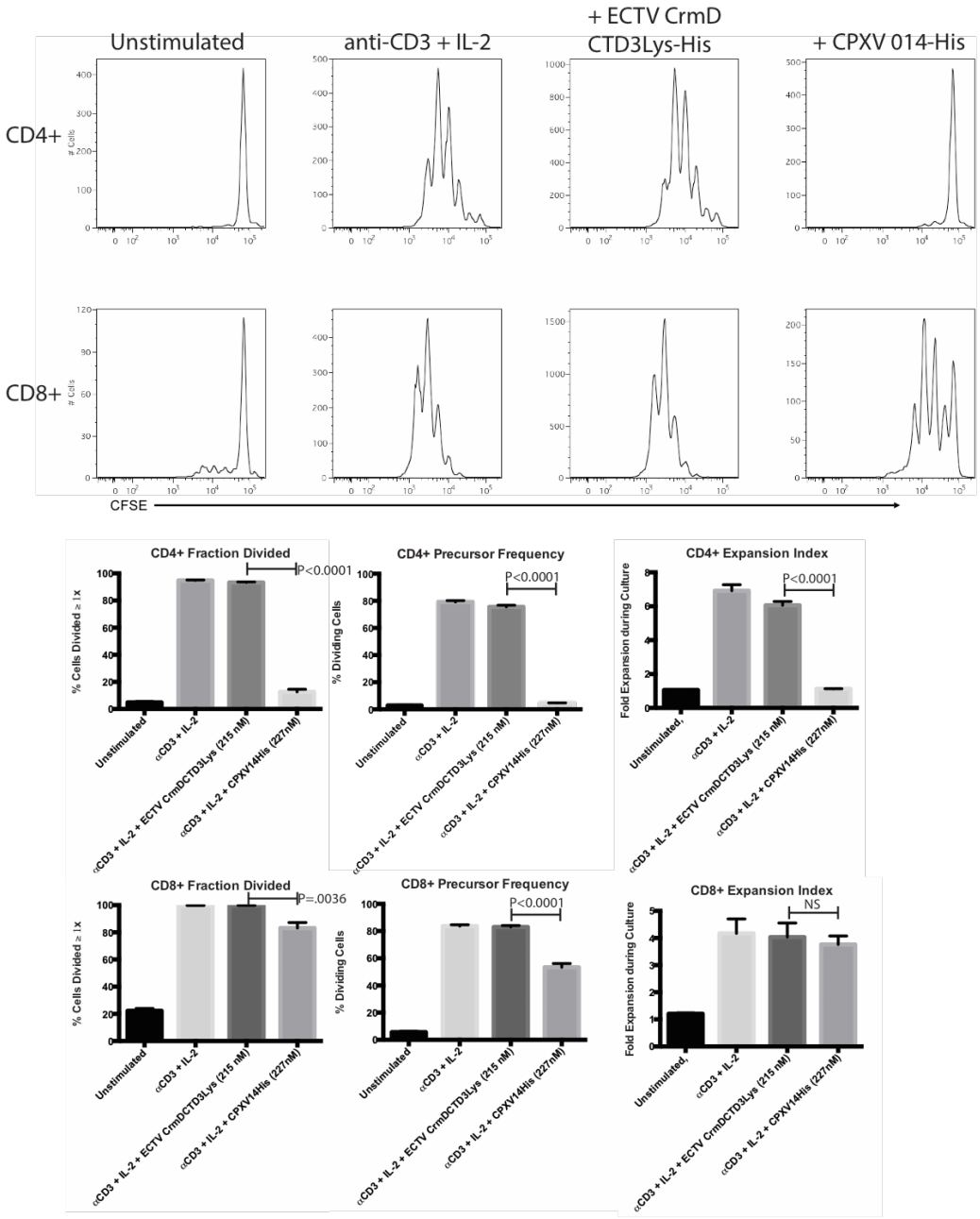


Figure 28 Recombinant CPXV 014 inhibits T cell proliferation driven by soluble anti-CD3 + IL-2. Whole mouse splenocytes were harvested from 5 age and sex matched BALB/cByJ mice and loaded with CFSE. They were then stimulated in the presence of anti-CD3 and recombinant murine IL-2 for three days in the presence or absence of the indicated recombinant proteins before being subjected to surface staining and flow cytometry. Upper histograms show data collected from one representative mouse. Data was analyzed using FlowJo v9.8. “Fraction divided” indicated the % of cells in the final culture that have divided at least one time. “Precursor frequency” indicated the % of cells from the original culture that have divided at least one time during the stimulation. “Fold expansion” indicated the overall population fold expansion during stimulation. Gating strategy was as outlined in **Figure 6** with the final parameter being CFSE (FITC) instead of ICS. Error bars indicate SEM, P values calculated using unpaired two-tailed Student’s T test

V. CPXV 014 Inhibits Activation of Memory T cells by Anti-CD3

We were interested to determine if CPXV 014 was able to inhibit the activation of memory T cells as well as naive T cells. Memory effector T cells have a much lower activation threshold and are not reliant on costimulatory molecules in order to be reactivated and exert their effects. To this end, we isolated splenocytes taken from mice infected intraperitoneally with 5 x 10^5 pfu of MCMV (strain "M45SL") one month prior. (These spleens were a gift from the laboratory of Dan Streblow, OHSU). These cells were stimulated in the presence or absence of CPXV 014-His by only plate-bound anti-CD3 for six hours. We then performed flow cytometry for surface markers and for TNFα and IFNγ. As shown in **Figure 29**, recombinant CPXV 014-His, but not the control protein, was able to inhibit the response of memory CD8+ T cells in this assay. We also observed an IFNγ response to stimulation in this assay, which provides further confirmation of the memory phenotype of these cells. These results were confusing to us at the time, because studies from our laboratory and others had already demonstrated that stimulation of poxvirus-specific $CD8^+$ T cells was restored when ORFs 012 and 203 (the MHC Class I downregulating genes) were deleted from CPXV [226, 228]. This was our first hint that perhaps the observed inhibition of T cell activation was an indirect effect and dependent on the antibody-driven model system we were using.

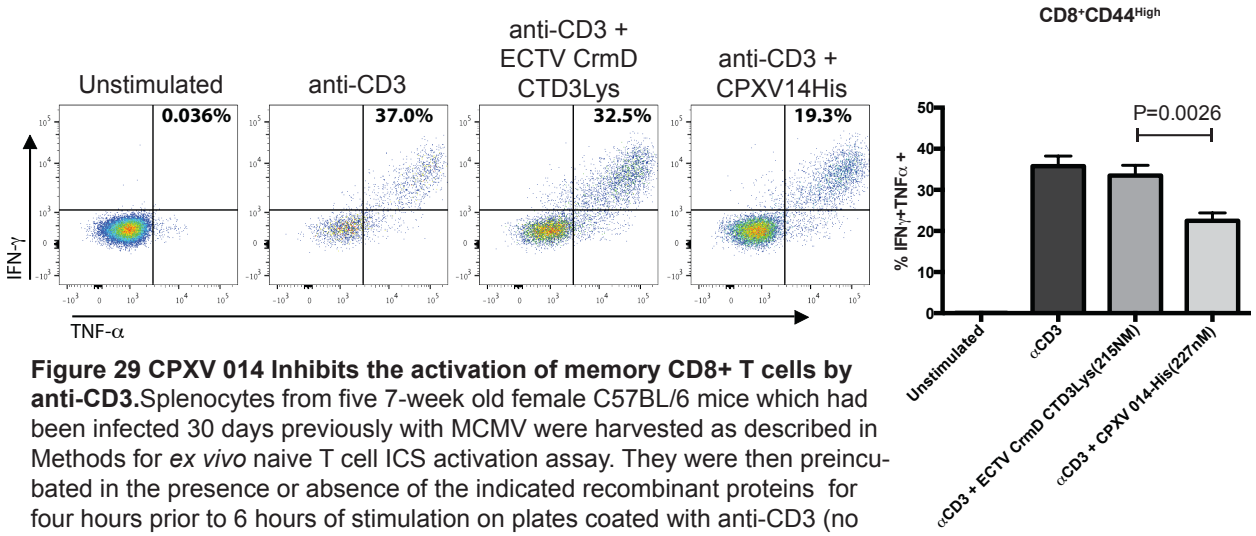


Figure 29 CPXV 014 Inhibits the activation of memory CD8+ T cells by anti-CD3. Splenocytes from five 7-week old female C57BL/6 mice which had been infected 30 days previously with MCMV were harvested as described in Methods for *ex vivo* naive T cell ICS activation assay. They were then preincubated in the presence or absence of the indicated recombinant proteins for four hours prior to 6 hours of stimulation on plates coated with anti-CD3 (no anti-CD28 included). Cells were then harvested and stained for surface markers and intracellular cytokines as previously descibed. Plots indicate data from one representative mouse, table displays pooled data from 2 independent experiments of 5 mice each. Error bars represent SEM, p values calculated using and unpaired two-tailed Students T test. T cells were gated on Time-> F/S scatter (lymphocytes) -> Single cells -> Live cells -> $CD3^+$ -> $CD8^+$ -> $CD44^{High}$ -> $IFN\gamma^+TNF\alpha^+$

VI. The Uninhibited Assays

The unexpected inhibition we observed of memory $CD8^+$ T cells stimulated with anti-CD3 led us to question whether CPXV 014 was able to inhibit T cell activation in assays driven by antigenic peptides or mitogens other than anti-CD3. We were also curious if CPXV 014 could inhibit the activation of T cells from other species. The hypothesis was that if CPXV 014 was unable to block T cell activation by other means, in particular T cells stimulated with their specific cognate antigens, those results would indicate that perhaps CPXV 014 was not acting directly on T cells as we suspected.

VI A. CPXV 014 and human T cells

Since CPXV is a zoonotic pathogen capable of making the jump from rodents into humans and other mammals, we studied if CPXV 014 is capable of inhibiting the *in vitro* activation of human T cells. Our collaborators in the Slifka lab tested if the recombinant CPXV 014-His protein could inhibit the anti-CD3-driven activation of either human PBMC (consisting of a large portion of memory and effector T cells) or T cells extracted from umbilical cord blood (which is considered a fairly naive population of T cells). An unrelated His-tagged protein (M3, a chemokine scavenger from murine gammaherpesvirus MHV-68) was used as the control protein for these experiments. As shown in **Figure 30**, CPXV 014-His exerted no significant effect on human T cell activation by plate-bound antibodies for either VACV-immunized adult PBMC (**30a**), cord blood cells (**30b**) or VACV-naive adult PBMC (**30c**). We concluded that CPXV 014 inhibition of T cells therefore is a mouse-specific phenomenon, although the existence of orthologous mechanisms that inhibit human T cells is possible. Unexpectedly, M3-His appears to inhibit human T cell activation, but this phenomenon was not pursued.

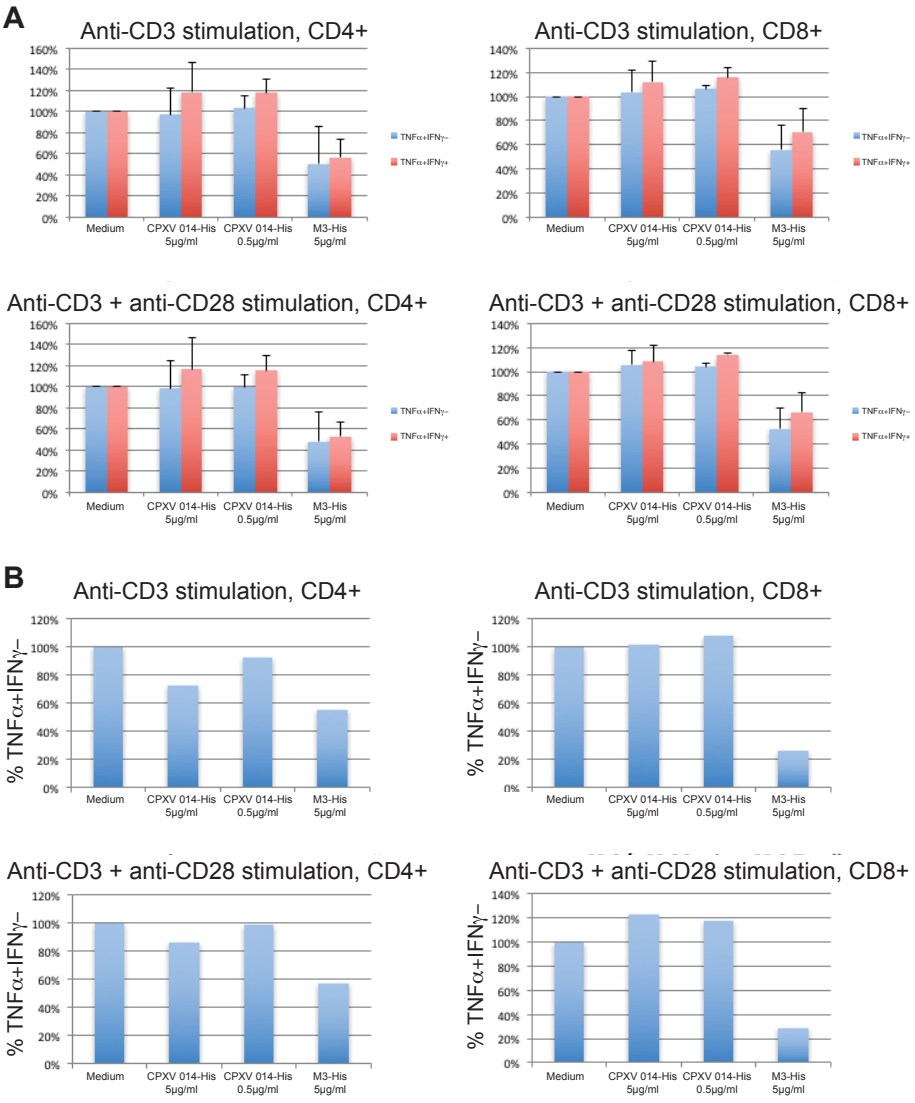


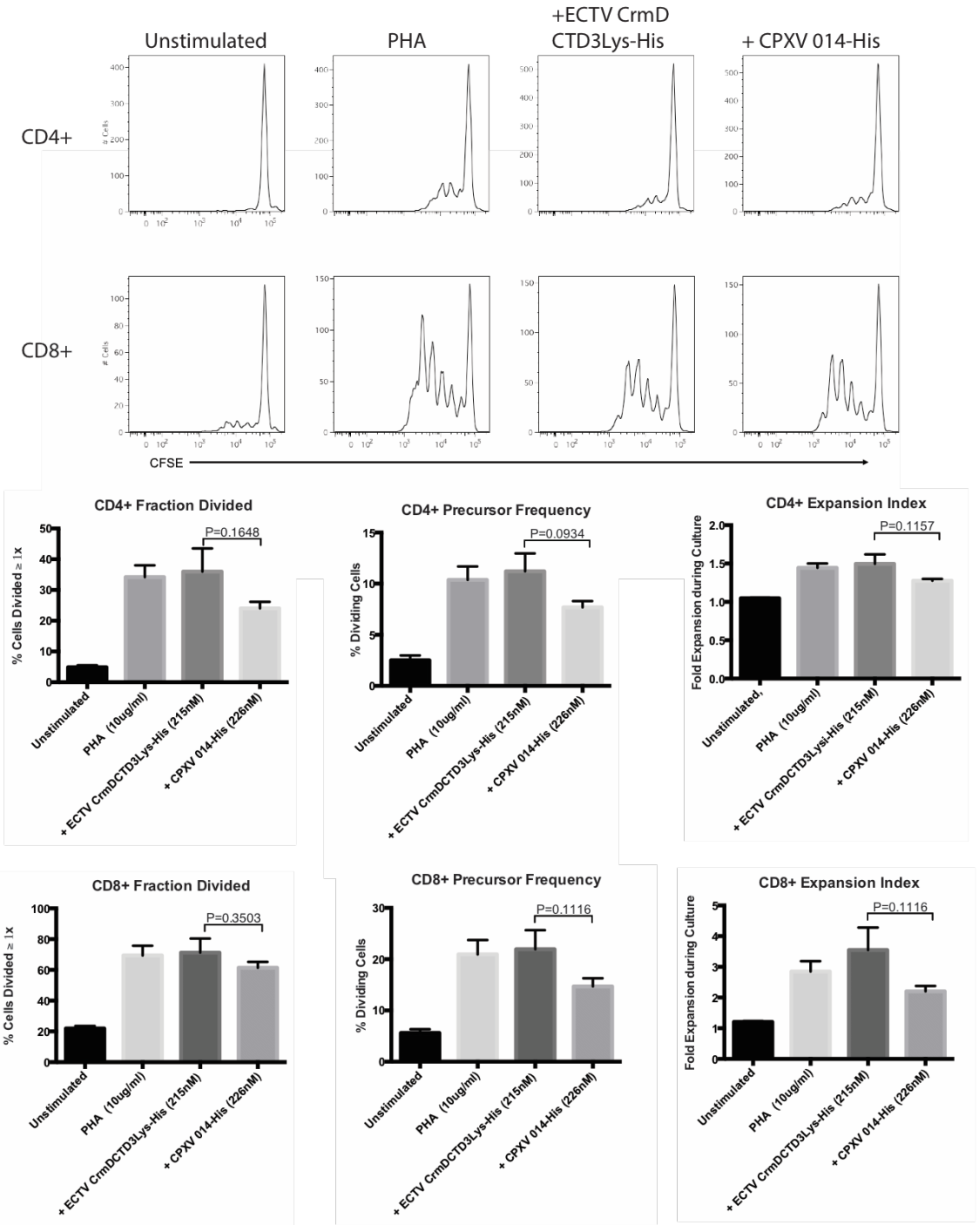
Fig 30 CPXV 014 does not inhibit human T cell activation by plate-bound antibodies. A) Average response of PBMC from 3 adult subjects to six hour stimulation with plate-bound anti-CD3 or anti-CD3 with anti-CD28 in the presence or absence of CPXV014 or unrelated-His-tagged protein (M3) **B)** Response of human PBMC harvested frojm umbilical cord blood to plate bound antibdy in the presence or absence of CPXV 014 or unrelated His-tagged protein (M3-His) **C)** Human PBMC from 3 VACV-naive subjects were infected with the indicated virus or left uninfected for twelve hours prior to six hour stimulation with plate-bound anti-CD3. Experiments performed by Erika Hammarlund.

VI B. CPXV 014 and PHA-mediated T cell activation

Phytohaemagglutinin (PHA) is a lectin found in several types of legumes (particularly red and white kidney beans) that actually consists of two different proteins, PHA-L and PHA-E. PHA acts as a mitogen by nonspecifically cross-linking a multitude of glycoproteins on the T cell surface (including TCR components), leading to their colocalization and subsequent T cell activation.

We tested the ability of our recombinant CPXV 014-His protein to inhibit T cell proliferation induced by PHA. Briefly, murine splenocytes were loaded with CFSE as described earlier, then cultured for three days in the presence of 10 μg/ml of PHA with recombinant CPXV 014-His or the control protein. On the third day, the cells were stained for T cell surface markers and viability by flow cytometry. Proliferative peaks were analyzed using FlowJo v9.8 as described above. As shown in **Figure 31** we saw no significant effect of the viral protein in T cell proliferation. Both $CD4^+$ and $CD8^+$ T cells were stimulated to divide by 10 μg/ml PHA, with $CD8^+$ T cells proliferating to a greater extent than the $CD4^+$ T cells. Both T cell populations appeared to exhibit slightly less cell division in the presence of CPXV 014-His but not an inactive orthologous protein from ECTV (described in section VII of this chapter). However, this difference was not found to be statistically significant for any of the proliferative metrics generated. These results indicated that CPXV 014 was not capable of inhibiting T cell activation by this mitogen.

Figure 31 Recombinant CPXV 014-His does not significantly inhibit T naive T cell proliferation driven by PHA. The naive T cell proliferation assay using PHA was performed as detailed in Methods. Briefly, Whole mouse splenocytes were harvested from 5 age and sex matched BALB/cByJ mice and loaded with CFSE. They were then stimulated by 10 μg/ml PHA for three days in the presence or absence of the indicated recombinant proteins before being subjected to surface staining and flow cytometry as described. Upper histograms are data collected from one representative mouse. Data was analyzed using FlowJo v9.8. "Fraction divided" indicated the % of cells in the final culture that have divided at least one time. "Precursor frequency" indicated the % of cells from the original culture that have divided at least one time during the stimulation. "Fold expansion" indicated the overall population fold expansion during stimulation. Error bars indicate SEM, P values calculated using a two-tailed unpaired students T test.



VI C. CPXV 014 and PMA/ionomycin-mediated T cell activation

Phorbol myristate acetate (PMA) and the Ca^{2+} ionophore ionomycin are two reagents commonly used to activate T cells *in vitro*. They act together in a synergistic manner to stimulate T cell activation, proliferation and cytokine secretion. PMA, an analog of DAG, acts by transiently activating protein kinase C (PKC), which is a key mediator in TCR signaling, and PKC activation leads to downstream activation of NFκB. Ionomycin is a calcium ionophore which allows Ca^{2+} to flow into the cell, leading to activation of the transcription factor NFAT as described in the introduction. These reagents therefore allow us to polyclonally activate T cells *in vitro* downstream of TCR ligation.

We wished to see if CPXV 014 was able to inhibit activation of T cells by these mitogens. We coincubated naive mouse splenocytes with either virus-free supernatants taken from CPXV-infected MC57 murine fibroblasts (prepared as described in Chapter 3 and in the Methods section) or recombinant CPXV 014-Fc for four hours prior to stimulating them with 50 ng/ml PMA and 1 μg/ml ionomycin for six hours in the presence of brefeldin A. Supernatants from uninfected cells, virus-free supernatants from VACV-infected cells and human IgG were used as negative controls. Analysis of activation was carried out by ICS and flow cytometry as previously described. As shown in **Figure 32**, neither recombinant CPXV 014-Fc, nor supernatants harvested from infected cells were able to inhibit the activation of naive T cells by PMA and ionomycin. Unexpectedly, the supernatants taken from both infected and uninfected MC57s had a suppressive effect on T cell activation in this assay, but this phenomenon was not pursued.

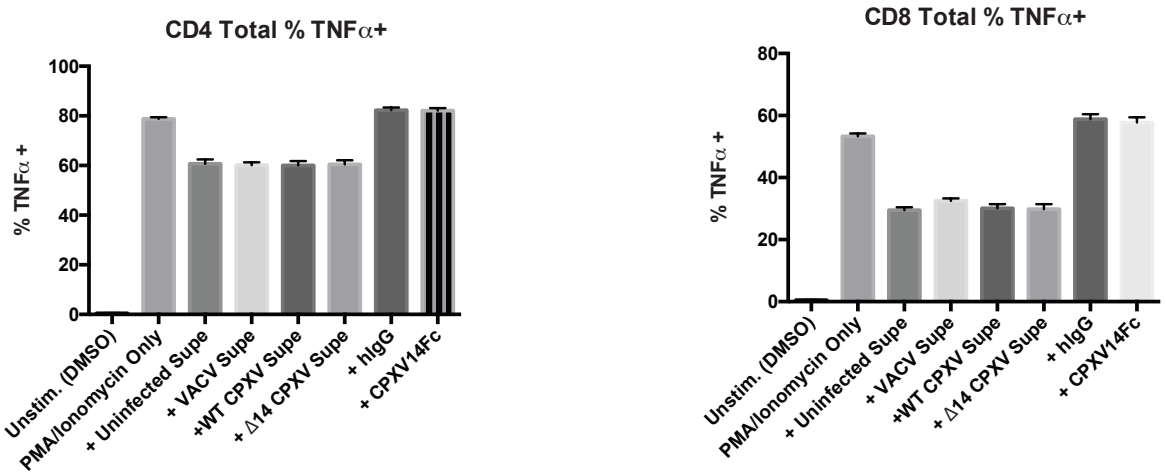


Figure 32 CPXV 014 does not inhibit naive T cell activation by PMA + ionomycin. The *ex vivo* naive T cell ICS activation assay using virus-free supernatant, recombinant protein and PMA/ionomycin was performed as detailed in Methods. Briefly, spleens from 5 naive female 6-10 week old BALB/cByJ mice were harvested and processed as above. Splenocytes were resuspended in stimulation media at 2 x 10^7 cells per ml and coincubated with either virus-free supernatants from infected MC57 fibroblasts in a 1:1 ratio with stimulation media, or with 5μg of either CPXV 014-Fc or control human IgG for 4 hours at 37°C. The cells were then treated with 50 ng/ml PMA, 1 μg/ml ionomycin and 4 μg/ml Brefeldin A for 6 hrs at 37°C. Following stimulation the cells were washed, then stained for surface markers, followed by fixation, permeabilization and ICS for TNFα and IFNγ as described. Gating strategy was as detailed in **Figure 6**. Error bars indicate SEM.

VI D. CPXV 014 and the activation of OT-I CTLs by SIINFEKL

CPXV 014 only appeared to suppress naive T cell activation in the anti-CD3-driven assays, but not with any of the other mitogens tested so far. This prompted us to test its ability to inhibit T cell activation in a model more closely approximating the conditions of T cell activation *in vivo*. Can CPXV 014 inhibit T cells activated by encountering their specific antigenic epitope in the contact of MHC presentation?

To this end, we utilized transgenic OT-I mice. $CD8^+$ T cells from this C57BL/6-based mouse strain express a transgenic TCR which is specific for H2-Kb carrying the SIINFEKL peptide of chicken ovalbumin. By incubating the OT-I splenocytes with SIINFEKL peptide we can mimic how T cells are activated by their cognate peptide *in vivo*. We incubated the OT-I splenocytes with either CPXV 014-His or hIgG for four hours prior to stimulating them with different concentrations of SIINFEKL peptide in the presence of brefeldin A for six hours. As shown in **Figure 33**, we saw no inhibition of

naive T cell stimulation by recombinant CPXV 014-His compared with the control hIgG under these conditions.

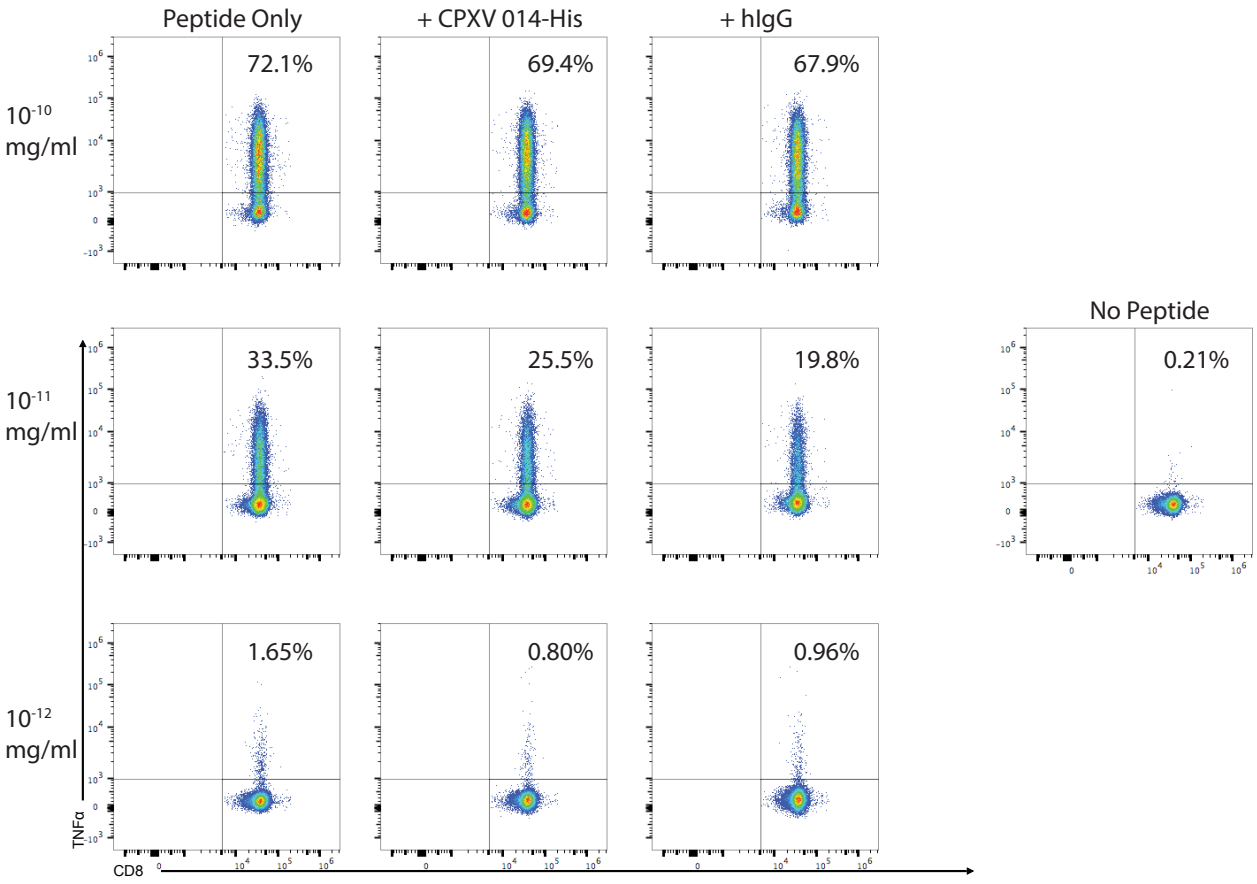
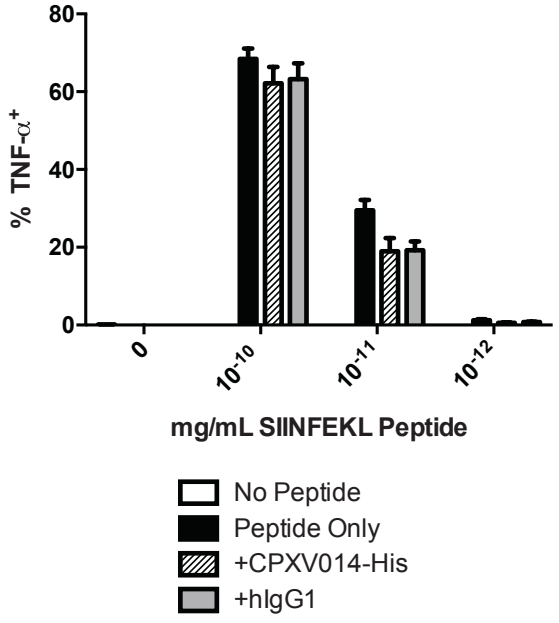


Figure 33 Recombinant CPXV 014 does not inhibit the stimulation of OT-1 CD8+ T cells by SIINFEKL. Splenocytes from naive OT-I mice (C57BL/6J background) were pre-treated with 5 μg/ml CPXV 014-His tagged protein (226 nM) or hIgG and then stimulated with SIINFEKL peptide at the indicated concentrations in the presence of BFA for 6 hours. Splenocytes were then surface stained and then subjected to ICS to quantitate production of OT-I specific TNFα. Plots show data from one representative mouse. Gating strategy was Lymphoctes (FSC/SSC)-> single cells -> live cells -> $CD3^+$ -> $CD8^+TNF\alpha^+$ Graphs depict the mean responses of 3 mice. Error bars indicate SEM. Data generated by David Edwards, Früh lab.

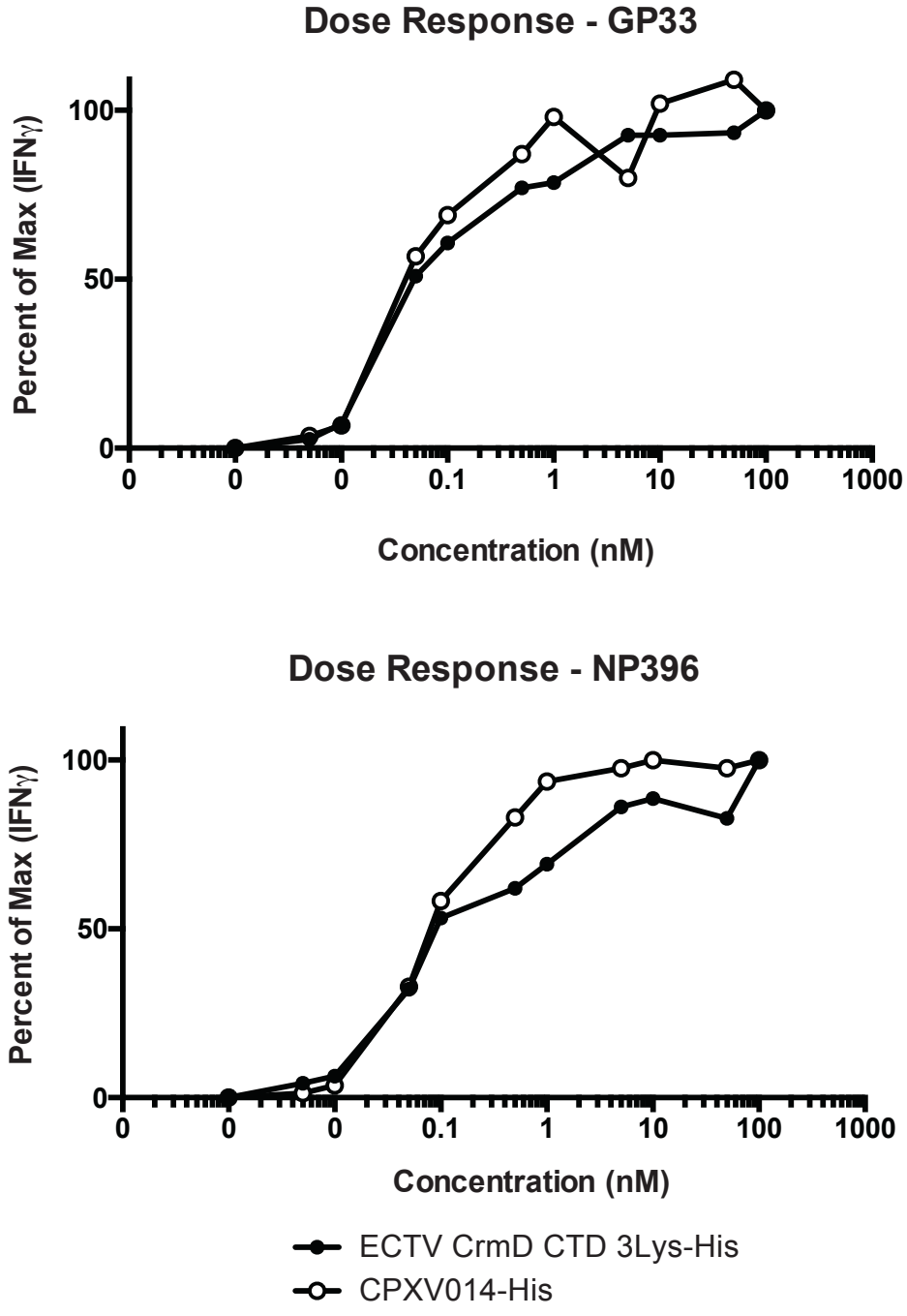


VI E. CPXV 014 and stimulation of LCMV memory T cells with immunodominant epitopes

A similar assay was conducted in the lab of our collaborator Jeff Nolz (OHSU) using memory T cells taken from mice infected with LCMV for at least 60 days. When these splenocytes were stimulated using specific LCMV peptides known to be immunodominant [84, 85], recombinant CPXV 014-His was unable to inhibit their production of IFNγ in a dose-dependent manner by specific antigen (**Figure 34**). An inactive ECTV ortholog of CPXV 014 (described in section VII of this chapter) was used as the negative control.

These results led us to question our initial conclusions that CPXV 014 was directly preventing naive T cell activation. When viewed in the light of results obtained using other poxviral orthologs of CPXV 014, we began to suspect that the inhibition we had been investigating was restricted to anti-CD3 driven T cell activation but not non-antibody-mediated activation.

Figure 34 Recombinant CPXV 014 does not inhibit the stimulation of LCMV memory T cells by specific immunodominant peptides. Memory T cells taken from C57BL/6 mice infected with LCMV at least 60 days previously were stimulated by the indicated concentrations of LCMV antigenic peptide for five hours in the presence of brefeldin A and either 5 μg/ml CPXV 014-His (226 nM) or ECTV CrmD CTD 3Lys-His (215 nM) negative control protein. Data generated by the laboratory of Dr. Jeff Nolz, OHSU.



VII. ECTV Orthologs of CPXV 014 Implicate FcγR Binding

As stated before, CPXV 014 belongs to a family of SECRET or PIE-like proteins distributed among several poxvirus species. Our collaborators in the Fremont lab had been studying the binding capabilities of CPXV 014 orthologs found in ECTV, particularly the C-terminal domain (CTD) of ECTV CrmD. Their (unpublished) data on ECTV CrmD CTD and CPXV 014 indicated that both were able to bind to both human and murine FcγRIIB and/or FcγRIII since they competed with anti-CD16/32 in binding to both human and mouse cell lines (data not shown). From them we obtained recombinant ECTV CrmD CTD and ECTV 008 (a close ortholog of CPXV 014) and tested these in our assay. As shown in **Figure 35**, not only did the close relative ECTV 008 inhibit naive T cell activation in our plate-bound anti-CD3/anti-CD28 model, but the ECTV CrmD CTD did as well. This was surprising to us because CPXV also possesses a version of CrmD with a C-terminal SECRET domain, but this protein is still present and expressed in our Δ14 CPXV. Since the Δ14 CPXV virus no longer inhibits T cell activation in our assay, we may conclude that the CPXV version of the CrmD CTD does not possess the same inhibitory activity as CPXV 014. This is despite the CPXV and ECTV CrmD proteins sharing 96% sequence identity. In contrast, CPXV 014 and ECTV CrmD CTD, which are both able to inhibit antibody-mediated T cell activation, share very little sequence identity **(Figure 36**).

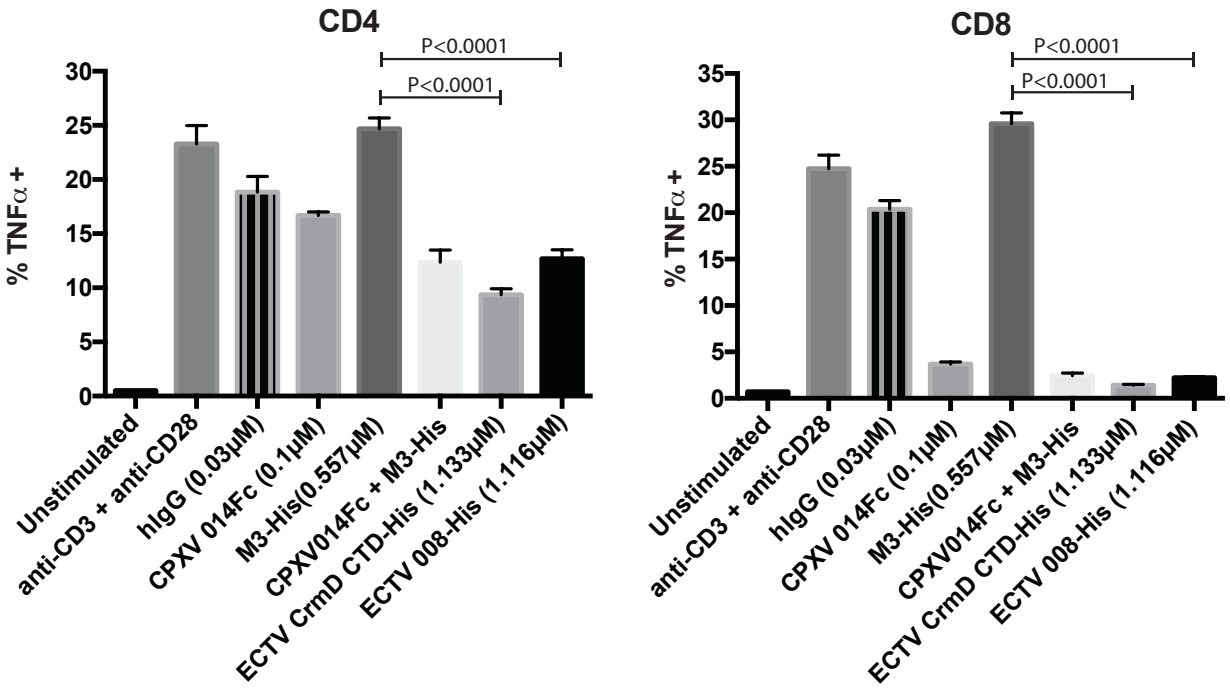


Figure 35 ECTV orthologs of CPXV 014 also inhibit naive T cell activation by plate-bound anti-CD3 and anti-CD28 antibodies. The *ex vivo* naive T cell ICS activation assay using both virus-free supernatants and recombinant protein was performed as described in Methods. Virus-free supernatants from infected MC57 fibroblasts or recombinant proteins were added in the concentrations indicated (all at 5 μg/ml) for four hours prior to the six hour stimulation. Data shows mean TNFα responses of five age and sex matched BALB/cByJ mice. Error bars represent SEM, P values calculated using unpaired two-tailed Student's T test.

Figure 36 Alignment of CPXV_BR-014 vs CPXV_BR-221 (CrmD) vs ECTV_-Mos-003 (CrmD).
The predicted start of the CrmD C-terminal domain is marked by the red S.
CPXV and ECTV CrmD share 96.25% Sequence identity.
Both CrmDs share approximately 37% sequence identity with CPXV 014.
Alignments performed using CLUSTAL O v1.2.1 [247].

```
CPXV_BR-014                     ------------------------------------------------------------
CPXV_BR-221                     MMNMTPSYILLVYMFVVVSGDVPYEHINGKCNGTDYNSNNLCCKQCDPGMYMTHSCNTTS
ECTV_Mos-003                    MMKMTPSYILLVYMFVVVSGDVPYTPINGKCNGTDYNSNNLCCKQCNPGMYMTHSCNTTS


CPXV_BR-014                     ---------------------------------------MININTNTILIFASLFVASF---
CPXV_BR-221                     NTKCDKCPDGTFTSIPNHIPTCLSCRGKCSSNHVETKSCSNTQDRVCVCASGYYCEFEGS
ECTV_Mos-003                    NTKCDKCPDDTFTSIPNHSPACLSCRGKCSSNQVETKSCSNTQDRVCVCASGYYCEFEGS
                                                          . . : : : ** : ..*


CPXV_BR-014                     ------------ANDYP--------------PPGFFEDKYITNTFNYISIDFELYPVNVS
CPXV_BR-221                     NGCRLCVPQTKCDSGYGVYGYSSKGDVICKKCPGNID--KCDLSFNSIDVEINMYPVNKT
ECTV_Mos-003                    NGCRLCVPQTKCGSGYGVYGYSSKGDVICKKCPGNID--KCDLSFNSIDVEINMYPVNKT
                                           . *               ** ::      :** *.:::::**** :


CPXV_BR-014                     SCNRLSTKQSSDVISTSELTITVNSTDCDPVFVTEYYSVKDKTAIAGLFTDTTKKQNTSK
CPXV_BR-221                     SCNSS--IGSSSTISTSELTITLKHEDCTTVFIGDYYSVVDKLATSGFFTNDKVHQDLTT
ECTV_Mos-003                    SCNSS--IGSSSTISTSELTITLTHEDCTPVFIGDYYSVVDKLATSGFFTNDKVHQDLTT
                                ***      **..*********:.  **  **: :**** ** * :*:**: . :*: :.


CPXV_BR-014                     MCTLNIEVKCNAETEPVLIGNFTRVPEKASTHAENFTLIGNCLSDLHLYIAYVNTDEEFE
CPXV_BR-221                     QCKINLEIKCNSGGESRQLTPTTKV--YFMPHSETVTVVGDCLSNLDVYIVYANTDAIYS
ECTV_Mos-003                    QCKINLEIKCNSGRESRQLTPTTKV--YFMPHSETVTVVGDCLSNLDVYIVYANTDAIYS
                                 *.:*:*:***:  *   :   *:*      *:*..*::*:***:*.:**.*.***  :.


CPXV_BR-014                     ED-TATVHIGNKLDINGIPPNMCATRTIN
CPXV_BR-221                     DMDVVAYHTSYILNVDHIPPNDCERD---
ECTV_Mos-003                    DMDVVAYHTSYILNVDHIPPNDCERD---
                                :  ..: * .  *::: **** *
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We also tested recombinant His-tagged M3 protein from murine gammaherpesvirus MHV68 to see if it would have an effect in this assay. M3 is a secreted protein which is capable of binding to and preventing signaling by a large swathe of both human and murine chemokines [255]. We wished to see if perhaps the analogous chemokine-binding ability of CPXV 014 [170] could potentially be responsible for the observed inhibitory phenotype. As shown in **Figure 35**, M3-His exerted no inhibition on naive T cells in our assay, nor did it alter the inhibition by CPXV 014-Fc, strongly suggesting that the chemokine scavenging ability of CPXV 014 is not responsible for the demonstrated inhibition. As such M3-His also functions as an unrelated -His tagged protein control for this experiment.

Further exploring the ECTV orthologs of CPXV 014, we obtained mutant versions of the recombinant ECTV CrmD CTD protein generated by our collaborators in the laboratory of Daved Fremont (Washington University, St. Louis). These consisted of

single point mutations either negating (3Ala) or reversing (3Lys) amino acid charges in the surface of the protein suspected to mediate binding to FcγR, such that both of these mutant proteins have lost the ability to bind to FcγR. We tested these in our plate-bound antibody driven ICS T cell activation assay **(Figure 37)** and found that these FcγR-nonbinding ECTV CrmD CTD proteins had lost their ability to inhibit T cell activation in these assays.

This result was perplexing to us at the time, because we could not envision a plausible way by which an FcγR-binding protein could inhibit activation of naive T cells, since they are not believed to express FcR [248]. We realized that CPXV 014 was perhaps not acting directly on naive T cells, but that the effect we saw could be the result of an artifact stemming from the experimental antibody-driven T cell activation model that we used. Perhaps the FcγR-binding capability of CPXV 014 was somehow inhibiting the ability of T cells to be activated by anti-CD3?

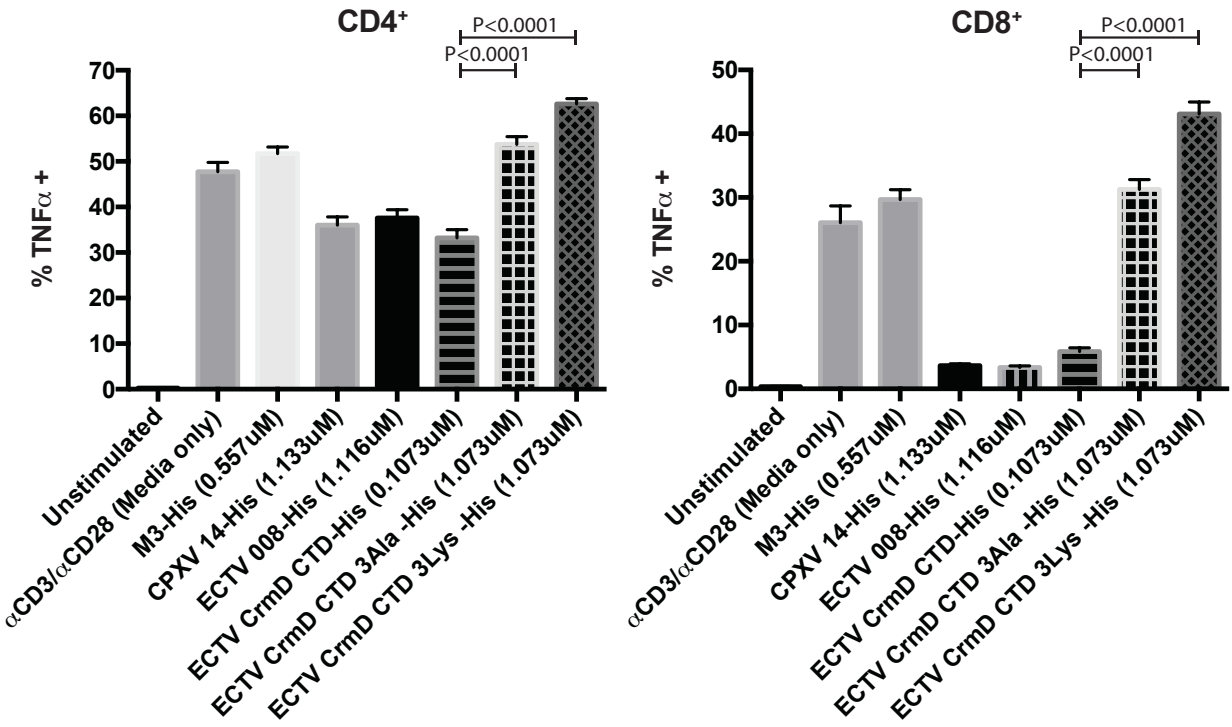


Figure 37 Non-FcγR binding ECTV ortholog mutants no longer inhibit naive T cell activation by plate bound antibodies. The *ex vivo* naive T cell ICS activation assay using recombinant protein was conducted as previously decribed. Briefly, splenocytes were preincubated with the recombinant proteins at the concentrations indicated for four hours prior to six hours of antibody stimulation. Data represents the mean TNFα responses from 5 age and sex matched BALB/cByJ mice. Error bars represent SEM, P values calculated using unpaired two-tailed Student's T test.

Chapter Five: Reliance of antibody-driven naive T cell assays on FcγR-bearing cells

Our studies made it clear that CPXV 014 was not a specific inhibitor of T cell activation, and that the initial phenotype we observed was selective for antibody-driven splenic T cell activation. Namely, that FcγR-bearing non-T "accessory cells" might be responsible for enhancing the level of activation delivered by the anti-CD3/CD28 agonist antibodies. CPXV 014 appears to have been interfering with this unexpected cofactor role of accessory cells. The following chapter details the role of FcγR in supporting antibody-driven T cell activation.

I. The activation of naive murine splenic T cells using anti-CD3 is dependent upon the presence of non-T splenocytes.

Splenocytes from naive SPF-housed BALB/cByJ mice were enriched for T cells to >98% CD3+ by negative selection using a Milteny Biotek PanT cell isolation kit II^{TM} and magnetic cell separation using the MidiMACS^{TM} system before being loaded with CFSE. Stimulation of these enriched T cells for three days with anti-CD3 and IL-2 resulted in less proliferation relative to the response of T cells in complete splenocyte populations **(Figure 38a and b).** This lack of a proliferative response was much more pronounced for $CD4^+$ than for $CD8^+$ populations. This indicates that the naïve T cell proliferative response to exogenously supplied anti-CD3 and IL-2 is largely dependent upon factors supplied by non-T cell splenocytes.

Seeing that accessory cells contribute to the proliferation of T cells in response to soluble anti-CD3 was unsurprising since this phenomenon was noted in some of the

earliest studies using anti-TCR [256-258]. However, the magnitude of the failure of $CD4^+$ T cells to proliferate in response to IL-2 in the absence of accessory cells was more dramatic than anticipated. We further tested if this effect would also be evident in the cytokine response of T cells to stimulation by plate-bound antibodies. We again enriched naive splenic T cells by negative selection, and then stimulated them using plate-bound anti-CD3 and anti-CD28 for six hours followed by intracellular cytokine staining for TNFα**.** As shown in **Figure 38c and 38d**, the TNFα response of the enriched T cells is significantly ablated compared with the same cells in complete splenocyte populations. These results indicate that the T cell response to even immobilized anti-CD3 and anti-CD28 is largely dependent upon non-T cell supplied factors.

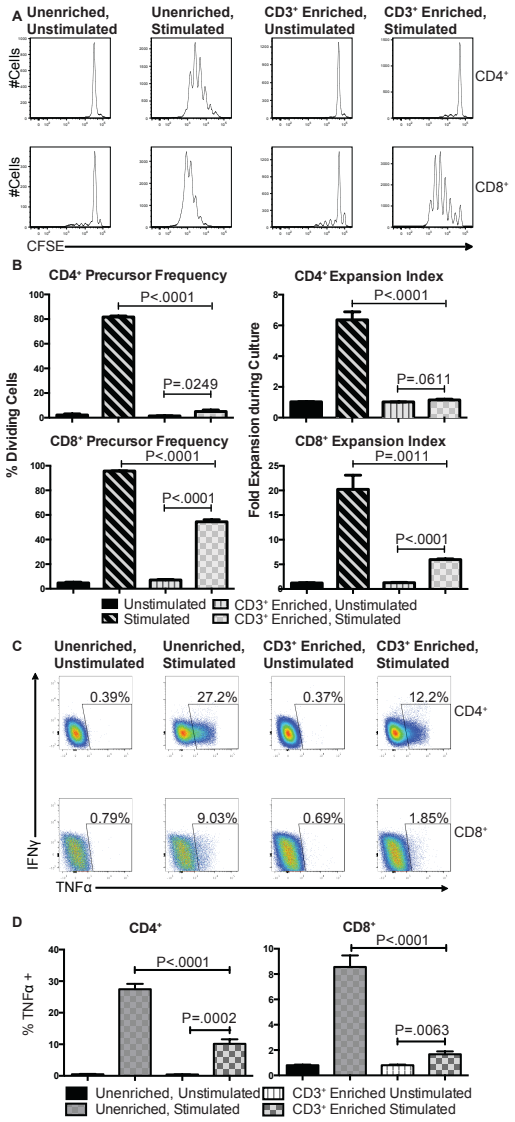


Figure 38 Splenic T cell response to anti-CD3 is dependent upon the presence of non-T splenocytes. Cells were either enriched to >98% $CD3^+$ (gated on live single cells) using a Milteny Biotek Pan T Cell Isolation kit II or allowed to remain in the presence of the other splenocyte populations. Error bars are SEM. P values calculated using unpaired two-tailed Student's T test. **A&B)** Splenic murine T cells were loaded with CFSE and stimulated for 3 days with anti-CD3 and recombinant IL-2. At the end of the 3 days the cells were stained for life/death, CD3, CD4 and CD8 and analyzed by flow cytometry. **A)** Histograms are representative plots shown from one mouse. **B)** Precursor frequency (the % of cells in the original culture that divided at least one time during the incubation period) and fold expansion plots are generated from 5 individual age- and sex-matched BALB/cByJ mice. **C&D)** $CD3^+$ enriched or unenriched murine spenocytes were stimulated for 6 hours using plate-bound anti-CD3 and anti-CD28, then stained for life/death, CD3, CD4 and CD8 and intracellular TNFα and IFNγ and analyzed by flow cytometry. **C)** Representative plots from one mouse. **D)** Mean TNFα responses generated from 5 individual age- and sex-matched BALB/cByJ mice.

To determine whether we could reconstitute the augmented response of T cells in the presence of accessory cells by introducing APCs back to the enriched $CD3^+$ T cell, we stimulated enriched murine T cells (>98% $CD3^+$ of live single cells) with plate-bound anti-CD3 and anti-CD28 in the presence of A20 cells (a syngeneic B cell lymphoma line). As shown in **Figure 39**, A20 cells were able to restore the TNFα response to plate-bound antibodies to levels surpassing those seen with unenriched splenocyte cultures.

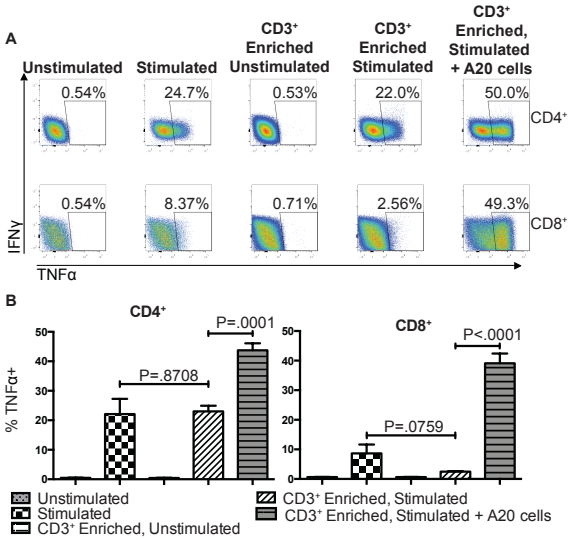
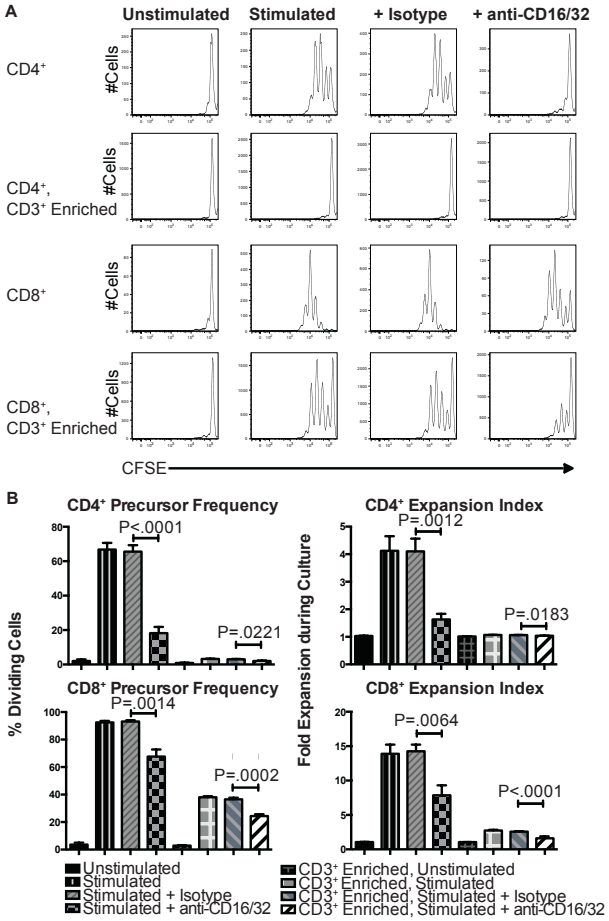


Figure 39 Adding A20 B cells to enriched T cell splenocytes greatly increases the T cell TNFα response to plate bound anti-CD3 and anti-CD28. A20 cells were added to purified splenic T cells (>98% $CD3^+$) at a ratio of 1:2 A20:T cells for two hours prior to 6 hour stimulation with plate-bound anti-CD3 and anti-CD28. Staining and flow cytometry were performed as before. Error bars are SEM. P values calculated using unpaired two-tailed Student's T test. **A)** Plots show representative responses from one mouse. **B)** Pooled data from 5 individual pairs of age- and sex-matched BALB/cByJ mice.

II. Anti-CD16/32 inhibits anti-CD3-driven T cell stimulation in whole splenocyte populations

The only FcγR reported to be expressed on the surface of murine B cells is the inhibitory FcγRIIB (CD32B) [109, 121]. The ability of A20 cells to restore antibody-driven activation of enriched T cells led us to suspect the involvement of this receptor in our assays. To test this hypothesis, we performed the above-described cytokine and proliferation T cell activation assays in the presence of "Fc block," (anti-CD16/32) a monoclonal rat antibody that binds and blocks mouse FcγRIIB and FcγRIII. We found that anti-CD16/32 was able to inhibit both the proliferative response **(Figure 40a and 40b)** to anti-CD3 and IL-2, as well as the cytokine response to plate-bound antibodies **(Figure 40c and 40d)**. We did not observe the same result with an isotype control indicating that the antibody is not simply affecting the binding of anti-CD3 and anti-CD28 to the plate. It is also noteworthy that while the inclusion of anti-CD16/32 leads to significantly reduced stimulation of T cells in whole splenocyte populations, this inhibition dwindles when the non-T cell populations are removed from the experimental

system. This indicates that the antibody is acting by specifically blocking FcγR on the accessory splenocytes, and that the enhancement of T cell responses to agonist antibodies is due to the ability of anti-CD3 and anti-CD28 to bind to FcR on neighboring accessory cells.



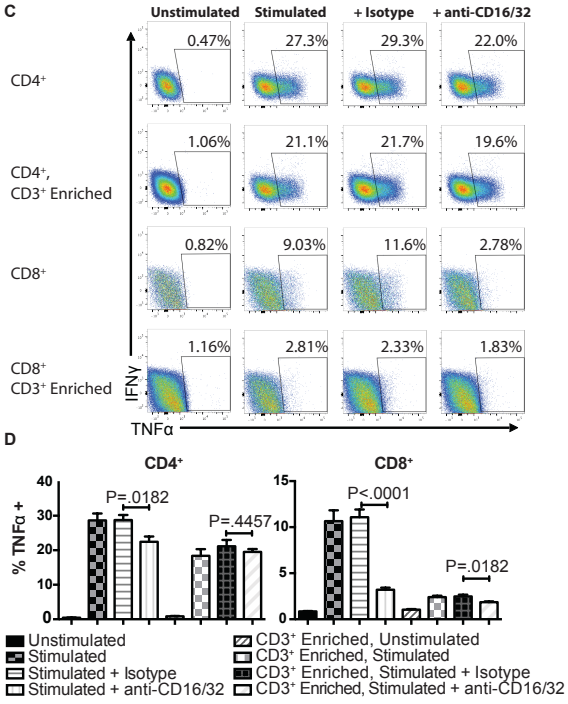


Figure 40 Blocking Fc receptors CD16 and CD32 inhibits the activation of splenic T cells by anti-CD3. Enriched populations were negatively selected to >95% $CD3^+$ as determined by flow cytometry gating on live single cells. Anti-CD16/32 or isotype control was supplied at 200 nM during the stimulation where indicated. Error bars are SEM. P values calculated using unpaired two-tailed Student's T test. **A&B)** Enriched or untouched splenic murine T cells were loaded with CFSE and stimulated for 3 days with anti-CD3 and IL-2. The cells were then stained for life/death, CD3, CD4 and CD8 then analyzed via flow cytometry. **A)** Histograms show proliferative response from one mouse. **B)** Pooled data from 5 age- and sex-matched mice. **C&D)** Anti-CD16/32 or isotype control was added to the cells at 10 nM for 2 hours prior to stimulation with plate-bound anti-CD3 and anti-CD28. **C)** Representative data from one pair of pooled mouse spleens. **D)** Pooled data from 5 pairs of age- and sex-matched mice.

Chapter Six: CPXV 014 as a Secreted FcγR Binding Protein

I. Fc Block Mimics CPXV 014 Activity

The data showing that mutating the CPXV 014 ortholog ECTV CrmD CTD so that it no longer bound FcR destroyed its ability to inhibit naive T cells in our antibody-driven assays, combined with the failure of CPXV 014 to inhibit T cell activation by other mitogens, drove us to examine the role of FcR in our assays.

To this end, we utilized a well-characterized rat-anti-murine FcR blocking antibody, anti-CD16/32, commercially marketed as "Fc block". There are two monoclonal antibodies sold as Fc block, clone 2.4G2 and clone 93. They possess the same specificity but 2.4G2 is rat IgG1 and clone 93 is rat IgG2a. Both are commonly used for blocking nonspecific antibody binding in flow cytometry and immunofluorescence. For all of the functional studies using anti-CD16/32 in this work we used clone 93 with nonspecific rat IgG2a as the isotype control. We wished to see if blocking FcγR during *in vitro* T cell activation assays would have an effect. As shown in **Figure 40**, anti-CD16/32 inhibits antibody driven T cell activation in both the plate-bound anti-CD3/anti-CD28 ICS assay and the anti-CD3 + IL-2 proliferation assay. Furthermore, the pattern of preferentially inhibiting CD8+ cytokine responses and CD4+ proliferation responses was strikingly similar to that seen when using CPXV 014-His.

II. Enriched $CD8^+$ T Cells Are Not Inhibited by CPXV 014

Our data clearly shows that the antibody-driven T cell assays are very dependent on the presence of FcγR bearing "accessory cells," and that the inhibition by anti-

CD16/32, which so closely resembles that of CPXV 014 becomes insignificant when non-T cells are removed from the experimental system.

We tested our recombinant CPXV 014-His protein on highly purified $CD8^+$ murine T cells to determine if inhibition still occurred. As shown in **Figure 41**, when murine T cells were purified to >97% $CD8^+$ by negative selection, not only did the level of T cell activation plummet, but the inhibition exerted by either CPXV014-His or anti-CD16/32 dwindled to insignificance. When whole splenocyte populations were used, we observed inhibition of T cell activation by both CPXV-014 His and anti-CD16/32, but not the non-FcγR-binding ECTV ortholog or an isotype control (**Figure 41a**). However, when $CD8^+$ population were purified by negative selection, the TNFα response of the CTLs was markedly attenuated and there was no difference in cells treated with either CPXV 014-His, anti-CD16/32 or the controls (**Figure 41b)**. We hypothesize that any residual inhibition seen is likely a result of the few remaining FcγR-bearing cells still present in the culture. An early version of this experiment where some of the T cell enrichments were not optimal suggested that the purer the T cell culture, the less T cell activation occurred (data not shown).

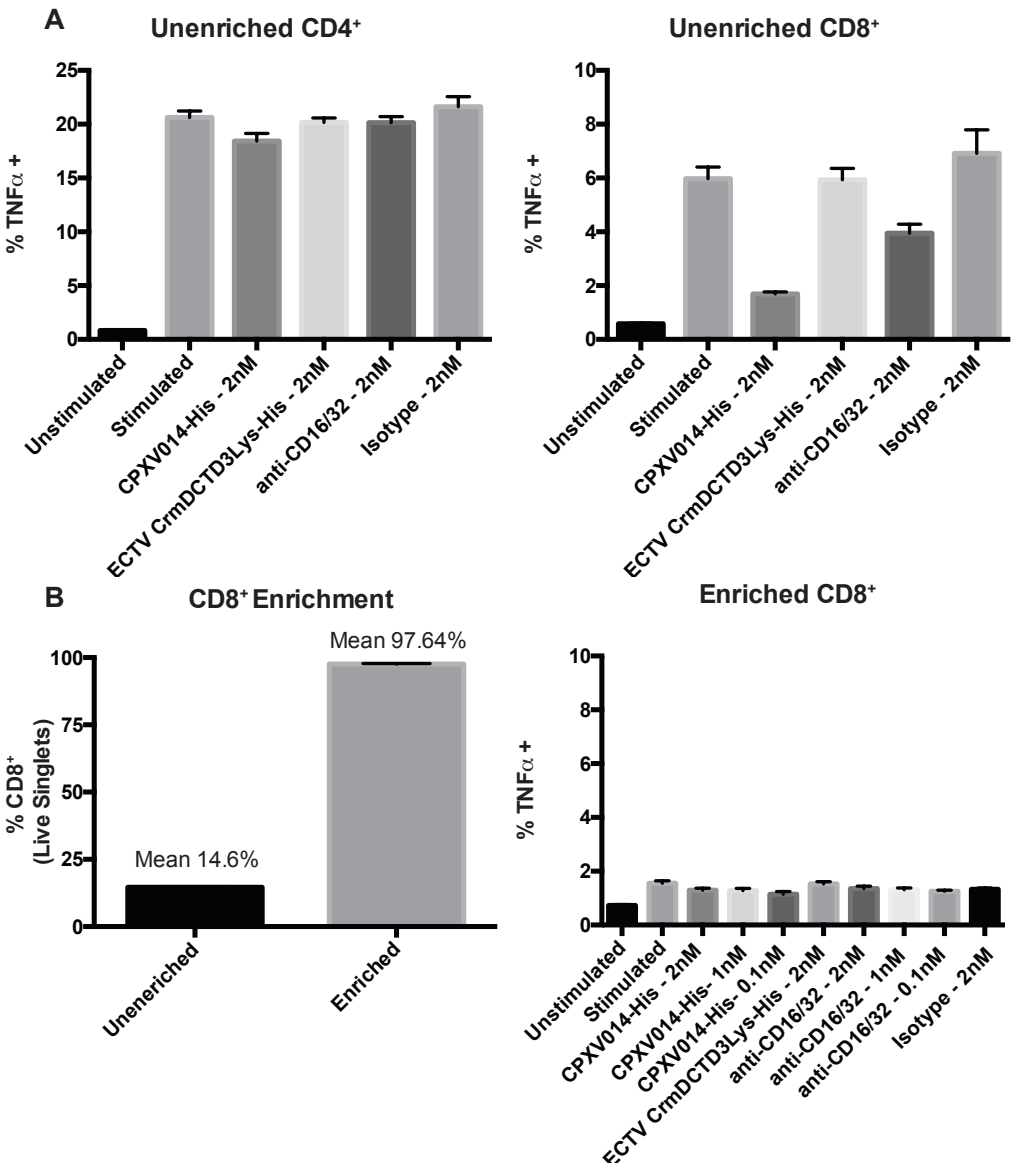


Figure 41 CPXV 014 inhibits the augmentation of CD8⁺ T cell activation by FcγR-bearing accessory cells. The *ex vivo* naive T cell ICS activation assay was performed as described in Methods using unenriched splenocytes (**A**) or enriched CD8⁺ T cells (**B**). Enriched populations were negatively selected to >95% CD8⁺ using a Milteny CD8⁺ cell isolation kit II and then plated at and stimulated with the same concentrations as used in the unenriched T cell assays. Anti-CD16/32 or isotype control was added to the cells at the indicated concentrations for 2 hours prior to stimulation with plate-bound anti-CD3 and anti-CD28. Tables show pooled data from 5 pairs of age- and sex-matched mice. Fraction CD8⁺ enrichment based on flow cytometry gated on live singlet cells. Error bars are SEM. P values calculated using unpaired two-tailed Student's T test.

III. CD32B KO T Cell Assay (Pilot)

Since Fc receptors appear to be responsible for the observed inhibition exerted by CPXV 014 on naive murine splenic T cells, a natural course of inquiry would be to examine the effect this protein has on T cells from mice lacking one or more Fc receptors. At the time of this writing, we have only managed to acquire splenocytes from one CD32B KO mouse for testing. As shown in **Figure 42**, our initial results with the one FcγRIIB KO mouse would appear to support the hypothesis that CPXV 014 only acts to

inhibit naive T cells in our model by blocking the abundant FcR on neighboring non-T splenocytes. Firstly, the proportion of T cells stimulated to produce TNFα from the CD32B KO mice are discernably less than those of the WT mouse. Furthermore, CPXV 014 shows no apparent exertion of T cell inhibition over that still exerted by anti-CD16/32. This is most pronounced for the $CD4^+$ T cells, where the CD32B KO T cells do not appear to be inhibited by either recombinant CPXV 014-His or anti-CD16/32 (**Figure 42a**). The $CD8^+$ T cells of the CD32B KO mice are still inhibited to some extent by both antibody and recombinant protein but not the non-FcγR-binding ECTV CrmD CTD (**Figure 42b**), and I hypothesize that this is due to a lingering scaffolding effect provided by the other FcγR targeted by Fc block, FcγRIII (CD16). Our future plans include more rigorous testing of both strains of mice lacking FcγR (the FcγRIIB KO mice, one of which was examined here, and the γ-chain KO mice which lack FcγRI, II, and IV) in both *in vivo* and *ex vivo* immune response assay.

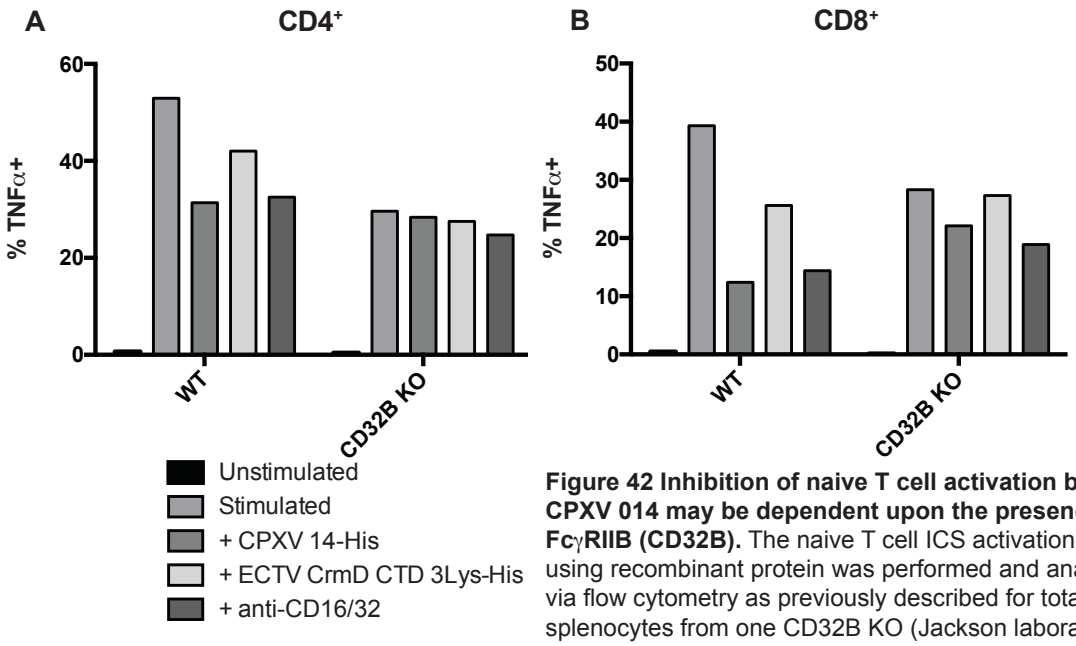


Figure 42 Inhibition of naive T cell activation by CPXV 014 may be dependent upon the presence of FcγRIIB (CD32B). The naive T cell ICS activation assay using recombinant protein was performed and analyzed via flow cytometry as previously described for total splenocytes from one CD32B KO (Jackson laboratory stock #002848 B6;129SFcgr2b^{tm1Trk}/J) mouse and one C57BL/66 control mouse. CPXV014-His, ECTV negative control protein or anti-CD16/32 antibody were all supplied at 100 nM.

IV. Models for CPXV 014 Activity in T Cell Assays

We interpret this data to indicate that CPXV014, like anti-CD16/32, is not actually inhibiting naive T cell activation, but is instead inhibiting the *enhancement* of T cell activation by FcR bearing accessory cells in these antibody-driven T cell activation models. This enhancement may occur via the "scaffolding" of the agonist antibodies by neighboring FcR, which enables better TCR clustering and activation as proposed in the previous chapter/paper. This mutual binding of APC FcγR and T cell CD3 could also be bringing the T cells into contact with costimulatory ligands on the APC surface (as these accessory cells are not inert substrates and bear many of their own surface molecules). In this case, anti-CD3 could be acting as a linker molecule between T cells and FcγR-

bearing APC (**Figure 43b**). CPXV 014 and anti-CD16/32 block the binding of agonist Ig to FcR, and block the generation of a greater T cell activation in this fashion (**Figure 43a**). The inhibition of naive T cell activation we used to identify this poxviral immunomodulator was actually a byproduct of the system used.

This realization, combined with the *in vivo* results generated in our laboratory, begs the question of what precisely this secreted chemokine and FcR-binding protein is doing to support cowpox virus *in vivo*? CPXV 014 appears to be the only FcγIIRB/IIIR-binding or blocking protein in the CPXV genome, since the Δ14 CPXV no longer inhibited the augmented T cell activation.

In order to address this issue, we shifted our focus to other cell types. Because of the striking enhancement we saw when we added a syngeneic B cell line to the culture **(Figure 39)**, and also because the only known surface FcR expressed by B cells is CD32B, the inhibitory FcγR (though some sources suggest human splenic B cells may express some neonatal FcR - FcRn as well [121]), we shifted our focus to examining the effects of CPXV014 on B cell activation and the humoral response to CPXV infection.

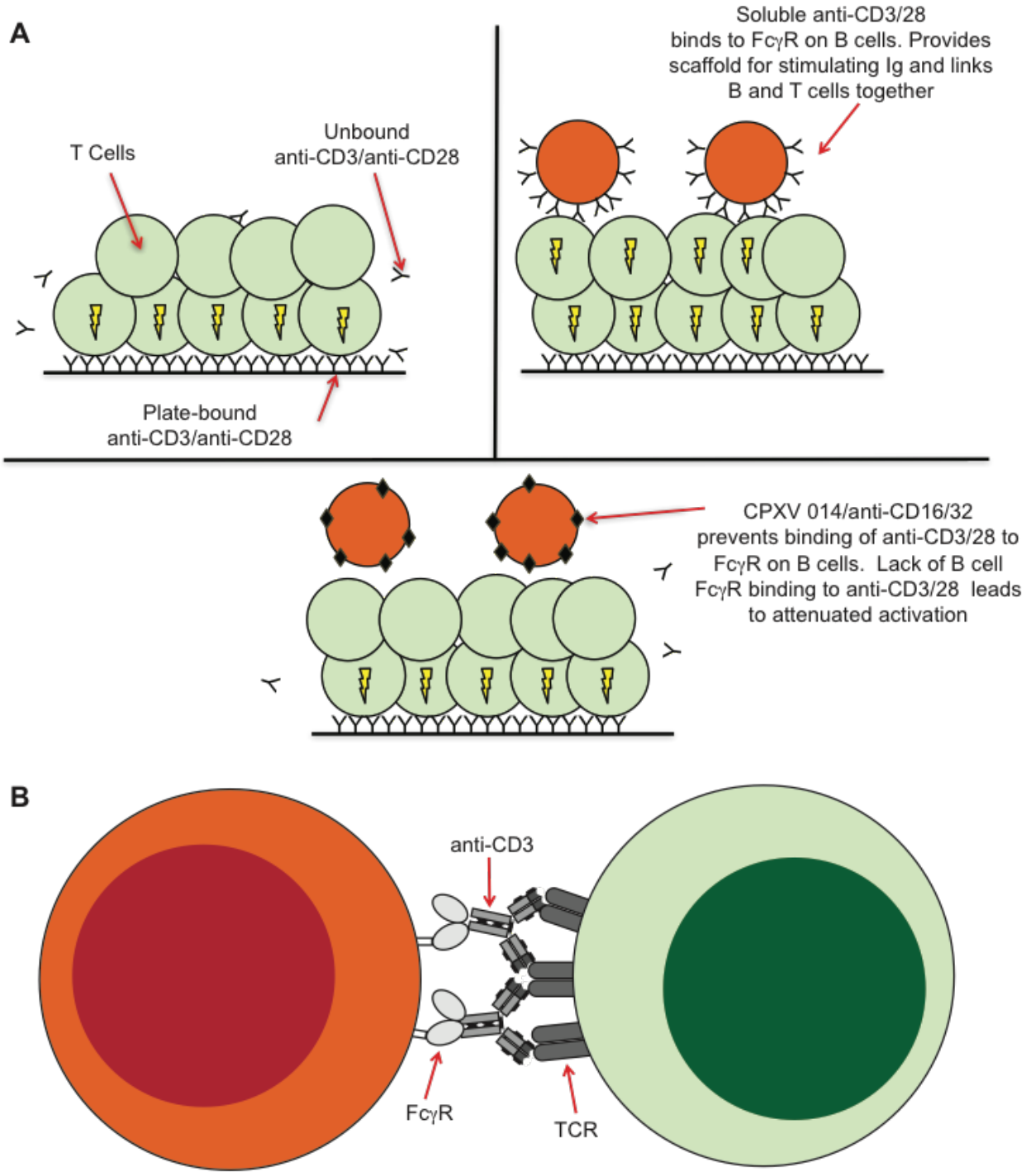


Figure 43 Model for augmented antibody-driven T cell activation by FcγR-bearing non-T splenocytes. A) CPXV 014 or anti-CD16/32 block the binding of agonist antibodies to FcγR-bearing non-T splenocytes, inhibiting the enhancement of T cell activation by these neighboring cells. B) Agonist anti-TCR antbodies provide a link between T cells and FcγR-bearing cells, creating a pseudo-immunological synspse, which leads to greater polyclonal T cell activation than that provided only by immobilized anti-TCR.

V. CPXV 014 Inhibits Murine B Cell Proliferation *in vitro*

With the realization that CPXV 014 does not directly inhibit naive T cells we directed our attention to other immune cell populations. As mentioned in the introduction, the crucial mediators of adaptive humoral immunity are the B cells. We decided to look at the effects of CPXV 014 on B cells because they are known to express the inhibitory CD32B receptor, which might be specifically targeted by the secreted cowpox protein.

Since it has been demonstrated previously that coligation of CD32B (FcγRIIB2, the sole reported FcR on murine B lymphocytes) with the BCR leads to attenuation of the B cell activation and proliferation signal [105, 123, 259], we decided to test if CPXV 014 could attenuate the B cell proliferative response. Splenic B cells obtained from naive BALB/cByJ mice were loaded with CFSE and then stimulated using plate-bound anti-mouse IgM $F(ab')_2$ in the presence of recombinant IL-2**.** As shown in **Figure 44a**, addition of recombinant CPXV 014, but not soluble anti-CD16/CD32 was sufficient to inhibit the B cell proliferative response. Mouse IgG was included as a positive control for inhibition, but since CD32B is a low-affinity FcγR, monomeric IgG does not exert any significant inhibition. This inhibition of B cell proliferation by CPXV 014 was significant in all three readouts of cellular proliferation. “Fraction divided” refers to the percentage of cells in the final culture that have divided at least one time. “Precursor frequency” indicates the proportion of cells from the original culture that divided at least one time during the incubation period. “Expansion index” refers to the overall fold expansion of cells in the culture during the incubation period. Furthermore, as shown in **Figure 44b**, the inhibition of B cell proliferation exerted by CPXV 014 was dose-dependent. This

contrasts with the all-or-nothing inhibition of T cell activation by CPXV 014 observed in the antibody-driven T cell assays (**Figure 27**). These results suggest that not only is CPXV 014 capable of blocking FcR similarly to anti-CD16/32 (as seen in our T cell assays), but that it is potentially signaling through FcγRIIB to attenuate the activation signal. We are currently extending these B cell activation and inhibition studies as well as looking into potential inhibition of dendritic cell maturation and cross presentation by CPXV 014. If so, this would represent a novel immunoevasion tactic employed by CPXV.

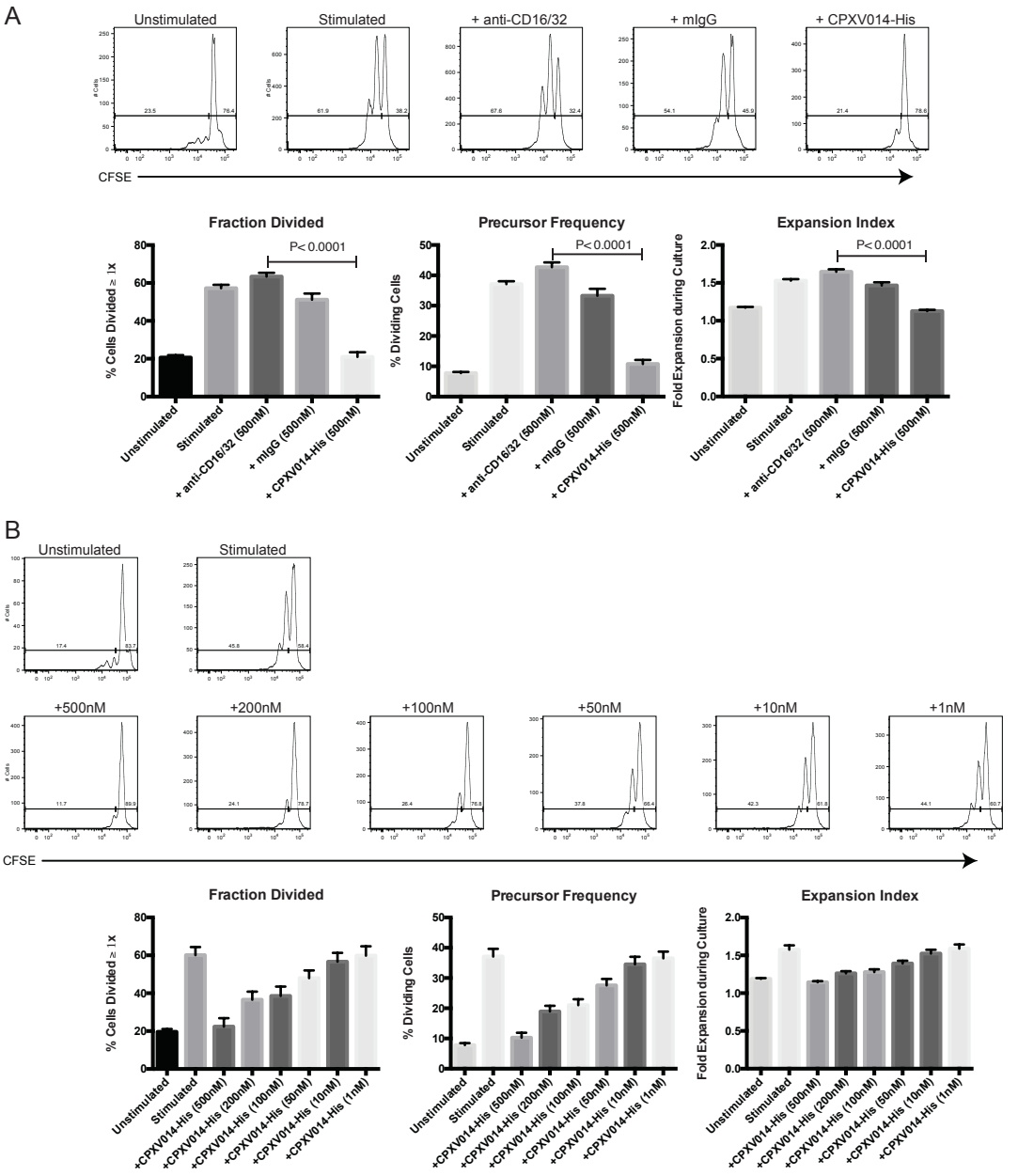


Figure 44 CPXV 014-His inhibits anti-IgM F(ab')$_2$-driven B cell proliferation. Spleens were harvested from naive female BALB/cByJ aged 6-10 weeks and loaded with 1 μM CFSE, as described previously for the T cell proliferation assays. before resuspension at the same cell concentration in B cell proliferation media. The cell suspensions were plated at a concentration of 1.25 x 10^6 cells/ml into a flat bottomed 96 well plate which had been coated with 50 μg/ml anti-IgM F(ab')$_2$ overnight at 4°C then washed twice. Recombinant CPXV 014-His or control proteins were added in the concentrations indicated along with 1 nM IL-2, and they were cultured for three days at 37°C 5% CO2. After this incubation they were washed, stained for life/death and surface molecules CD3, and B220 and CD19, then analyzed on a BD LSRII cytometer as described above. Data was interpreted using TreeStar FlowJo software (v9.8). **A** and **B** represent two independent experiments each using 5 mice. Histograms show responses from one representative mouse. Tables represent the mean values as calculated using FlowJo. Error bars represent SEM, P values calculated using unpaired two-tailed Student's t test. Cells are gated on Time -> Lymphocytes (FSC/SSC) -> Single cells -> Live cells -> $CD3^-$ -> $B220^+CD19^+$

Chapter Seven: Discussion

I. Overview

Scientific research rarely travels in a straight line towards a known endpoint, and our investigations into the nature and activities of CPXV 014 provide a striking example of this fact. The idea for this project initially stemmed from the observation that MPXV- and CPXV-infected cells were able to inhibit the activation of human (but not mouse) $CD4^+$ and $CD8^+$ T cells *in trans* by anti-CD3 using B22 proteins (MPXV 197 and CPXV 219) [231, 232]. These results prompted us to ask the question if CPXV, possessing the largest number of genes among OPXV, possessed any additional activities for the inhibition of T cell activation. We initially identified CPXV 014 as an inhibitor of murine naive splenic T cell activation, but have subsequently shown that this was the result of the antibody-driven *in vitro* T cell activation assays used. Instead, what we discovered is a secreted CPXV protein that binds to FcγR and interferes with T cell activation by agonistic antibodies, but not with other T cell stimuli. This led us to examine how this protein might affect other immune cells such as B lymphocytes. As such, our pursuit of CPXV 014 has led us on a journey through T cell activation models, Fc receptors and into B cell activation and inhibition.

II. CPXV 014

II A. Identification of CPXV 014

We used plate-bound anti-CD3 and anti-CD28 to stimulate naive murine splenic T cells in the presence of infected or uninfected cells. We observed that CPXV-, but not VACV-infected cells inhibited the polyclonal activation of these T cells to produce TNFα. This was not due to direct infection of the T cells by cowpox, but it should be noted that

inclusion of the antipoxviral drug ST-246 in the assay did reduce the level of inhibition exerted. This was likely due to ST-246 limiting the extent of A20 cell infection, leading to a reduced amount of viral inhibitor being produced. We repeated this *in vitro* assay using a series of overlapping genomic deletion CPXV mutants (generated in the lab of David Pickup, Duke) to look for a loss of inhibition. The results of these mapping experiments indicated that the gene CPXV 014 was responsible for the observed effects.

As demonstrated by our subsequent data, this initial inhibition by which we identified CPXV 014 was an indirect effect dependent upon the presence of FcγR-bearing non-T cells in the culture. Even if we had performed these first studies using purified $CD3^+$ splenic T cells, our use of infected A20 cells (which express FcγRIIB) ensured that T cell activation by agonist antibodies would be amplified, and CPXV 014 would have interfered with this amplification and appeared to inhibit naive T cell activation. Later *in vivo* results demonstrated that CPXV 014 does have an effect on T cell priming in multiple mouse models of infection, though we now realize that this is not due to any direct activity of CPXV 014 on T cells, contrary to our initial assumptions.

II B. Generating Δ014 CPXV

In order to confirm that CPXV 014 was responsible, I generated a disruption mutant virus in which most of CPXV 014 is replaced by an E/L_EGFP_7.5K_GPT selection cassette by homologous recombination. For reasons remaining unclear to this day, replacing CPXV 014 was very inefficient and required the construction of many different constructs before effective recombination occured. A revertant virus has not

been made due to similar problems with the reverse recombination. We instead opted to sequence the resulting virus, and realized that the initial isolate also had a gene-killing point mutation in CrmA, an antiapoptotic viral serpin and another CPXV immunomodulatory gene. It is worth mentioning that had we successfully made a revertant virus and not performed genomic sequencing of the initial ∆014 CPXV isolate, this defect in CrmA would probably have gone unnoticed.

The point mutation in CrmA was simply corrected (at the suggestion of Scott Wong) by coinfecting cells with both the WT and ∆014∆CrmA CPXV, allowing the mutant virus to recombine with wild type. Subsequent analysis confirmed the final ∆014 CPXV virus to have an intact CrmA while retaining the selection cassette replacing CPXV 014. We then tested this ∆014 virus in our T cell assay in order to confirm that CPXV 014 was responsible for the observed inhibition. We found that ∆014 CPXV no longer inhibited the polyclonal naive T cell activation exhibited by WT CPXV, indicating that CPXV 014 was necessary for the observed inhibition in these antibody-driven *in vitro* naive T cell activation assays.

II C. Generating recombinant CPXV 014

We next wished to determine if CPXV 014 was sufficient by itself to inhibit naive T cell activation. We generated a recombinant, exogenously expressed and purified version of CPXV 014 with a C-terminal human Fc tag (CPXV 014-Fc). Later on in the project I switched to producing a His-tagged construct (CPXV 014-His) created by our collaborators in the Fremont lab. The primary reason for this switch was to enable faster recombinant protein production in greater quantities. While it takes upwards of one

month to generate a couple of hundred micrograms of the Fc-tagged protein from stably transfected CHO cells, the His-tagged protein may be generated in milligram quantities within one to two weeks by transient transfection of HEK293F cells. Furthermore, we had become concerned that the human IgG Fc tag on CPXV 014-Fc could exert steric effects on the activity of the protein (this tag being roughly the size of the CPXV 014 protein itself) or obscure any results stemming from the FcγR-binding capabilities of the protein, which we had just learned about. We engineered a thrombin cleavage site upstream of the hIgG1 Fc tag, so that the tag could be removed by enzymatic treatment, but cleavage was inefficient (probably due to the aforementioned steric hindrance) and the greater ease of protein production using the His-tagged construct won out.

Testing the Fc and His-tagged proteins in our plate-bound anti-CD3/anti-CD28 naive T cell activation assay showed that both versions of recombinant CPXV 014 were sufficient to recapitulate the observed inhibition by CPXV. We additionally tested recombinant protein in another naive T cell assay, this time looking at the response of splenic T cells to soluble anti-CD3 and exogenously supplied IL-2, which induces them to proliferate. We found that CPXV 014 was able to suppress the proliferation of the T cells, particularly the $CD4^+$ populations, in contrast to the plate-bound antibody and ICS T cell assays (wherein the inhibition of $CD8^+$ T cells was more pronounced). We additionally tested if CPXV 014 could inhibit non-naive T cell responses. We took splenic T cells from mice that had been infected with murine cytomegalovirus, (which generates a massive MCMV-specific T cell population), and stimulated these with plate bound anti-CD3 only. Since these populations are largely memory and effector T cells

they are able to respond with both TNFα and IFNγ to only TCR ligation without the requirement for CD28 costimulation. We found that recombinant CPXV 014 was able to prevent T cell reactivation by anti-CD3. This result was surprising to us because it has been previously demonstrated that deleting CPXV 012 and CPXV 203, the genes responsible for the downregulation of MHC Class I in infected cells, restored the stimulation of poxvirus-specific $CD8^+$ T cells by infected A20 cells [226, 228]. If CPXV 014 was truly able to inhibit the activation of memory and infected cells, this inhibition would have still been apparent with the Δ012Δ203 CPXV. In retrospect, this inhibition of the effector and memory T cell response to anti-CD3 was a sign that CPXV 014 was only effective *in vitro* against T cell activation which is driven by agonist antibodies.

We initiated a collaboration with the laboratory of Daved Fremont (specializing in structural biology), which was studying CPXV 014 and several orthologous proteins (particularly those in ECTV). They showed us their (unpublished) work demonstrating these proteins competing with anti-CD16/32 (clone 2.4G2) for binding to the surface of human monocytic and murine macrophage cell lines. Since 2.4G2 is specific for FcγRIIB and FcγRIII, their results indicated that CPXV 014 was binding to one or both of these receptors. The capacity of CPXV 014 to bind FcR initially seemed to us unlikely to mediate the effects on T cells we observed, as naive T cells are not known to express these or any other FcR [248]. This realization still helped prompt us to switch from the Fc-tagged protein to the His-tagged CPXV 014 construct in order to avoid any confounding effects the human Fc tag might have in our assays. Murine FcR are known to be very promiscuous for the Fc of other species [256, 260, 261], and we wished to avoid any artefactual effects stemming from any interactions between the tag and FcR.

II D. CPXV 014 in non-antibody-driven T cell activation assays

In order to get at the mechanism of how CPXV 014 inhibits T cells, we began exploring other models of T cell activation and testing if CPXV 014 could inhibit in those contexts. Initially we used PMA and ionomycin to polyclonally activate naive T cells by targeting the activation pathway downstream of TCR ligation. We found no inhibition of the naive T cell TNFα response in this model. Then we tested if naive T cell activation by PHA could be inhibited. Again, CPXV 014 had no effect on T cell activation in this model. Then we tested whether a more "natural" T cell activation model could be inhibited: the stimulation of transgenic OT-I $CD8^+$ T cells by the antigenic SIINFEKL peptide for which the OT-I TCR is specific. Again, we saw no inhibition of OT-I $CD8^+$ T cell activation by CPXV 014. The laboratory of Jeff Nolz also tested the CPXV 014-His protein in an analogous assay based on stimulating the memory T cells generated during LCMV infection with known specific immunodominant antigen and again saw no inhibition by CPXV 014.

It was clear at this point that the only models in which we could see T cell activation inhibited by CPXV 014 were those driven by anti-TCR agonist antibodies. This realization led us to question whether the FcR binding capacity of CPXV 014, which we initially discounted, was actually responsible for the observed effects. Even though naive murine T cells are not believed to express any known FcR [248], perhaps other splenic cell types which do bear FcR could be mediating the apparent inhibition? We took a step back from CPXV 014 and decided to revisit our assumptions about the antibody-driven activation assays and the potential roles of FcR and "accessory" cells.

III. FcγR blocking antibody and CPXV 014 inhibit the augmentation of T cell activation by FcγR-bearing non-T splenocytes.

We decided to test if "Fc block", anti-CD16/32, could recapitulate the inhibition seen by CPXV 014 in the plate-bound antibody T cell activation assay. As demonstrated, anti-CD16/32 is able to inhibit naive T cell activation in both the plate bound anti-CD3/anti-CD28 assay looking at TNFα induction as well as the soluble anti-CD3 with IL-2 T cell proliferation assays. Notably, the pattern of inhibition mirrors that exerted by CPXV 014 in that both preferentially inhibit the $CD4^+$ proliferative response and the $CD8^+$ cytokine response, to the same level when supplied in equimolar concentrations.

In order to confirm the hypothesis that T cell inhibition by either anti-CD16/32 or CPXV 014 was due to simply blocking FcR on neighboring non-T splenocytes we performed these T cell activation assays using splenocyte populations purified to >97% $CD3^+$ or $CD8^+$ by negative selection. In multiple experiments we saw that when T cells are purified away from other cell types the levels to which they are activated drops precipitously. Also, the greater level of stimulation normally seen by T cells in mixed splenocyte cultures could be reconstituted in our $CD8^+$-enriched cultures by adding in a syngeneic B cell line to the assay. Furthermore, the inhibition exerted by either CPXV 014 or anti-CD16/32 dwindled to insignificance when non-T cells were removed from the assay.

The activation of T cells using anti-CD3 is a common technique used to study T cell activation responses, differentiation and signaling pathways. Our data demonstrates that non-T accessory cells are needed for efficient proliferative and cytokine responses of naive mouse T cells to anti-CD3 and costimulation. Furthermore, blocking specific FcγR on accessory cells using anti-CD16/32 during naive splenic T lymphocyte stimulation is sufficient to significantly inhibit the proliferative response to soluble anti-CD3 and IL-2 as well as the cytokine response to plate-bound anti-CD3 and anti-CD28. However, the inhibition by anti-CD16/32 antibody is greatly diminished when non-T cells are removed from the experimental system. While the inhibition of anti-CD3 and IL-2 driven proliferation by anti-CD16/32 has been previously mentioned in the literature as unpublished observations [256] this was the first time to our knowledge that anti-CD16/32-mediated inhibition had been demonstrated in a model utilizing plate-bound activating antibodies. It has since been brought to our attention that the inhibition of anti-CD3-mediated T cell activation by FcR blocking antibody has been formally demonstrated in previous work [262].

The augmentation of T cell responses to stimulating antibodies by "accessory cells" was recognized when the first monoclonal anti-TCR antibodies were developed, and the potential role of FcR on neighboring cells as a mechanism for crosslinking and aggregating the TCR on naive T cells was also commented upon [256-258]. In the 1980s it was discovered that immobilizing the agonist antibodies on a solid surface leads to superior T cell stimulation [263, 264]. This was the model we used for our initial identification of CPXV 014. We had assumed that if the stimulating antibodies were

plate-bound that any effects of bystander cells would be obviated, but the inability of CPXV 014 to inhibit in non-antibody driven T cell assays forced us to reconsider this.

Our results here indicate that the FcR on neighboring cells are also major factors in assays using immobilized antibodies. We postulate that this is due to the murine FcR on APC, providing an additional scaffold for anti-CD3 from which TCR (and costimulatory receptor) clustering can occur. How the supposedly immobilized antibody is scaffolded by FcR is still unclear, but I postulate that either the cells bind and accumulate whatever fraction of soluble anti-CD3 and anti-CD28 is present, or that these FcR expressing cells are able to pull the antibody off from the plate surface in a sort of solid-state trogocytosis event. Moreover, it is possible that the simultaneous binding of APC FcR and TCR by anti-CD3 is bringing the cells together into a pseudo-immunological synapse where the T cell is brought into contact with costimulatory molecules on the other cell's surface. If anti-CD3 is essentially acting as a linker molecule between T cells and FcγR-bearing APC, it seems likely that APC surface molecules may influence T cell activation. Note this does not preclude the likelihood that the "accessory" cells can play additional roles in modulating the T cell response during these assays such as by secreting cytokines such as IL-1 or IL-6 [265].

Agonist antibodies are derived from a species different than the model organism, but cross-reactivity between the immunoglobulins and FcR of different species does occur and some FcR have been demonstrated to be quite promiscuous in their ability to bind antibodies from other species [260, 261]. The hamster anti-mouse-CD3 used to stimulate T cells in our assays (clone 145-2C11) is known to have a high affinity for murine FcR [256]. The role that FcR on "accessory" cells can play in augmenting the

response to anti-CD3 was initially realized in part due to cross-reactivity between mouse antibodies and human FcR. In that study, human PBMC were stimulated *ex vivo* using a mouse antibody directed against human CD3. Most donor cells responded with robust proliferation, but cells from roughly 30% of the donors did not multiply. Investigation of the differences between the responder and non-responder cells revealed that the non-responders all exhibited a polymorphism in an FcR that prevented its ability to bind the Fc portion of the mouse antibody [266, 267].

In recent years, this scaffolding ability of FcR has again become a topic of some interest for immunologists designing and characterizing various antibodies for cancer therapy. Recent studies on antibodies as possible therapies for cancer have reported requirements for CD32B engagement by anti-TNFR [268] and anti-CD40 [269] in order to see effects of binding *in vivo*. In the case of the anti-CD40, the researchers additionally demonstrated that this requirement was averted by multimerizing the antibody such that it could crosslink its ligand efficiently on its own. It has also been reported that removing the Fc portion of several different cancer therapeutic antibody species abrogates their activity [270].

A final example which demonstrates the importance of recognizing FcR interactions with therapeutic antibodies is the catastrophic Phase 1 clinical trial of TGN1412, a superagonist anti-human CD28. Earlier studies in both mice and non-human primates had suggested TGN1412 was nontoxic and a potential therapy for certain autoimmune disorders [271-274]. However, when the drug was given to six study participants, all of them rapidly developed a life threatening cytokine storm [275]. A recent post-mortem of the ill-fated study examined if TGN1412 could be bound by

FcR, leading to an increased CD28 clustering and an exaggerated cytokine response. They found that the inclusion of CD32B-bearing B cells (or other cell types transfected with CD32B) greatly increased the T cell response to the superagonist [276]. This is similar to the effect we saw when we added A20 B cells into enriched T cell cultures and confirms the role of FcR-expressing bystander cells in the activation of naive T cells by agonist antibodies.

Our data further underscores the importance of neighboring FcR in the polyclonal activation of T cells by agonist antibodies. This dependence upon FcR-bearing "accessory" cells should be taken into consideration by anyone using these assays to study T cell activation. A failure to control for the augmented activation of T cells in the presence of other cell populations bearing FcR may lead to a misinterpretation of results and the pursuit of spurious artifacts. One may be led to believe that a particular molecule (such as CPXV 014) directly inhibits T cell responses when it actually disrupts the interactions between the stimulatory antibody and FcR on neighboring APCs. These studies reinforce the need for a complete understanding of the myriad effects of FcR during investigations utilizing antibodies as either reagents *in vitro* or therapies *in vivo*. The data presented here indicates that both CPXV 014 and anti-CD16/32 interfere with the binding of stimulating anti-CD3 antibody to FcR-bearing "accessory cells," depriving them of the cross-linking scaffold they normally provide.

Our studies made it clear to us that even plate-bound agonist antibody T cell activation models are highly susceptible to the presence of FcR-bearing accessory cells. Regardless of the specific mechanism of how the augmentation of antibody-driven T cell activation by FcR-bearing cells occurs, these results led us to conclude that

CPXV 014 is not acting directly on T cells, and that the inhibitory effect we witnessed in our initial experiments was simply a side effect stemming from its ability to block antibody binding to neighboring FcR and the augmentation of T cell activation by these accessory cells.

IV. CPXV 014 as a Secreted FcγRIIB Agonist

This conclusion leads to two questions. Firstly, if CPXV 014 is not directly inhibiting T cells, what is the immunomodulatory role of this protein in the context of CPXV pathogenesis? Secondly, how could we explain the increased priming of T cells *in vivo* when mice are infected with the Δ014 mutant compared with the WT virus? Since FcγRIIB (CD32B) is the only FcR known to be expressed on B cells, and since adding a B cell line into the enriched $CD8^+$ T cell assay led to T cell activation levels comparable to those seen in mixed splenocyte cultures, we decided to focus on this receptor as a potential target for CPXV 014 activity. Perhaps CPXV 014 is capable of signaling through FcγRIIB instead of simply blocking it? If so, this could represent a novel tactic of viral immunomodulation.

IV A. FcγRIIB background

FcγRIIB is an important cell surface receptor for feedback inhibition of B cell activation. When FcγRIIB is colligated along with the BCR (by immune complexes for example) it limits the level of the B cell response [104, 105, 123-125, 259]. It does this by recruiting SHIP-1 phosphatase which counteracts the BCR-induced protein tyrosine signaling cascade [112-114] **(Figure 2)**. FcγRIIB is considered to be a "low affinity" FcγR

in that is does not bind monomeric IgG, but rather functions by binding with avidity to immune complexes. Signaling through FcγRIIB has been demonstrated to be an important source of feedback inhibition for B cells [123-125]. Furthermore, signaling thorough this receptor has been shown to inhibit maturation and cross presentation of DCs stimulated by immune complexes [112, 128].

There are not many examples in biomedical literature showing this inhibitory pathway to be targeted by pathogens, but one notable exception is by measles virus nucleoprotein (NP). Ravanel *et al* demonstrated that recombinant measles NP was able to compete with anti-CD16/32 for binding to FcγRIIB bearing B cells, but not a FcγRIIB KO cell line. Furthermore, the measles NP was able to significantly reduce Ig production by activated B cells [277]. This indicates that the FcγRIIB receptor is a valid target for viral immune evasion.

IV B. CPXV 014 and B cell activation

In order to get at the question of whether CPXV 014 can signal through FcγRIIB, we tested if the recombinant protein can inhibit the proliferative response of murine splenic B cells to anti-IgM $F(ab')_2$ in the presence of IL-2. Learning from earlier lessons, we opted to use $F(ab')_2$ antibody fragments (generated by pepsin digestion) **(Figure 2)** to stimulate the cells in order to avoid confusion that could result from the Fc portion of whole agonist antibody binding to FcγRIIB on the B cells and delivering its own inhibitory signal. We found that CPXV 014 strongly inhibited B cell proliferation in this model while the anti-CD16/32 did not. These results support the hypothesis that CPXV 014 signals though FcγRIIB while anti-CD16/32 merely blocks it.

IV C. Potential roles of CPXV 014 and FcγRIIB agonism for CPXV

As of the time of writing, our working hypothesis is that CPXV 014 is acting as a specific agonist for FcγRIIB in addition to its already documented chemokine-binding abilities. There are many ways in which such an immune evasion molecule could work to the advantage of the virus.

As discussed in the introduction, a strong antibody response is critical for preventing infection by CPXV and other OPXV. An FcγRIIB agonist could serve to limit the humoral response to CPXV and limit virus neutralization.

Another conceivable way in which an FcγRIIB agonist could help CPXV is if it can limit maturation and cross presentation by dendritic cells that would normally be activated by immune complexes, as this receptor has been reported to have this effect [112, 128]. FcγRIIB is also expressed by macrophages and neutrophils, and CPXV 014 may be found to limit the activation of these cells. In these ways, we can envision how a viral FcγRIIB agonist could limit both the innate and adaptive immune responses to CPXV.

V. Explaining CPXV 014 Effects on T Cell Priming *in vivo*

Concurrent studies performed by ours and other laboratories tested the differences between WT and Δ014 CPXV during *in vivo* mouse infection. It was found that T cell priming was inhibited by WT but not the mutant virus in two different infection models.

During these experiments, we naturally questioned how CPXV 014 inhibits T cell activation. Literature searches combined with conversations with other poxvirus researchers revealed that CPXV 014 has been examined as a member of a family of secreted scavengers of chemokines. This family of poxviral proteins possess the SECRET domain which enables them to bind and sequester a wide range of mouse and human chemokines [170]. Although disruption of immune cell chemotaxis and trafficking has been demonstrated to affect the priming of $CD8^+$ T cells [225], it seems unlikely that chemokine sequestration could have an effect during *in vitro* T cell activation models where the cells are concentrated in a single well of a 96 well plate. During *in vivo* CPXV infection studies with the wild type and CPXV 014 disruption mutant virus, we have observed that CPXV is inhibiting T cell priming, and that inactivating CPXV 014 helps reverse this effect. Initially, when we conducted these studies we interpreted the results to mean that CPXV 014 was directly inhibiting T cells. Now that we know that CPXV 014 is not actually acting directly on T cells, it begs the question of how this effect on T cell priming might be mediated.

One explanation is suggested by our initial results showing inhibition of B cell proliferation. CPXV 014 could act to suppress B cell activation, leading to a blunted antibody response. The diminished antibody response could then lead to fewer immune complexes and less uptake of antigen and presentation by APCs, leading to a reduction in T cell priming.

Another explanation could be that CPXV 014 chemokine-scavenging properties are able to disrupt normal APC and lymphocyte trafficking such that the T cells no longer are able to efficiently encounter their cognate antigens. There is evidence in the

literature indicating that interfering with the generation of certain chemokine gradients can lead to a defect in T cell priming [225]. However, this explanation seems unlikely as CPXV contains numerous other SECRET domain / chemokine scavenging proteins **(Figure 4)**. The existing redundancy makes it seem improbable that disrupting the chemokine binding activity of CPXV 014 would have such a noticeable effect on T cell priming *in vivo.*

It has been shown that signaling through the inhibitory FcγRIIB on dendritic cells functions to inhibit their maturation and cross presentation [112, 128]. It seems reasonable to hypothesize that CPXV 014 is functioning as a high-affinity FcγRIIB agonist, and that binding to DCs during *in vivo* CPXV infection could be serving to counteract their activation and cross-presentation pathways, which in turn would lead to less efficient T cell priming.

VI. Potential Applications & Significance

We began these studies in a search for T cell inhibitors that could potentially lead to new therapies for T cell-mediated autoimmune disorders such as multiple sclerosis or graft vs. host disease. Our discovery that CPXV 014 may be operating as a specific FcγRIIB agonist means that this protein could instead potentially lead to therapies for B cell mediated autoimmune disorders, such as rheumatoid arthritis or systemic lupus erythrotosis. We also predict that studying the effects of this putative FcγRIIB agonist in various *in vitro* and *in vivo* assays will shed light on the role of this receptor in the immune response to viral infection.

Chapter Eight: Future Directions

There still is much work to do on this project in order to properly define the activities of CPXV 014 and its role in viral pathogenesis. However, the data presented within this dissertation strongly supports our working hypothesis that CPXV 014, instead of directly inhibiting naive T cell activation, may actually exert immunosuppressive effects by signaling through the inhibitory FcγRIIB receptor. The following planned experiments we intend to perform should validate or refute that hypothesis.

Our experience with the antibody-driven T cell assays has demonstrated the importance of testing for lymphocyte inhibition in multiple models. To this end we intend to examine B cell stimuli and readouts for activation other than the antibody-mediated BCR ligation and proliferation studies performed thus far. Although the *in vitro* B cell proliferative assays shown here use $F(ab')_2$ anti-mIgM fragments and should not be susceptible to the same artefactual issues encountered with the T cell assays this project began with, the inhibitory capability of CPXV 014 should be tested in other models.

Both LPS and 8-mercaptoguanosine are mitogens that have been used to stimulate B cells. If CPXV 014 is indeed acting as an FcγRIIB agonist, however, we would not expect it to inhibit in these models as the SHIP-1 phosphatase recruited by the receptor likely does not play into these pathways. A better model of B cell activation would utilize transgenic mice whose BCR is specific to a particular antigen, similar to the OT-I and SIINFEKL model used for CD8+ T cells, and to see if CPXV 014 still inhibits B cells stimulated in this manner. Unfortunately, the development of such an

analogous mouse model has yet to be achieved as the recombinant BCR are not expressed on the surface of B cells *in vivo* [278]. Another model to be tried could be to isolate memory B cells from immunized mice and to stimulate them with specific antigen in the presence or absence of CPXV 014. In addition to proliferation as a readout for B cell activation, antibody secretion can be measured by ELISA from the supernatants of cultured B cells. Looking for specific B cell maturation markers via flow cytometry is another potential readout for B cell activation. We further intend to look at B cell FcγRIIB signaling in the presence or absence of CPXV 014 by blotting for specific pathway members such as phospho-SHIP-1 or NFκB activation status. These experiments will be initially attempted using the A20 cell line since we possess the FcγRIIB KO version of these cells (A20 IIA1.6).

As mentioned before, it has been demonstrated that FcγRIIB signaling can inhibit the activation of dendritic cells which have been stimulated by immune complexes [112, 128], using flow cytometry for specific maturation markers (CD40, CD80, MHC Class II) as the readout. We have performed some preliminary experiments using the DC2.4 cell line and have not yet seen any evidence of inhibition by CPXV 014 when these cells were stimulated by LPS (data not shown), but some technical hurdles with their stimulation by immune complexes need to be overcome before any conclusions can be reached. We also intend to look into developing assays for examining the role of CPXV014 ligation of FcγRIIB on primary macrophages and monocytes.

We will attempt to create a non-FcR-binding mutant version of the CPXV014-His recombinant protein. The Fremont lab's work on ECTV CrmD CTD has enabled us to identify the homologous residues of CPXV 014 suspected to be involved in FcR binding.

We will use the same strategy that they used by making a mutant version with charge-neutralization or charge-reversal mutations in these residues. This will enable us to confirm or disprove that the immune effects of CPXV 014 are due to the FcR binding activity, as well as providing us with another control construct for future assays. Furthermore, the protein FGL-2 has been shown to act on FcγRIIB receptors on lymphocytes [279], and if we can obtain the recombinant version of this protein it could potentially be used as a positive control.

Our lab is currently developing models to test the effects of CPXV 014 on B cell activation *in vivo.* We hope to infect mice with either WT or Δ14 CPXV and perform anti-CPXV antibody titers to look for differences. Also, we hope to use proliferative stains such as BrdU to assay for B cell division *in vivo*. Furthermore, we are currently breeding FcγRIIB KO mice to use for both *in vivo* and *ex vivo* studies of CPXV 014 inhibition. If CPXV 014 really is an agonist of this receptor, then any differences between the WT and Δ14 CPXV should be abrogated in these mice.

Finally, we would like to test if CPXV 014 is able to inhibit the activation of human B cells and determine if its activity is solely restricted to mouse B cells.

We anticipate that by conducting these studies we will elucidate the mechanism and significance of CPXV 014 for the virus.

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