VACCINIA VIRUS BINDING AND INFECTION OF PRIMARY HUMAN B CELLS

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DEDICATION

I would like to dedicate this work my mother for her ceaseless support, endless love, and true definition and image of perseverance against all the odds. Additionally, my family for their help in keeping me resilient through the toughest of times. Also, to the professor and lab, Randall Roper and the Roper lab, that revealed the delights of research and lead me down this path.

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Vaccinia virus (VACV), the prototypical poxvirus, was used to eradicate smallpox worldwide and, in recent years, has received considerable attention as a vector for the development of vaccines against infectious diseases and oncolytic virus therapy. Studies have demonstrated that VACV exhibits an extremely strong bias for binding to and infection of primary human antigen-presenting cells (APCs) including monocytes, macrophages, and dendritic cells. However, very few studies have evaluated VACV binding to and infection of primary human B cells, a main type of professional APC. In this study, we evaluated the susceptibility of primary human peripheral B cells at different developmental stages to VACV binding, infection, and replication. We found that VACV exhibited strong binding but little entry into *ex vivo* B cells. Phenotypic analysis of B cells revealed that plasmablasts were the only subset resistant to VACV binding. Infection studies showed that plasma and mature-naïve B cells were resistant to VACV infection, while memory B cells were preferentially infected. Additionally, VACV infection was increased in larger and proliferative B cells suggesting a bias of VACV infection towards specific stages of differentiation and proliferative ability. VACV infection in B cells was abortive, and cessation of VACV infection was determined to occur at the stage of late viral gene expression. Interestingly, B cell function, measured by cytokine production, was not affected within 24 hours post-infection. In contrast to *ex vivo* B cells,

stimulated B cells were permissive to productive VACV infection. These results demonstrate the value of B cells as a tool to aid in deciphering the intricacies of poxvirus infection in humans. Understanding VACV infection in primary human B cells at various stages of differentiation and maturation is important for the development of a safer smallpox vaccine and better vectors for vaccines against cancers and other infectious diseases.

Andy Qigui Yu, Ph.D., Chair

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Ab	Antibody
AD	Atopic dermatitis
AF	Alexa fluor
Ag	Antigen
ALVAC	A recombinant canarypoxvirus
APC	Allophycocyanin
APC(s)	Antigen presenting cell
Bax	Bcl-2-associated X protein
Bcl-2	B cell lymphoma 2
BCR	B cell receptor
B-LCL	B-lymphoblastoid cell lines
BV	Brilliant violet
Calcein AM	Calcein acetoxymethyl
CCR6	C-C chemokine receptor type 6
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CEV	Cell-associated enveloped virus
cGAS	Cyclic GMP-AMP synthase
ChIP-seq	Chromatin immunoprecipitation with DNA sequencing
CHO	Chinese hamster ovary
CTB	Cholera toxin subunit B
CpG	Cytosine-phosphate-guanine

CS	Chondroitin sulfate
CXCR4	C-X-C chemokine receptor type 4
CXCR5	C-X-C chemokine receptor type 5
Cy	Cyanine
DAPI	4’,6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DRM	Detergent-resistant membrane
dsRNA	Double stranded ribonucleic acid
EEV	Extracellular enveloped virus
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
eIF2α	Eukaryotic initiation factor 2 alpha
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EV	Eczema vaccinatum
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCM	Flow cytometry
FITC	Fluorescein isothyocyanate
G3BP	Ras-GTPase-activating protein SH3 domain-binding protein
GAG	Glycosaminoglycan
GC	Germinal center

GM1	Ganglioside M1
HIV-1	Human immunodeficiency virus 1
h	Hour(s)
hpi	Hours post-infection
HS	Heparan sulfate
Hsp90	Heat shock protein 90
IEV	Intracellular enveloped virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMV	Intracellular mature virus
IRF-3	Interferon regulatory factor 3
M phase	Mitotic phase
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MV	Mature virus
MVA	Modified vaccinia virus ankara
ODN	Oligodeoxynucleotides
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell(s)
PBS	Phosphate-buffered saline

PE	Phycoerythrin
PerCP	Peridinin chlorophyll
PFA	Paraformaldehyde
PFU	Plaque-forming unit
PHL	Primary human leukocyte(s)
PI	Propidium iodide
PKR	Protein kinase R
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute 1640 medium
RT-PCR	Reverse transcription polymerase chain reaction
S phase	Synthesis phase
STING	Stimulator of interferon genes
TLR	Toll-like receptors
V region	Variable region
VACV	Vaccinia virus
VARV	Variola virus
VIG	Vaccinia immune globulin
VITF	Vaccinia intermediate transcription factor
VLTF	Vaccinia late transcription factor
WR	Western Reserve
YFP	Yellow fluorescent protein
YY1	Yin yang 1

Chapter I - Introduction

Virus Tropism

In spite of the advancement in preventing and treating infectious diseases, there are many emerging and re-emerging pathogens, of which the majority are viruses. The multitude of emerging and re-emerging viruses include Filoviruses (Ebola and Marburg), Flaviviruses (hepatitis C, Dengue, and Zika), Coronaviruses (MERS-CoV), Bunyaviruses (hantavirus), Retroviruses (HIV-1), and Poxviruses (monkeypox) (1). Despite the advances in science, medicine, and technology, intervention to control viral diseases mostly depends on vaccines, novel medical practices, and antivirals. Antiviral drug development has progressed relatively slower when compared to that of antibiotics. Regardless of these advances, emerging and re-emerging pathogens remain as a primary concern for global healthcare (1). Emergence of a virus as a causative agent of human disease may occur by zoonosis, transfer of disease from another species to humans, which is limited by host tropism.

Viral tropism determines the permissiveness of a species to a virus and can be viewed and described from a microscopic to macroscopic level. Tropism is a tri-leveled barrier system involving cellular, tissue, and host specificities (2). All three levels of viral tropism are very interdependent; however, each level has unique features that interact to regulate the virus-host interactions thus regulating specificity. Host tropism, which is largely affected by cellular and tissue tropism, is organism specificity and defines whether the whole species supports either

abortive or permissive viral infections. Variola virus (VARV), the causative agent of smallpox, infection demonstrates an example of host tropism because of its human-specificity and lack of natural reservoir. Tropism is defined by the symptoms of disease, possible range of effects from viral pathogenesis, and the ability to infect other individuals. Certain hosts or organisms with no overt disease can be a reservoir and can still infect other individuals or species.

The second level of viral tropism is tissue specificity which influences organism tropism and is influenced by cellular tropism. This level of specificity is determined by tissue-specific antiviral responses and largely dependent on the patterns of virus distribution and dissemination within the host. It also affects the ability of the virus to spread between hosts (2). For example, hepatitis B virus infection is strictly species and tissue specific, as humans are the only natural hosts of hepatitis B virus infection, and the liver is the only tissue where viral replication takes place.

The final and microscopic level is cellular specificity. On a cellular level, virus replication for certain cell types of specific species can either be permissive or abortive (2). Permissive infection is represented by success in entering and replicating in the cell. Obligate intracellular pathogens, such as viruses and bacteria, require specific host factors on the cell surface and in the cytoplasm, to gain entry into a cell, replicate, and disseminate to other cells. Viruses must bind to their receptors on the target cell's surface to initiate or induce entry of the virus particle either by a form of endocytosis or membrane fusion, depending on the pH, virus, and other various factors. Abortive infections are often the result of

disruptions in virus replication. Interspecies and intercellular barriers, such as intracellular factors and disruptions in virus-receptor interactions, can contribute to non-productive viral infections. HIV-1 demonstrates an example of cellular tropism by exclusively entering and productively infecting cells expressing CD4 and CXCR4 or CCR5.

Poxvirus tropism

Poxviruses are a family of complex, large, enveloped DNA viruses with a wide range of species specificities. The brick-shaped sub-family Chordopoxvirinae specifically infects vertebrates and includes four genera that contain viruses that can infect humans. These four genera are yatapoxvirus, molluscipoxvirus, parapoxvirus, and orthopoxviridae. Orthopoxviruses are mammal specific, morphologically indistinguishable, replicate exclusively in the cytoplasm, and have approximately 250 genes. This genus includes Variola major, camelpox, monkeypox, cowpox, and vaccinia.

Variola virus, the causative agent of smallpox, was a tremendously deadly virus that killed hundreds of millions of people and is likely the deadliest pathogen in history (2). The first vaccine was discovered by Edward Jenner in the late 1700's and developed against VARV. The initial smallpox vaccine was live cowpox virus which provided cross protection against smallpox, among many of the Orthopoxviruses, due to the extreme sequence similarity and relatively slow rate of mutation of poxviruses (3, 4). Over the years, the smallpox vaccine was altered to increase the safety by now using a live, attenuated vaccinia virus

(VACV), the prototypical poxvirus. Attenuation of VACV provided a near certainty of developing an acquired immunity against smallpox with relatively benign symptoms following percutaneous inoculation and infection. The development of the attenuated VACV vaccine allowed for a global smallpox eradication campaign in 1967 which lead to the complete eradication of smallpox by 1979 (5).

While smallpox has been eradicated, various poxviruses are emerging as primary concerns for global healthcare, such as molluscum contagiosum, cowpox, and monkeypox (6-12). These emerging poxviruses are causing natural infections in several countries and can lead to mortality in certain cases of monkeypox (6-8, 12). Additionally, smallpox still remains a threat as a bioterrorism agent due to the unaccounted-for stocks of VARV in decommissioned labs and vast majority of the population lacking vaccination with VACV (13).

For poxviruses, failure or success at the various stages of the viral life cycle determines cellular tropism. Orthopoxviruses first bind to host receptors and can enter into a cell by one of two ways. Poxviruses enter by membrane fusion at the cell surface at a neutral pH, while entry at a low pH follows an endocytic pathway with virus fusion inside the vesicle membrane. The viral capsid then uncoats by degradation and early genes encoding factors necessary for viral DNA replication and immunomodulation are transcribed (Fig.1). VACV DNA replication occurs exclusively in a cytoplasmic endoplasmic reticulum (ER)-enclosed mini-nucleus (14). These mini-nuclei, also known as viral replication factories, are comprised of a section of the rough ER organized to allow for

assembly of viral particles. After viral DNA replication, two more waves, intermediate and late, of viral mRNA and protein synthesis occurs before subsequent morphogenesis of infectious particles. These infectious viral particles exit the viral replication factory as intracellular mature virions (IMV), one of two distinct infectious virus particle types, that are transported along microtubules (15). IMVs exist as single enveloped virions in the cytoplasm; however, some IMVs are transported to the Golgi apparatus where they are wrapped in a double membrane derived from the Golgi and referred to as triple-enveloped intracellular enveloped virus (IEV) (15). IEVs are then transported once more along microtubules to the surface to fuse with the plasma membrane to release cell-associated enveloped virus (CEV) from the cell. CEVs, double membraned virions, either stay associated with the cell surface or are released as the other type of infectious virus particle known as extracellular enveloped virus (EEV). CEVs are released by various mechanisms, and commonly launched from the surface to neighboring cells via intracellular actin polymerization. Additionally, IMVs can bypass the IEV form and directly bud from the cell to form EEVs (16-18).

On a cellular level, several restriction events involving regulatory factors that control key intracellular steps have been discovered to control poxvirus infection. Four main regulating events are signal transduction, cell lineage and differentiation state, cell cycle control, and complementing host factors. Signal transduction focuses on the various signaling pathways involved in coordinating the cellular response to viral infection (2). The antiviral responses are one of the

most studied examples of this event, and the interferon (IFN) response is key to controlling poxvirus infections. Poxviruses employ several strategies to inhibit the IFN response, which include receptor mimics, phosphatases to block signal transduction, and inhibitors of IFN induction and mediators (2). Infection by some poxviruses has been shown to be dependent on the differentiation state of the host cell potentially due to the cellular factors required for viral replication (2). Although poxviruses are less S-phase-dependent than most other viruses, they secrete homologues to growth factors responsible for stimulating mitosis to control the cell cycle (2). The final event regulating poxvirus replication is the necessity for hijacking trans-acting factors to complete viral replication (2). One example is molecule chaperone Hsp90 that regulates viral replication (19). Failure of poxviruses to employ these strategies leads to the antiviral response aborting or halting the infection.

Another key factor in poxvirus tropism is the viral host-range genes, which is important despite highly similar genomes between poxviruses. These host-range genes, divergent between the poxviruses, are essential for allowing poxviruses to specifically infect and thrive in certain species, thus leading to restriction in host tropism. Myxoma virus causes lethal disease specifically in rabbits only (20), while VARV expresses host-range genes leading to strict human-specific infection (2). The first host-range genes, K1L and C7L, were identified in VACV (21, 22). The inability of VACV to infect Chinese hamster ovary cells has been shown to be rescued by cowpox virus gene CP77, which compensated for K1L and C7L in a restrictive host cell (23). While many host-

range genes have been discovered, K3L and E3L are the best characterized genes in VACV. The genes prevent PKR from activating the IFN response and inducing apoptosis. Poxvirus infections, among other viral infections, produce double-stranded RNA (dsRNA) that is detected by the host cell leading to the initiation of innate immunity. PKR is responsible for detecting dsRNA in humans. VACV has evolved to utilize two strategies to circumvent detection of dsRNA by PKR. One strategy, involving K3L, is to encode a decoy PKR or pseudo-substrate to avoid discovery and inhibit PKR by mimicking eIF2α (24). The other strategy is to disguise the RNA with E3L by binding to dsRNA and sequestering it from PKR and other pattern recognition receptors to prevent activation (24). Reduced rates of infectivity have been noted when K3L and E3L were deleted in VACV (24).

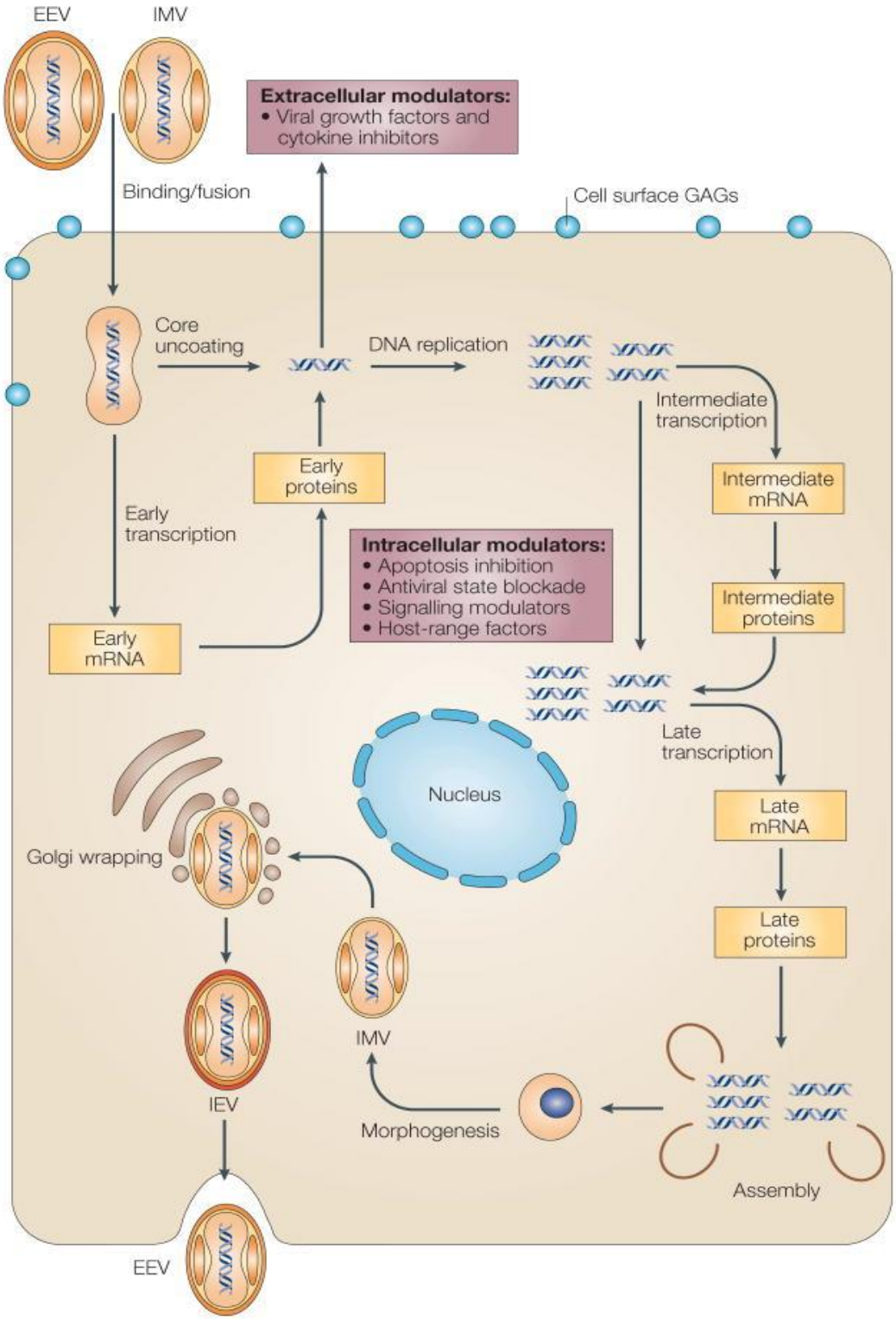

Figure 1. VACV life cycle. Overview of the VACV progression of infection from

McFadden G. 2005. Poxvirus tropism. Nat Rev Microbiol **3:**201-213.

Poxvirus binding and entry

Virus binding often begins with largely non-specific and electrostatic interactions between viral surface components and host cell surface factors such as glycolipids and glycoprotein attachment factors. These initial interactions provide an initial attachment to the host cell which allows the virus to subsequently recruit specific receptors to drive the entry reactions. After binding to their receptors, viruses need to induce entry of viral particles by either a form of endocytosis or membrane fusion. Receptors can either efficiently target the viruses for endocytosis or be used to activate specific signaling pathways to facilitate entry or drive fusion/penetration events. These signaling pathways can also be used to elicit fusion/penetration not only at the host cell surface level but also be involved in altering the endocytic compartments by inducing conformational changes in virus surface structures. This pH-independent route involves capsid degradation to uncoat the virus. The virus capsid degrades once the virus has entered the host cell cytoplasm in the presence of specific intracellular host factors. The use of specific host cell surface components with restricted expression patterns is one form of viral tropism. Membrane fusion often occurs in a pH-dependent manner via the induction of lower pH environments as with virus containing endosomes which are endocytosed.

Although advances in understanding poxvirus cellular tropism has occurred using cell lines, no specific cellular receptor for any poxvirus had yet been identified for cell lines or primary cells. Several ubiquitous carbohydrate-based molecules have been suggested as receptors for VACV; however, these

molecules have been recently shown to be involved in the first interactions needed between virus and host cell to drive receptor binding. The first of these discovered were heparin sulfate (HS) and chondroitin sulfate (CS), which are highly negatively charged, complex, unbranched polysaccharides called glycosaminoglycans (GAGs) that are associated with numerous membrane proteins (25). Two VACV envelope proteins, A27 and H3, have been reported to bind HS and VACV D8 protein binds to CS (26-29). An additional VACV protein, A26, has been shown to bind to extracellular matrix laminins, which are complexes of glycoproteins with multiple functional isotypes (30). Soluble HS as well as soluble A27, H3, and D8 are able to reduce VACV attachment and infection suggesting that GAGs play an important role in virus infection at the attachment and binding stage. Additionally, GAG deficit cells are more resistant to VACV binding (31). Thus, GAGs are important to VACV binding, providing the initial interaction between host cell and VACV, but are not essential to VACV infection. In addition to the GAG-dependent VACV binding, GAG-independent binding has been noted in GAG-deficient cells and was pinpointed to the involvement of L1. A soluble, truncated L1 was shown to bind to GAG-deficient cells and lead to inhibition of binding and entry (32). Although VACV has redundancies for most processes including binding and entry, VACV has a strictly sequential, temporal viral gene expression process.

Poxvirus Gene Expression

Among the various natural occurring viruses, viral gene expression can vary between a simpler system of regulation to a more complex form of regulation. Viral transcription is usually controlled by interactions between pre-existing cellular transcription factors and virally encoded transcription factors and regulatory proteins. These interactions can in part be regulated by the cellular factors present in certain cell types and can drive the outcome of infection in the host cell (33). Importantly, these virus-host transcriptional regulatory interactions are fundamental to the permissivity of infection. Simple viral gene expression, often seen in smaller viruses such as polyomaviruses and papillomaviruses, tends to occur in two defined stages with the necessity of early gene expression for subsequent late gene expression (33). Additionally, simple viral gene expression is usually regulated by the host cell type due to greater dependence on host factors for replication (33). Larger viruses, like herpesviruses and adenoviruses, tend to have more complex gene expression with additional temporal phases compared to smaller viruses. These viruses are also affected by the cell type and supplementary regulatory protein encoded genes (33).

Since poxviruses replicate in the cytoplasm compared to most viruses that replicate in the nucleus, poxviruses encode most of their own transcription and replication factors to regulate viral gene expression. VACV contains approximately 250 genes, which are involved in regulating gene expression or regulated by viral gene expression, that can be classified temporally as early, intermediate, or late (34). However, these genes are not exclusively expressed

during these times, i.e. intermediate genes can also be expressed during the late gene phase and thus having a dual promoter (35). Transcription in VACV, as well as other Orthopoxviruses, is mediated by a multi-subunit DNA-dependent RNA polymerase along with temporal stage-specific transcription factors that recognize affiliated promoters (36).

Poxvirus early genes encode proteins involved in intermediate gene expression (37), DNA replication (38), and nucleotide biosynthesis (39). Host defense evasion genes are also included in the early genes (40, 41). These genes are transcribed with the assistance of RNA synthesis machinery packaged within the core of new infectious virus particles (42-44). Early transcription occurs within minutes of infection, before the viral core loses considerable structural integrity (45, 46). This data suggests that VACV early transcription involves no host factors and is exclusively performed by viral proteins (47). Intermediate and late viral genes are transcribed following DNA replication and occurs in the cytoplasm outside of the viral core. Since intermediate and late gene expression occur in the cytoplasm, specific cellular factors, such as G3BP, p137, TATA-binding protein, and YY1, have been reported to participate in intermediate and late gene expression (48-52). Genes of the intermediate stage encode transcription factors for late gene expression. Intermediate genes G8R, A1L, and A2L encode proteins required for late gene transcription (53-55). Finally, late genes encode structural proteins and necessary proteins for transcription of early genes. Viral gene expression has been shown to play an important role in abortive poxvirus infections (56).

Abortive poxvirus infection in primary human leukocytes

Poxviruses infect a wide range of organs, tissues, and cell types; however, the infection can be abortive, the termination of infection due to various factors, or productive, the generation of new progeny. VACV is among the most promiscuous poxviruses, as it is able to infect the majority of cell lines. Some of these cell lines are infected, but result in abortive infection, such as Chinese hamster ovary (CHO) cells and certain B lymphocyte cell lines. The abortive VACV infection in CHO cells was shown to occur at the stage of intermediate viral protein synthesis (57). B lymphocyte cell lines demonstrate differences in permissivity of VACV infection depending on the stage of differentiation (58, 59). Although VACV displays promiscuous infection in cell lines, infection in primary human cells has been shown to be a different case (56, 60, 61). Monocytes and B cells are able to be infected by VACV, but resting T cells display resistance to VACV infection (61). Upon activation of T cells, T cells exhibit a loss of resistance and a gain of susceptibility to VACV infection and permissive infection (56). Additionally, dendritic cells and macrophages are susceptible to VACV infection. Antigen presenting cells (APCs) including monocytes, dendritic cells, and B cells are preferentially infected, but the majority result in abortive infection (56, 62). Infection in primary monocytes and B cells displayed abortive infection during the viral gene expression stage (56). While VACV infection has been thoroughly described in most primary APCs, the pathway for B cell infection by VACV remains less clear.

VACV infection in B lymphocyte cell lines and primary B cells

Few studies have focused on VACV infection in B cells, whether it be in cell lines or primary human B cells. The majority of B cell-VACV interaction studies have been completed using either peripheral blood mononuclear cells (PBMCs) or various B lymphocyte cell lines. VACV infection studies in PBMCs have found that B cells display strong VACV binding but minimal infection (60, 61), and the infected cells exhibit abortive infection (56). However, these studies mainly focus on monocytes and monocytic cell types and have not studied VACV infection in B cells in any depth. In addition to PBMCs, VACV infection has been studied in B lymphocyte cell lines to not only examine the abortive infection but also the B cell function of antigen presentation. When the immature B lymphocyte line WEHI-231 was infected, VACV infection was abortive and induced apoptosis by downregulating Bcl-2 which protects from cell death (59). In contrast, a B lymphoblastoid cell line (B-LCL) displayed a productive VACV infection (58). When the effect of VACV infection on B cells was assessed, the same B lymphocyte cell line displayed no changes in expression levels of MHC class II, but MHC class II-mediated antigen presentation was dramatically disrupted by the alteration of the peptide association (63). In addition, this same ability to modulate the immune response to the infection in B cells was demonstrated in individuals naturally infected with VACV (64). Infected individuals revealed reduced levels of B cells when compared to uninfected individuals (64). Additionally, these B cells in infected individuals were less activated when CD80 and CD86 expression was examined (64).

B cell function and development

B cells, bursal or bone marrow-derived cells, were discovered and characterized the 1960s and 1970s around the time that T cells were discovered (65). The discovery of B cells began with discovering and identifying the immunoglobulin (Ig) protein, leading to the identification of antibody-producing cells (66). The remaining B cell subsets of the highly heterogeneous B cell population were discovered and identified after these pivotal discoveries. In 1980, the first B cell-specific characterizing molecule, CD20, was described (67). Since the identification of immunoglobulins or antibodies being produced by antibody-producing B cells, several additional functions of B cells have been revealed. Antigen (Ag) processing and presentation to T cells is another crucial function of B cells. Ag presentation in B cells occurs when Ag is bound and internalized by the B cell receptor (BCR), processed, and presented on MHC (68-70). B regulatory cells have described and regulate T cell-mediated inflammatory responses by producing anti-inflammatory cytokines (71, 72). Additionally, cytokine production to influence T cell differentiation and providing T cell costimulation are other functions of B cells. In addition to having a variety of functions, B cells display a range of cell surface phenotypes and locations.

B cell development begins in the bone marrow from common lymphocyte progenitors (Fig. 2). Common lymphocyte progenitors differentiate into pro-B cells and progress from pro- to pre-B cells by Ig heavy chain rearrangement (73, 74). After positive selection for appropriate heavy chain rearrangement and light chain rearrangement, pre-B cells differentiate into immature B cells expressing

IgM that undergo negative selection to eliminate self-reactive cells (74, 75). Immature B cells then migrate from the bone marrow to the peripheral lymphoid tissue and gain expression of IgD to mature into transitional B cells (76). Transitional B cells undergo maturation into mature-naïve B cells migrating to lymphoid follicles before becoming activated by encountering Ag. These activated follicular B cells can then become germinal center (GC) B cells after receiving T cell help. GC B cells proliferate and class-switch the BCR constant region from IgM and IgD to IgM, IgG, IgA, or IgE (77). Moreover, the V region gene can undergo somatic hypermutation to change antibody (Ab) or BCR affinity (78, 79). These GC B cells can either become memory B cells or plasma cells. Long-lived plasma cells start as plasmablasts, while short-lived plasma cells can arise directly from activated B cells (80-82).

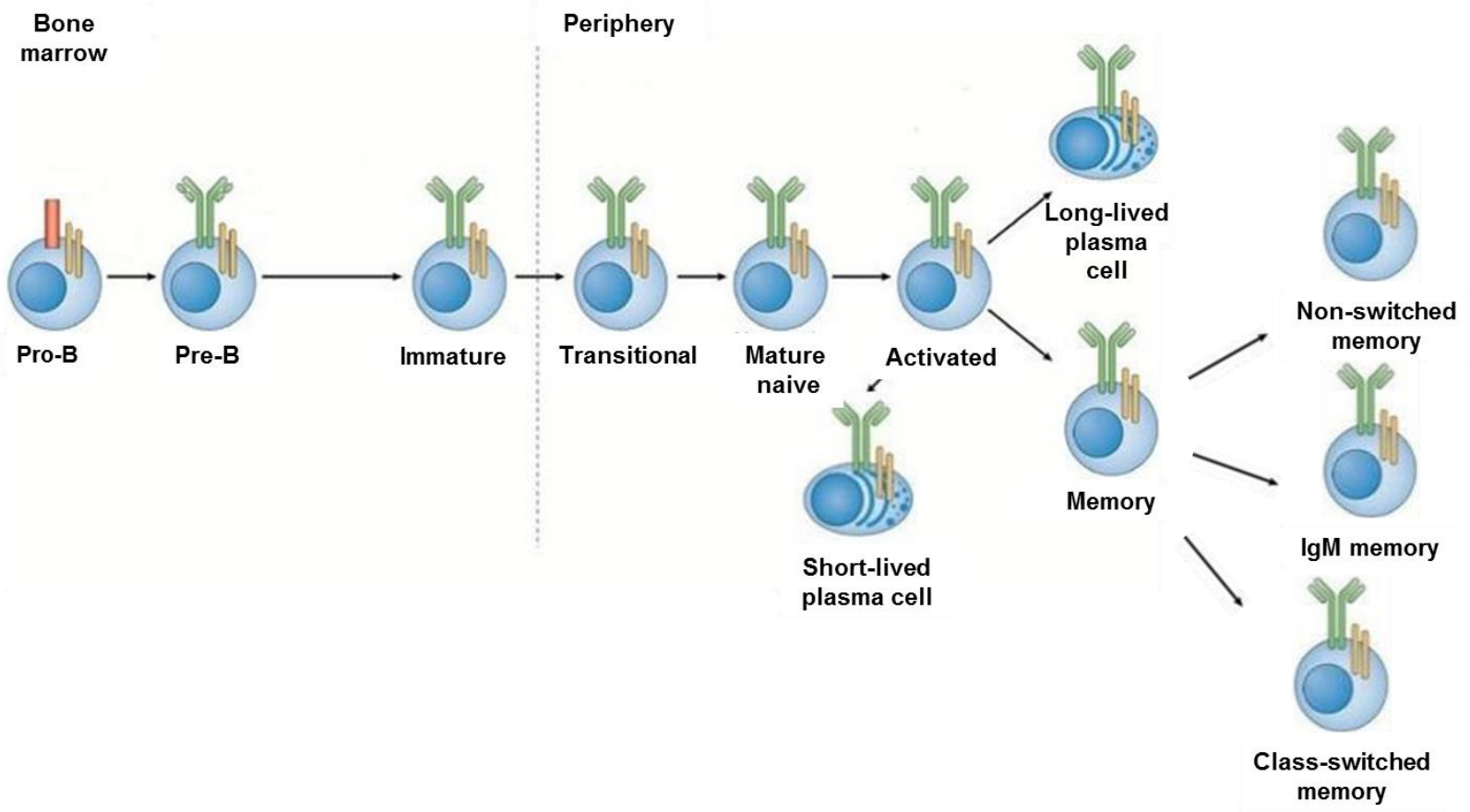

Figure 2. B cell development. Overview of B cell development adapted from Cambier JC, Gauld SB, Merrell KT, Vilen BJ. 2007. B-cell anergy: from transgenic models to naturally occurring anergic B cells? Nature Reviews Immunology **7:**633.

B cells and the smallpox vaccination

Memory B cells and long-lived plasma cells are responsible for Ab production and release contributing to long-term immunity, following pathogen exposure or vaccination. A rapid recall Ab response is driven by memory B cells upon re-exposure to an Ag, and these cells aid in replenishing the pool of long-lived plasma cells (83, 84). Meanwhile, long-lived plasma cells provide continuous serum Ab level maintenance (83, 85). The smallpox vaccination has been shown to generate a strong neutralizing Ab response that declines within the first 3-5 years, and the CDC recommends revaccination every 3 to 10 years, depending on exposure to specific poxviruses, to provide long-term protection (86, 87). However, studies have revealed long-term B cell memory to the smallpox vaccine (86, 88). Additionally, one study discovered vaccinia-specific IgG and neutralizing Abs at smallpox protective levels in individuals vaccinated more than 88 years earlier (89). These same memory cells are concentrated in the spleen (88).

While the generation of a long-term humoral response was remarkable for the smallpox vaccination, the eradication of smallpox and multiple adverse reactions lead to the termination of vaccine distribution to the public. Flu-like symptoms, generalized vaccinia, and myopericarditis were among the mild or moderate adverse reactions (90). The rare but potentially life threatening reactions include postvaccinal encephalitis, progressive vaccinia, and eczema vaccinatum (90). Eczema vaccinatum is a serious local or disseminated rash caused by widespread infection of the skin in individuals with eczema or atopic

dermatitis post-vaccination. Due to this potential reaction, individuals with eczema or atopic dermatitis are suggested to forego VACV vaccination. Additionally, 18 to 30 million individuals have eczema in the United States (91). Among other alterations in the immune system, these individuals have significantly higher frequencies of transitional B and chronically activated memory B cells (92).

Summary of findings

Here, I investigated VACV binding to and infection of primary human B cells at various stages of cell maturation and differentiation in peripheral blood. I found that plasmablasts were resistant to VACV binding, while B cells in other maturation stages exhibited no defects in VACV binding. Plasma and mature naïve B cells were resistant to VACV infection, while memory B cells were susceptible to VACV infection. VACV infection in *ex vivo* B cells was aborted at the late stage of viral gene expression. Additionally, VACV binding and infection was increased in larger and proliferative B cells suggesting a bias of VACV infection towards either specific stages of differentiation and proliferative ability or cell surface area. In contrast to *ex vivo* B cells, stimulated B cells were permissive to productive VACV infection. Additionally, this work highlights the potential of B cells as natural cellular models to identify VACV receptors or dissect the molecular mechanisms underlying key steps of the VACV life cycle such as binding, penetration, entry, and replication in primary human cells. The understanding of VACV biology in human primary cells is essential for the

development of a safe and effective live-virus vector for oncolytic virus therapy and vaccines against smallpox, other infectious diseases, and cancer.

Chapter II - Materials and Methods

Antibodies and flow cytometry (FCM)

Table 1 shows anti-human monoclonal antibodies conjugated with fluorochromes that were purchased from BD Biosciences (San Jose, CA) or Biolegend (San Diego, CA). These panels of fluorochrome-conjugated antibodies were used for cell surface staining of isolated B cells to analyze the B cell phenotypes noted in Table 2. Some of these fluorochrome-conjugated antibodies such as $CD69^{FITC}$, $CD83^{PE}$, $CD80^{PE\text{-}Cy7}$, and $CD86^{BV510}$ were also used for cell surface staining to evaluate B cell activation. All four panels include CD20, CD38, CD27, and IgD to allow for rudimentary phenotyping of B cell subsets. Panel 1 explores chemokine receptor expression, and panel 2 examines activation as well as B cell maturation. Panel 3 includes markers to further define memory B cells and their activation state. Plasma cells are the focus of panel 4. Additional anti-human monoclonal antibodies conjugated with fluorochromes were purchased from BD Biosciences (San Jose, CA): anti-$CD69^{FITC}$, anti-$CD83^{PE}$, and matched-isotype control antibodies conjugated with FITC and PE. Anti-human CD80 and CD86 antibodies were purchased from Biolegend (San Diego, CA). Unstimulated and stimulated B cells were subjected to surface staining with different combinations of antibodies focused on B cell phenotypic markers and/or activation markers. Proliferation was assessed by Ki-67 (BD Biosciences, San Jose, CA) intracellular staining with cold 70% ethanol used to fix and permeabilize the cells. Appropriate isotype controls were used at the same

protein concentration as the test antibodies for control staining, which was performed during every flow cytometric analysis. After cell surface staining, B cells were subjected to fixation by 2% paraformaldehyde (PFA) and followed by flow cytometric analysis using the BD FACSCalibur (BD Biosciences, San Diego, CA) or BD LSRFortessa (BD Bioscience, San Diego, CA). The data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

B cell viability and functionality were detected using calcein AM (Invitrogen, Carlsbad, CA). Calcein AM is a non-fluorescent, hydrophobic compound readily absorbed by cells. Upon hydrolysis of the compound by endogenous intracellular esterases, calcein AM becomes calcein, a hydrophilic fluorescent compound, and remains in the cytoplasm of cells with intact membranes. Briefly, B cells were labeled at 37°C for 30 min with 1 μM calcein AM. The cells were washed three times with PBS to remove free calcein AM and then resuspended with 2% FBS in PBS to run FCM. The control sample was prepared by mixing live with dead cells, generated by treating an aliquot of cells with 70% methanol for 30 minutes before washing thoroughly with PBS.

Propidium iodide (PI) was used for cell cycle analysis. For this assay, isolated B cells were fixed in ice cold 70% ethanol in a dropwise fashion to ensure complete fixation for 30 min at 4°C. Cells were then washed and treated with RNase H (Invitrogen, Carlsbad, CA) to prevent PI binding to RNA. After RNase treatment, cells were stained with 200ul of 50ug/ml PI (BD Pharmingen, San Jose, CA). B cells were resuspended with 2% FBS in PBS to run FCM using the BD Accuri C6 Plus (BD, Biosciences, San Diego, CA).

Table 1. B cell phenotyping antibody panels.

Panel 1	Panel 2	Panel 3	Panel 4
CCR7^{PE}	CD23^{PE}	CD86^{PE}	BCMA^{PE}
PI	PI	PI	PI
HLA-DR$^{PerCP-Cy5.5}$	CD69$^{PerCP-Cy5.5}$	IgM$^{PerCP-Cy5.5}$	CD10$^{PerCP-Cy5.5}$
CXCR4^{PE-Cy7}	CD40^{PE-Cy7}	CD80^{PE-Cy7}	CD138^{PE-Cy7}
CXCR5^{AF647}	CD21^{AF647}	CD226^{AF647}	TACI^{AF647}
IgD^{AF700}	IgD^{AF700}	IgD^{AF700}	IgD^{AF700}
CD20$^{APC-Cy7}$	CD20$^{APC-Cy7}$	CD20$^{APC-Cy7}$	CD20$^{APC-Cy7}$
CD38^{BV421}	CD38^{BV421}	CD38^{BV421}	CD38^{BV421}
CD27^{BV510}	CD27^{BV510}	CD27^{BV510}	CD27^{BV510}
CCR6^{BV605}	CD25^{BV605}	CD45RA^{BV605}	CD24^{BV605}

Four panels of antibodies used for examining B cell phenotypes in uninfected and VACV-infected B cells.

Table 2. Phenotypes of primary peripheral B cell subsets.

B cell subset	Phenotypic markers	Reference
transitional	$CD19^{+}CD38^{+}CD24^{+}$	Morbach *et al*
mature naïve	$CD19^{+}IgD^{+}CD27^{-}CD21^{+}$	Morbach *et al;* Kaminski *et al*
memory	$CD19^{+}CD27^{+}$	Morbach *et al*
non-switched memory	$CD19^{+}CD27^{+}IgD^{+}IgM^{+}$	Morbach *et al*
IgM only memory	$CD19^{+}CD27^{+}IgD^{-}IgM^{+}$	Morbach *et al;* Kaminski *et al*
class-switched memory	$CD19^{+}CD27^{+}IgD^{-}IgM^{-}$	Morbach *et al;* Kaminski *et al*
plasmablasts	$CD19^{+}CD20^{lo}CD138^{-}CD38^{+}CD27^{+}$	Morbach *et al*
plasma cells	$CD19^{+}CD20^{+}CD138^{+}$	Morbach *et al;* Kaminski *et al*

Cell surface markers used to define the specific B cell subsets.

VACV production, titration, and infection protocols

The primary VACV strain used in this study was Western Reserve (WR). The EGFP reporter virus, denoted as VV-EGFP hereinafter, is a WR strain containing a chimeric gene including the influenza virus nucleoprotein, the ovalbumin SIINFEKL peptide, and enhanced green fluorescence protein (EGFP) regulated by the P7.5 early/late promoter that localizes to the nucleus (93). Both VACV WR and VV-EGFP were obtained from Dr. Jonathan Yewdell (NIH, Bethesda, MD). vA5L-YFP, obtained from Dr. Bernard Moss (NIH, Bethesda, MD), is a recombinant WR VACV constructed with the viral core protein A5L fused to yellow fluorescence protein (YFP) suitable for visualizing individual virions (94). All viral stocks were generated and titrated in the monkey kidney cell line CV-1 (ATCC, Manassas, VA) in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin (complete RPMI-1640). After approximately 3 to 4 days of infection, cells were lysed in a dounce homogenizer. Culture supernatants and cell lysates were then subjected to ultracentrifugation at 25,000 g for 80 min through a 36% sucrose cushion, and pellets were resuspended and subjected to virus plaque assays to determine the viral titers. Briefly, CV-1 cells were grown in 12-well plates to over 90% confluency and overlaid with various dilutions of virus stock. After 2 h of incubation, cells were washed and overlaid with complete RPMI-1640 containing 1% carboxylmethylcellulose to prevent *de novo* plaque formation. After 3 days of culture, cells were washed and stained with a 0.01% crystal violet with 15%

ethanol solution and then washed to visualize plaques for counting to calculate virus plaque-forming units (PFU).

This study used the single-enveloped IMV particles of all VACV strains (vA5L-YFP, VV-EGFP, and WR) due to the abundance (>98%) and stability of the membrane even after freezer storage (15, 95). The double-enveloped forms of VACV, EEV and CEV, are difficult to maintain for long periods on time due to the deceased stability of the secondary membrane, not found on the IMV form.

For infections involving virus plaque assay analysis, stimulated or unstimulated primary B cells in 24-well plates (approximately 1 million cells per well) were incubated with VACV WR at a multiplicity of infection (MOI) of 2 for 1 h, washed three times with PBS, and cultured for 2 days in complete RPMI-1640. Cells and culture supernatants were harvested at various time points, and cells were lysed by three rounds of freezing and thawing, followed by cup horn sonication. Cell lysates and supernatants were either analyzed separately or mixed together for determination of virus titers.

For VACV binding assays, stimulated or unstimulated primary B cells were chilled on ice to 4°C and incubated with vA5L-YFP at a MOI of 0.5 on ice for 30 minutes with gentle mixing. Cells were washed three times with ice-cold PBS, fixed with 2% PFA, and YFP-positive cells were quantitated using FCM. VV-EGFP was used to monitor viral early gene expression in B cells as an indication of VACV infection. After either 12 or 24 h, cells were fixed with 2% PFA and EGFP-positive cells were quantitated using FCM.

For infections involving RT-PCR analysis, stimulated or unstimulated primary B cells in 48-well plates (approximately 1 million cells per well) were incubated with VACV WR at a MOI of 2 for 1 h, washed three times with PBS, and cultured for various time points in complete RPMI-1640. Cells were harvested at various time points and subjected to RNA isolation. Multiplex analysis VACV infection were performed by stimulated or unstimulated primary B cells in 24-well plates (approximately 0.5 to 1 million cells per well) being incubated with VACV WR at a MOI of 2 under the aforementioned VACV binding conditions, washed three times with PBS, and cultured for various times in complete RPMI-1640. Culture supernatants were harvested at various time points and underwent multiplex analysis to assess cytokine production.

Ethics statement

Peripheral blood or leukapheresis samples from healthy blood donors were obtained under an IRB protocol approved by the Indiana University School of Medicine Institutional Review Board for Human Research (Indianapolis, IN). Written informed consent was obtained from each participant before specimen collection.

Preparation of human PBMCs and B cell isolation and activation

Whole blood samples from healthy donors or leukapheresis products from the Indiana Blood Bank were obtained from healthy blood donors with written consent obtained from each participant. Investigational protocols were approved

by Institutional Review Boards for Human Research at the Indiana University School of Medicine (Indianapolis, IN). Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood or leukapheresis products using the gradient centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Isolated PBMCs were subjected to immunomagnetic positive selection of B cells using Human CD19 Microbeads (Miltenyi Biotec, Auburn, CA). Resulting cell preparations contained more than 97% purity of the B cells when assessed by CD19 staining of an alternate epitope and FCM.

Purified B cells were cultured in the presence of 20 ug/ml AffiniPure F(ab')$_2$ Goat Anti-Human IgG + IgM (H+L) (Jackson Immunoresearch Laboratories, West Grove, PA) and 50 nM TLR9 agonist CpG ODN 2006 (InvivoGen, San Diego, CA) in complete RPMI 1640 medium for 24 h at 37°C. Supernatants were subjected to enzyme-linked immunosorbent assays (ELISAs) of IL-6 and IL-10 to evaluate B cell activation. Human IL-6 DuoSet (R&D Systems, Minneapolis, MN) and IL-10 (Life Technologies, Carlsbad, CA) ELISA kits were used according to the manufacturer's instruction.

Confocal microscopy

For binding assays, B cells were incubated with vA5L-YFP VACV at a MOI of 10 under the aforementioned binding conditions. Infections for entry assays were performed by incubating B cells with VV-EGFP at a MOI of 10. We have previously shown that VACV binds to proteins within or near lipid rafts (61), and hence lipid rafts were used to mark the cell surface to indicate the location of the

virus. For lipid raft staining, cells were incubated with cholera toxin subunit B (CTB) conjugated with Alexa Fluor 647 (Life Technologies, Carlsbad, CA) at 4°C for 20 min to stain ganglioside M1 (GM1).To detect extracellular virions, cells were incubated with rabbit polyclonal antiserum NR-631 against the VACV WR-encoded L1R protein (obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH) followed by secondary antibody staining of donkey anti-rabbit IgG (H+L) conjugated to Alexa Fluor 546 (Life Technologies, Carlsbad, CA). Cells were mounted onto glass slides using ProLong Gold Antifade reagent (Life Technologies, Carlsbad, CA) containing 4',6-diamidino-2-phenylindole (DAPI) dye for DNA staining. Slides were viewed using an Olympus FV1000-MPE confocal/multiphoton microscope fitted with a 60X water objective. Images were processed using ImageJ version 1.47 software (NIH, Bethesda, MD).

RT-PCR analysis of VACV gene expression analysis

Stimulated or unstimulated primary B cells were incubated with VACV WR at a MOI of 2 for 1 h, washed with PBS, and cultured for various time points in complete RPMI 1640 medium. Cells were harvested, washed two times in PBS, and subjected to RNA extraction using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was subjected to cDNA synthesis using the Superscript III First Strand synthesis kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR was performed using the *Taq* PCR Master Mix kit (Qiagen, Hilden,

Germany) with primers against VACV early, intermediate, and late genes, including: A23R forward, 5'-CGTTAGTAACGCCATATGGATAATCTATTTACC-3' and A23R reverse 5'-ACCCTAGTCGTTGGATCCATTTCTGAATC-3'; C11R forward, 5'-CAGATCATTCGCCGATAGTGGTAAC-3' and C11R reverse 5'-GGTAGTTTAGTTCGTCGAGTGAACCT-3'; A2L forward, 5'-TCGTGTCCATAATCCTCTACCAT-3' and A2L reverse 5'-TCCACGGATGATGTAGATGCAAA-3'; G8R forward, 5'-GGCGGATCTGTAAACATTTGGG-3' and G8R reverse 5'-TCCTCGTAGTTTGTTGAGAGACG-3'; A17L forward, 5'-GGGCCATGGCTTATTTAAGATATTACAATATGCTT-3' and A17L reverse 5'-GGGGGATCCTTAATAATCGTCAGTATTTAAACT-3'; B7R forward, 5'-TATCGGATCCAATAATGAGTACACTCCG-3' and B7R reverse 5'-GAGCGAATTCTTAAAAATCATATTTTGA-3' (Life Technologies, Carlsbad, CA). Expression data was analyzed for presence of gene expression at particular time points as an indication of VACV infection progression.

Multiplex analysis

Stimulated or unstimulated primary B cells from 6 individual healthy blood donors were incubated with VACV WR at a MOI of 2 under the aforementioned VACV binding conditions, washed with PBS, and cultured for various times in complete RPMI 1640 medium. Culture supernatants were harvested at various time points and subjected to multiplex analysis to assess cytokine production. The concentrations of 45 human cytokines, chemokines, and growth factors

(BDNF, Eotaxin/CCL11, EGF, FGF-2, GM-CSF, GRO alpha/CXCL1, HGF, NGF beta, LIF, IFN alpha, IFN gamma, IL-1 beta, IL-1 alpha, IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12 p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, IP-10/CXCL10, MCP-1/CCL2, MIP-1 alpha/CCL3, MIP-1 beta/CCL4, RANTES/CCL5, SDF-1 alpha/CXCL12, TNF alpha, TNF beta/LTA, PDGF-BB, PLGF, SCF, VEGF-A, and VEGF-D) in the supernatants of B cells cultured for various times with and without VACV were simultaneously measured using the ProcartaPlex Human Cytokine/Chemokine/Growth Factor Panel 1 45plex kit (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. The standards were performed on each plate in duplicate, and all samples from a particular donor were assayed concurrently on the same plate to avoid interassay variability. Data were acquired using a Luminex-100 system and analyzed using Bio-Plex Manager Software (Applied-Cytometry).

Statistical analysis

Data from two groups were analyzed using the Student t test or paired t test, and data from three or more groups were analyzed using either ANOVA with Tukey's post hoc test or paired ANOVA with Tukey's post hoc test. Two-way ANOVA with Bonferroni post-test was used to analyze subset or marker data between the two groups within the isolated cells. P values of < 0.05 were considered statistically significant.

Chapter III – Results

B cell subset frequency in *ex vivo* B cells

As B cells were the main focus of this study, the cell subsets in *ex vivo* isolated B cells were examined to confirm that our blood donors displayed similar frequencies as other studies. The surface markers for the five main subsets and three memory subtypes came from a combination of Morbach *et al* and Kaminski *et al*, with Morbach *et al* providing the main surface markers and Kaminski *et al* providing refinement and further delineation of specific populations (Table 2) (96, 97). The $CD27^{-}IgD^{+}$ naïve B cell subset was further narrowed down to mature naïve B cells, which express CD21 (97), due to transitional B cells also displaying these markers (97). Additionally, $CD24^{-}CD38^{hi}$ plasmablasts display markers found on both plasmablasts and plasma cells, therefore CD138, typically found on plasma cells, was used to differentiate between the two cell types. The frequencies of the different B cell subsets in this study were comparable to Morbach *et al* (Fig. 3a, 3b) (96). Additionally, over 80% and 60% of B cells expressed CXCR5 or CXCR4, respectively (Fig. 3c).

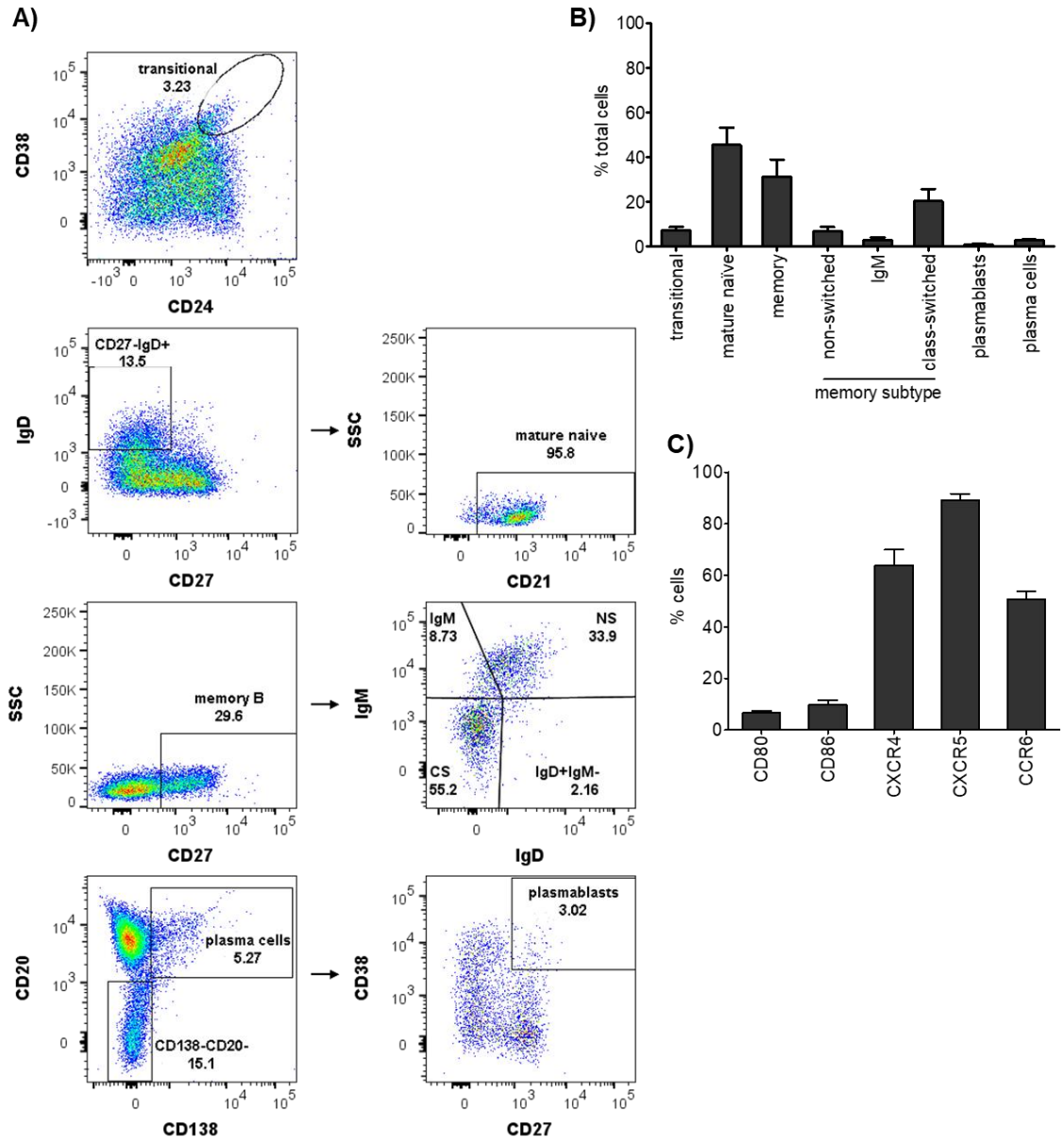

Figure 3. B cell subset frequency in *ex vivo* B cells. A) Representative FCM plots for the evaluation of specific B cell populations using the four B cell phenotyping panels from Table 1. B) Distribution of B cell subsets in *ex vivo* B cells. C) Percentage of cells expressing specific surface molecules. Graphs represent mean ± SEM; n= 5. NS, non-switched memory; CS, class-switched memory; IgM, IgM only memory.

VACV robustly bound to but weakly infected *ex vivo* B cells

Previous studies using primary human PBMCs have shown that APCs, including B cells, displayed strong VACV binding (56, 61), while only minimal viral entry and infection was seen in B cells (56, 60, 61, 98). To better understand this difference between binding and infection, we first examined if this disparity was recapitulated in isolated B cells by assessing VACV binding and infection in isolated B cells. *Ex vivo* B cells were incubated with a MOI 0.5 of vA5L-YFP particles at binding conditions, 4°C for 30 min, to study VACV binding to isolated B cells, purity >97% CD19+ (data not shown). VACV bound robustly to isolated B cells, 61.8 ± 3.3% of total B cells (Fig. 4a, 4b). These values were the results of the mean ± standard error of the mean (SEM) from seven healthy blood donors. These data demonstrated that isolated B cells displayed similar levels of virus binding as seen in PBMCs (Fig. 4b) (61). VACV infection was minimal , 12.6 ± 1.5% of total B cells, compared to binding which corresponds with previous studies in PBMCs showing low infection rates of B cells (Fig. 4a, 4b) (61). Upon further analysis, we found VACV binding displayed a bias towards $CD19^{+}CD20^{hi}$ rather than $CD19^{+}CD20^{lo}$ B cells, while VACV infection exhibited no statistically significant increase in infection of $CD19^{+}CD20^{hi}$ B cells (Fig. 4c). However, VACV infection displayed a bias towards $CD19^{+}CD20^{hi}$ rather than $CD19^{+}CD20^{lo}$ B cells when assessed on an individual donor basis. Binding assessed by viral encoded YFP gene expression suggests that $CD20^{hi}$ B cells are more sensitive to VACV binding, whereas $CD20^{lo}$ B cells are more resistant to VACV binding. Thus, VACV binding and infection in isolated B cells closely reflected the results seen

in PBMCs (56, 61) and showed a bias towards $CD20^{hi}$ transitional, mature naïve, and memory B cells compared to $CD20^{lo}$ or $CD20^{-}$ B cells, typically plasmablasts and plasma cells.

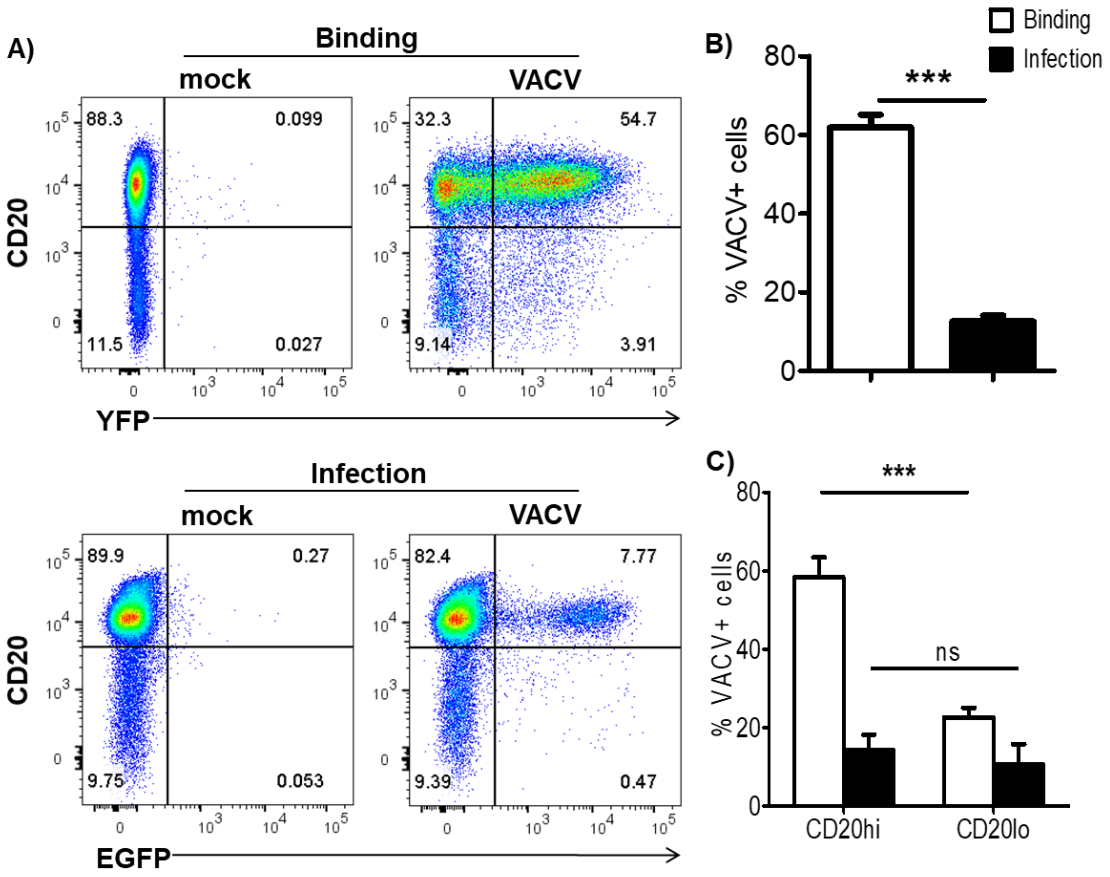

Figure 4. Limited VACV infection in primary B cells despite robust VACV binding. Analysis of VACV binding and infection (24h) at MOI 1 in isolated primary human B cells. A) Representative FCM plots for VACV binding and infection. B) Pooled data of VACV binding and infection of primary B cells from seven healthy donors. C) Analysis of VACV binding (vA5L-YFP) and infection (VV-EGFP) in $CD19^{+}CD20^{hi}$ and $CD19^{+}CD20^{lo}$ B cells. Graphs represent mean ± SEM. ***, $p < 0.001$. HD, healthy donor; mock, uninfected B cells.

Plasmablasts displayed resistance to VACV binding

B cells are highly heterogeneous with many subtypes and fully defining the various subtypes in humans is still in progress. CD19 and CD20 are only two of many markers that are used to differentiate between the different B cell subsets. CD19 is considered a pan-B cell marker and widely expressed during all B cell differentiation and maturation phases found in peripheral blood except some terminally differentiated B cells. CD20 is commonly expressed on most mature B cell subsets except for plasmablasts and most plasma cells. To better understand VACV infection in primary B cells, VACV binding to specific B cell subtypes was evaluated by FCM (Fig. 5). VACV displayed robust binding to the majority of the main peripheral B cell subsets when comparing bound and unbound cells within each specific B cell subset (Fig. 5a, 5b). Plasma, transitional, mature naïve, and memory B cells exhibited the most robust VACV binding. In contrast, plasmablasts exhibited minimal VACV binding (Fig. 5a, 5b). Amongst the memory B cells subtypes, class-switched memory displayed decreased VACV binding compared to non-switched and IgM memory cells. Although the receptors for poxviruses have yet to be discovered, one study suggested that poxviruses use chemokine receptors to bind to and enter into cells (99). Since poxviruses have been shown to have an association with chemokine receptors, we examined VACV binding to B cells expressing CXCR4 or CXCR5. VACV displayed no preferential binding to CXCR4 or CXCR5 expressing cells (Fig. 5c). Additionally, VACV binding to B cells expressing activation markers, such as CD80 and CD86, revealed large percentages of

VACV-bound cells (Fig. 5c). B cells can express many of these chemokine receptors and activation markers concurrently; however, this work focused on individual receptors or markers.

In addition to evaluating VACV binding to specific B cell populations, expression of markers for specific B cell subsets, activation, or potential VACV receptors were evaluated in YFP-positive, VACV-bound cells. The greater part of the VACV bound B cells were mature naïve and memory B cells, whereas plasma cells and plasmablasts made up only a small portion of the VACV-bound cells (Fig. 6a). As expected, the distribution of the B cell subsets in the VACV-bound cells (Fig. 6) was similar to the distribution seen in the mock infected B cells (Fig. 3). Evaluation of specific activation markers and possible poxvirus receptors in VACV-bound cells revealed greater than 90% of VACV-bound cells were positive for CXCR5, and more than 55% of VACV-bound cells were CXCR4+ (Fig. 6b). In contrast, less than 15% of the VACV-bound cells were CD80- or CD86+ (Fig. 6b). These results resemble the distribution noted in mock-infected B cells, suggesting that chemokine receptors, especially CXCR4, may not play a major role in VACV binding.

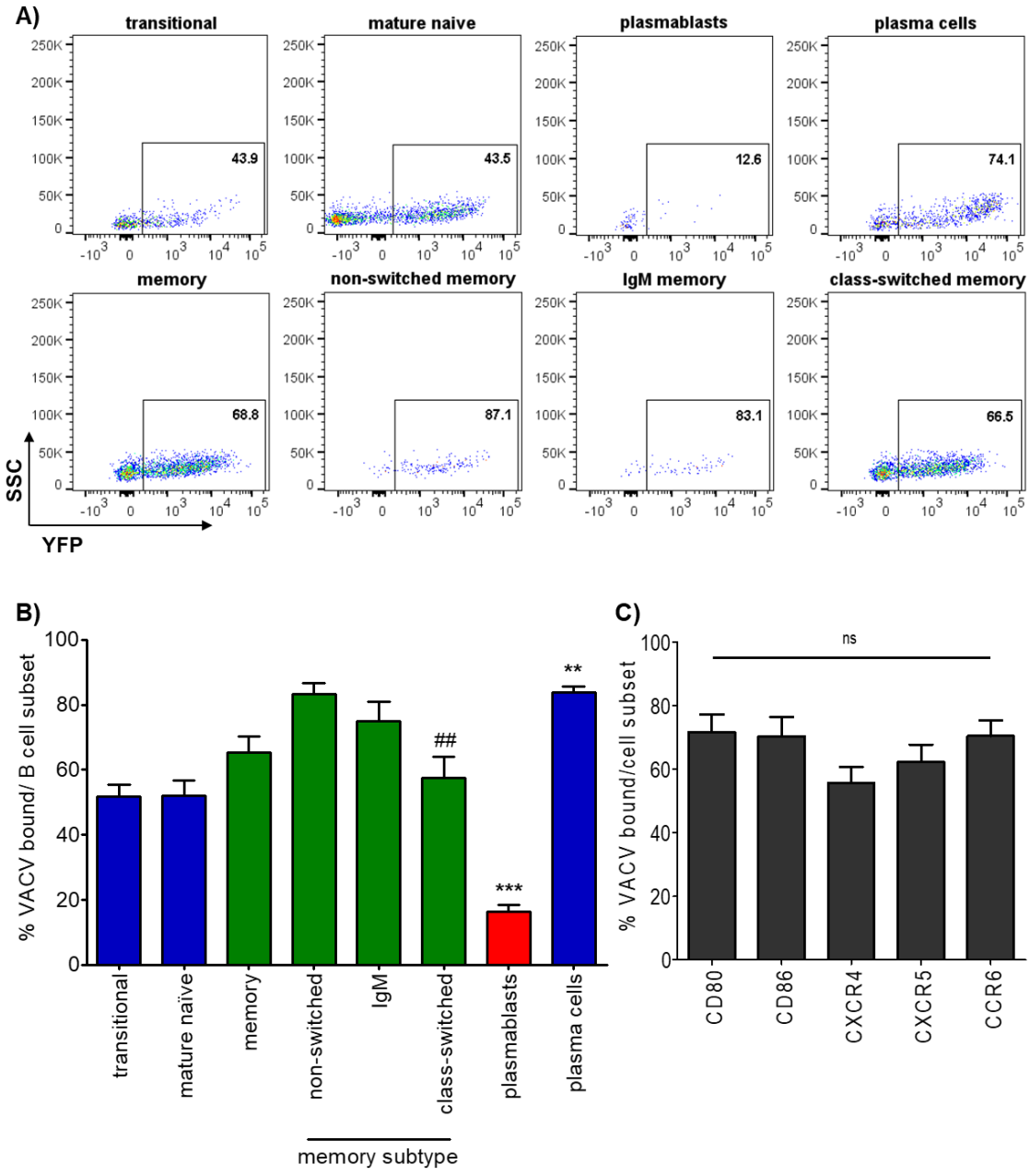

Figure 5. Plasmablasts displayed resistance to VACV binding. Evaluation of VACV binding (vA5L-YFP at MOI 0.5) in specific B cells populations and B cells expressing certain markers using the four B cell phenotyping antibody panels from Table 1. A) Representative FCM of VACV binding in five major B cell subsets found in peripheral blood and three subtypes of memory B cells. B)

Pooled data of VACV binding in B cell subsets. The three colors represent different groups when comparing binding and infection result patterns. Blue, high binding and low infection; red, low binding and low infection; green, high binding and high infection. C) Analysis of VACV binding to B cells expressing common activation markers and chemokine receptors. Graphs represent mean ± SEM. **, $p < 0.01$; ***, $p < 0.001$; ##, $p < 0.01$. *, denotes ANOVA results for the five B cell subsets (transitional, mature naïve, memory, plasmablasts, and plasma cells); #, denotes ANOVA results for the three memory subtypes (non-switched, IgM, and class-switched). ns, not significant.

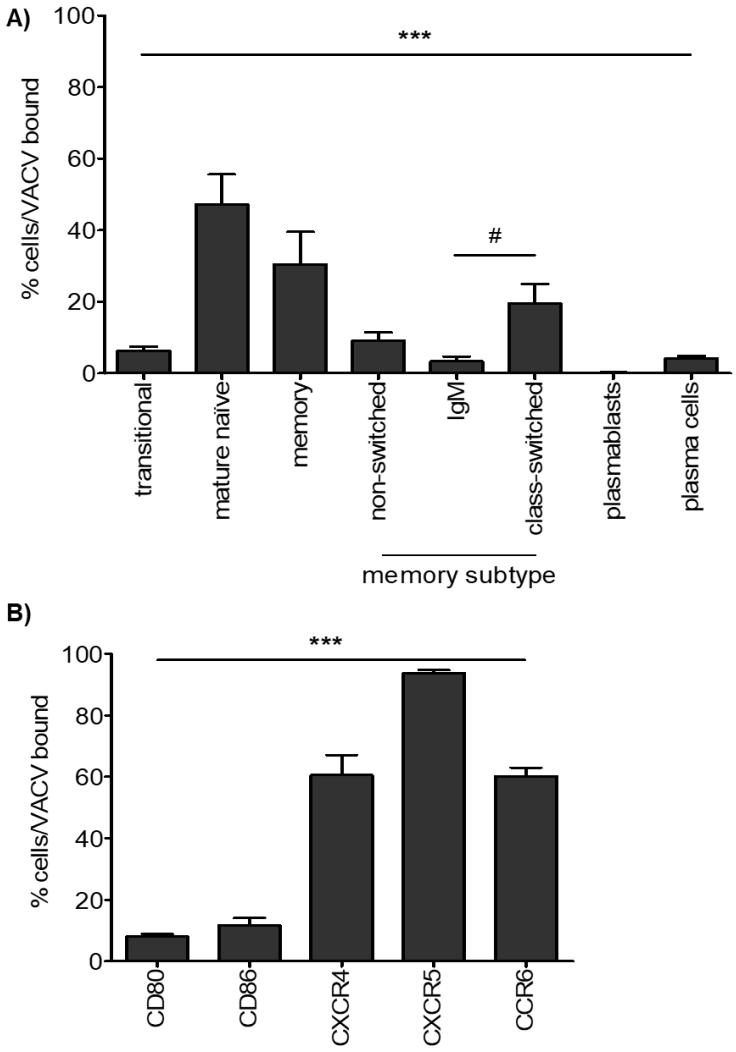

Figure 6. Higher frequency of mature naïve and memory B cells in VACV-bound cells. Evaluation of B cell subsets and surface molecule in the VACV-bound population. A) Percentage of cells displaying specific B cell phenotypes in the VACV-bound cells. B) Percentage of cells displaying specific markers in the VACV-bound cells. Graphs represent mean ± SEM; n=5. *, $p < 0.05$; ***, $p < 0.001$; #, $p < 0.05$. *, ANOVA results for the five B cell subsets (transitional, mature naïve, memory, plasmablasts, and plasma cells); #, ANOVA results for the three memory subtypes (non-switched, IgM, and class-switched).

B cell subset frequency in incubated B cells

Along with measuring the frequencies of B cells subsets *ex vivo*, we also determined subset frequencies in the mock infected cells that had been incubated for 12 h in complete RPMI-1640, the same length of time as the VACV infection. These experiments were conducted with the three donors with a sufficient number of cells for both the *ex vivo* and 12 h infection experiments. The majority of the subsets, including plasmablasts, mature naïve, transitional, and IgM only memory B cells, remained relatively similar post-incubation (Fig. 7a) compared to *ex vivo* (Fig. 3b) B cells (Fig. 8a). Although no significant changes in frequency of certain B cell subsets was noted, plasma cells displayed a slightly increased frequency, while memory, non-switched and class-switched, B cells showed a slight decrease (Fig. 8a). Additionally, the percentage of cells expressing CXCR4 was increased and CCR6 expressing cells decreased 12 h post-incubation (Fig. 7b, 8b). Of note, the percentage of CD80 and CD86 expressing cells was not increased despite VACV infection, but the frequency of CXCR4 expressing cells increased, suggesting that incubation of B cells could lead to activation (Fig. 8b).

Plasma and mature naïve B cells displayed a resistance to VACV infection

Since VACV displayed a disparity between binding and infection of B cells as well as a difference in binding to specific subsets, we sought to assess VACV infection in specific B cell subsets by FCM. Three patterns emerged when binding and infection were compared to each other. Memory B cells and their

subtypes permitted a large portion of the bound virus to enter into the cells, and there was no difference between the different memory subtypes (Fig. 9a, 9b). These memory B cells and subtypes displayed the first pattern, which was relatively high VACV binding rates and high VACV infection rates. However, memory B cells comprised less than 32% of the total B cells in this study. Taking this into account, the robust infection of memory B cells was moderated when considering VACV infection in total B cells because of the reduced percentage of this population compared to the rest of the resistant subtypes. VACV binding to plasma, transitional, and mature naïve B cells was also robust, but few of these VACV-bound cells displayed viral entry (Fig. 9a, 9b), which suggests these cells were either resistant to VACV entry or suppressed viral gene expression. The final pattern observed was noted in plasmablasts which displayed low VACV binding as well as low viral infection. Additionally, there was no difference in VACV infection between cells expressing chemokine receptors (Fig. 9c). Of the CD80+ or CD86+ B cells, the percentage of VACV-infected cells was below 10% and just above 30% of the population, respectively (Fig. 9c).

Examination of B cell populations in VACV-infected cells revealed memory B cells, specifically class-switched memory, were the dominate B cell subtype in the VACV-infected cells (Fig. 10a). The remaining B cell subtypes were minimal in the VACV-infected cells (Fig. 10a). These data correspond to the pattern of percentages of these memory subtypes in incubated cells (Fig. 7a, 8a). Of note, the percentage of VACV-infected B cells with a mature naïve phenotype (Fig. 10a) did not correspond to the pattern of mature naïve B cell percentage among

other B cells (Fig. 7a, 8a). These data may reflect a resistance to VACV infection or an alteration of cell surface markers by VACV infection.

Although >90% of the VACV-infected cells expressed CXCR4 and CXCR5 (Fig. 10b), less than 15% of B cells expressing CXCR4 or CXCR5 were infected with VACV (Fig. 9b), suggesting that the expression of chemokine receptors CXCR4 and CXCR5 is not the only factor involved in VACV entry. However, despite the decrease in the percentage of CCR6 expressing cells (Fig. 8b), a higher percentage, compared to the other chemokine receptors, of CCR6 positive cells were VACV infected (Fig. 9c).

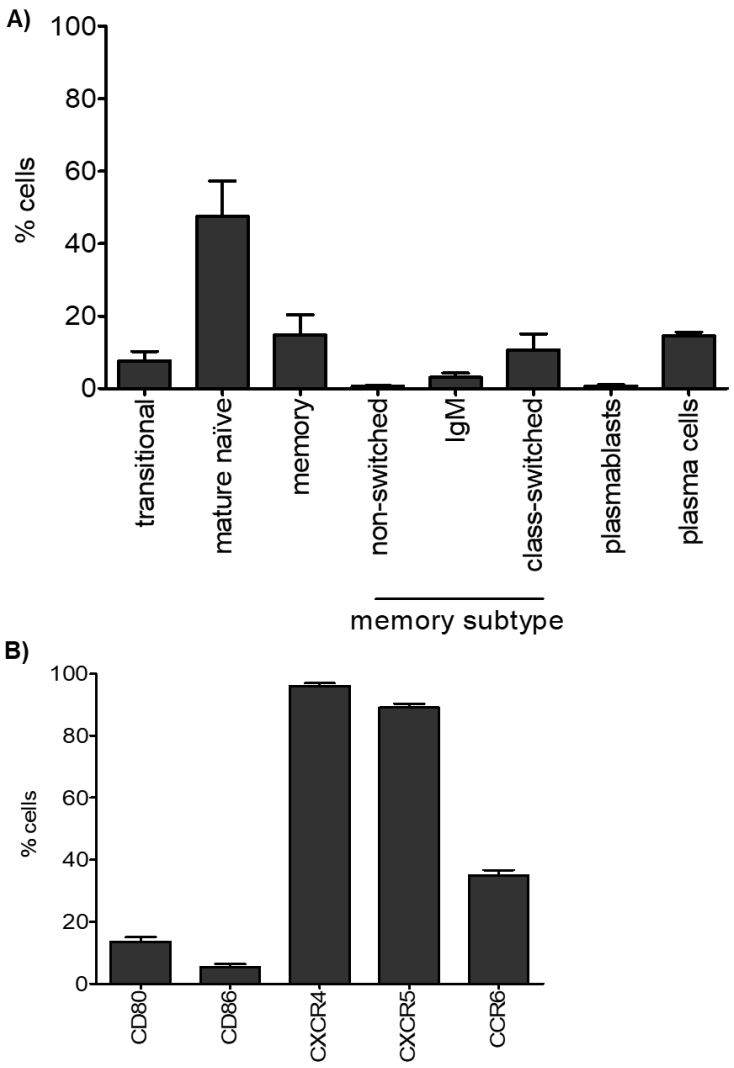

Figure 7. B cell subset frequency in incubated B cells. A) Distribution of B cell subsets in B cells incubated for 12 h. B) Percentage of cells expressing specific surface molecules. Graphs represent mean ± SEM; n= 3.

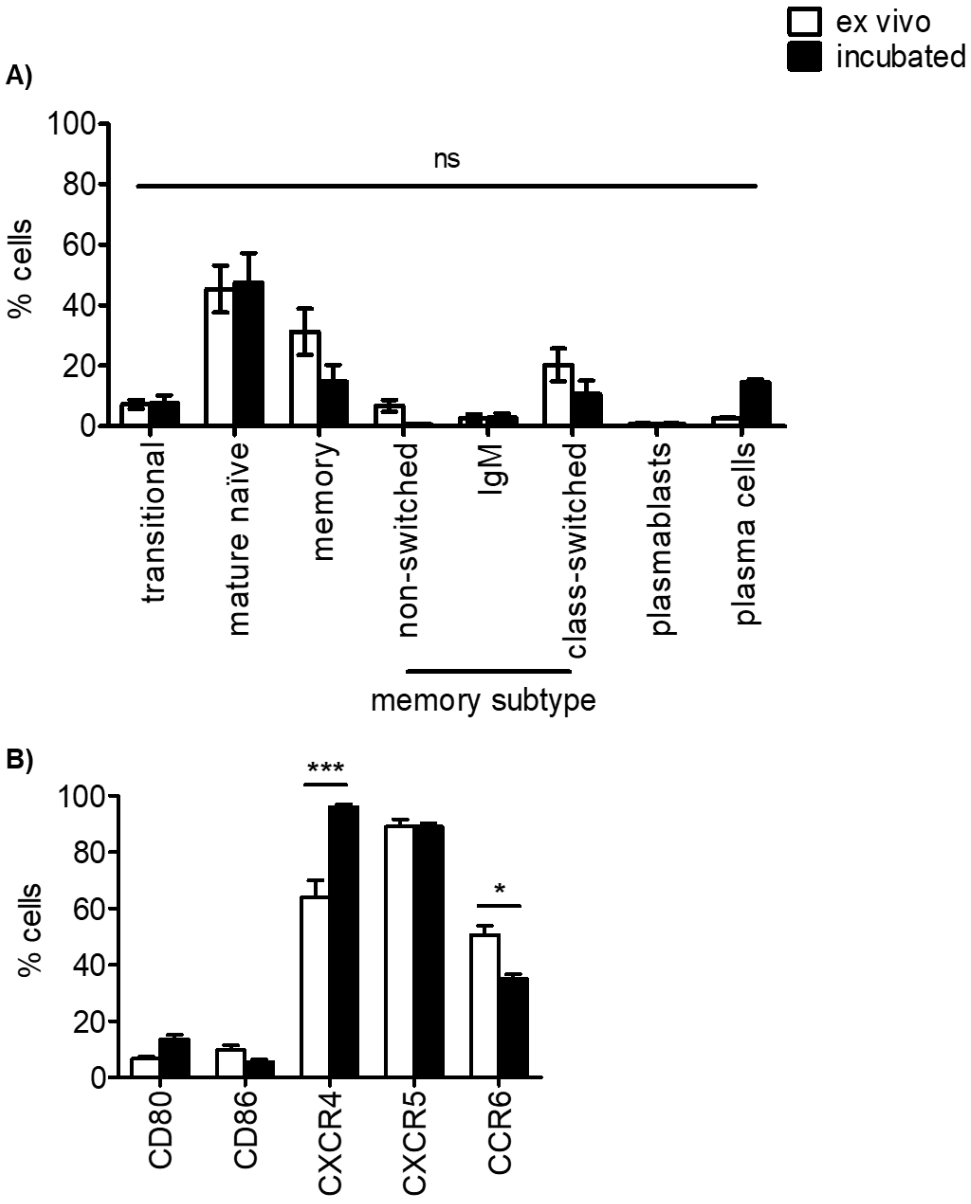

Figure 8. Comparison of B cell subset frequency between *ex vivo* and incubated cells. A) Distribution of B cell subsets in B cells either *ex vivo* or incubated for 12 h. B) Percentage of cells expressing specific surface molecules between *ex vivo* or incubated for 12 h B cells. Graphs represent mean ± SEM; n= 5 for *ex vivo* and n=3 for incubated. *, $p < 0.05$; ***, $p < 0.001$. ns, not significant.

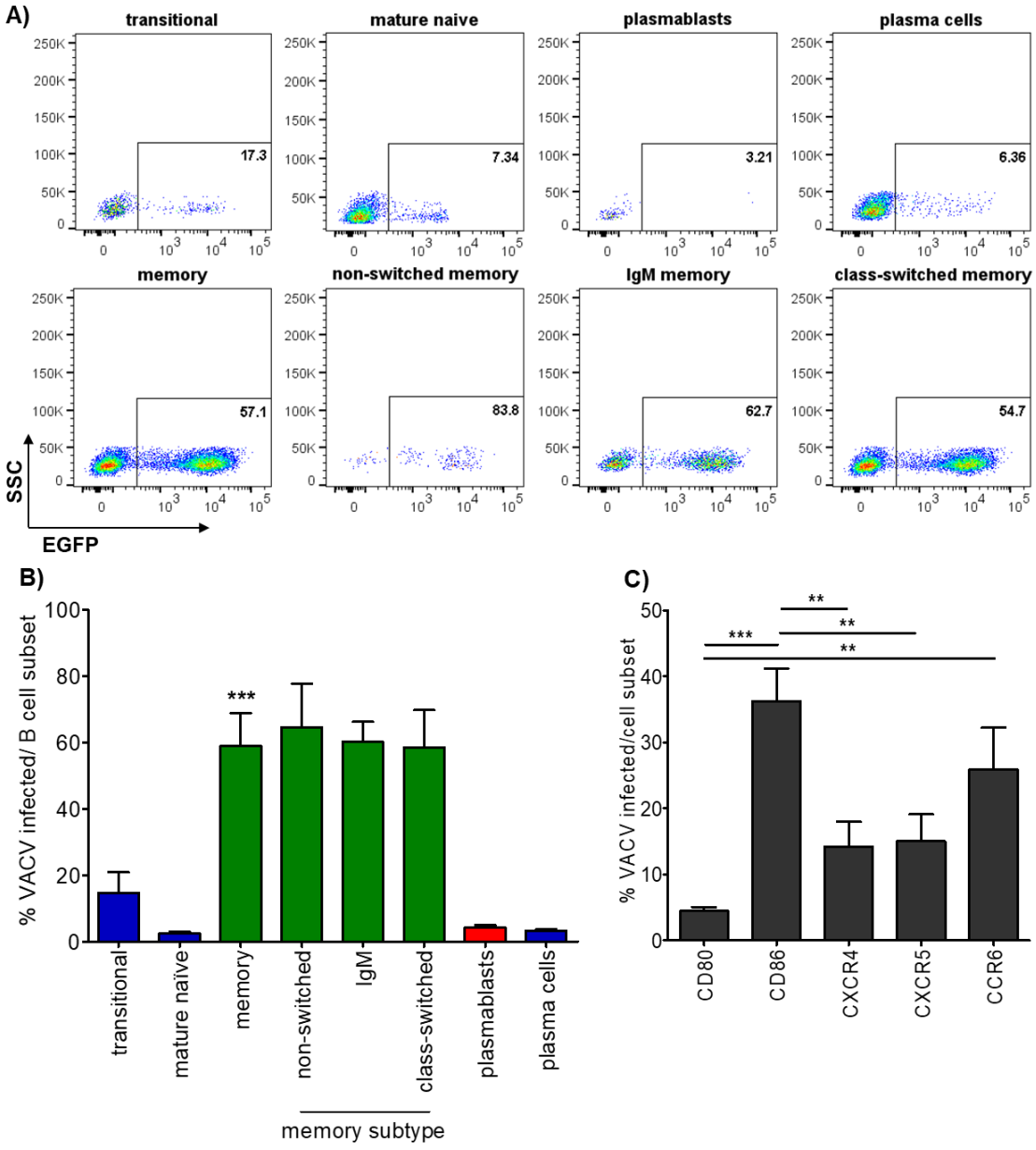

Figure 9. Plasma and mature naïve B cells displayed resistance to VACV infection. Evaluation of VACV infection (VV-EGFP at MOI 0.5; 12 h.p.i.) in specific B cells populations and B cells expressing certain markers using the four B cell phenotyping antibody panels from Table 1. A) Representative FCM plots for VACV infection (12 h.p.i.) in specific B cell subsets. B) VACV infection (12 h.p.i.) in five major B cell subsets found in peripheral blood and three subtypes of

memory B cells. The three colors represent different groups when comparing binding and infection result patterns. Blue, high binding and low infection; red, low binding and low infection; green, high binding and high infection. C) VACV infection (12 h.p.i.) in cells expressing common activation markers and chemokine receptors. Graphs represent mean ± SEM. **, $p < 0.01$; ***, $p < 0.001$. *, ANOVA results for the five B cell subsets (transitional, mature naïve, memory, plasmablasts, and plasma cells).

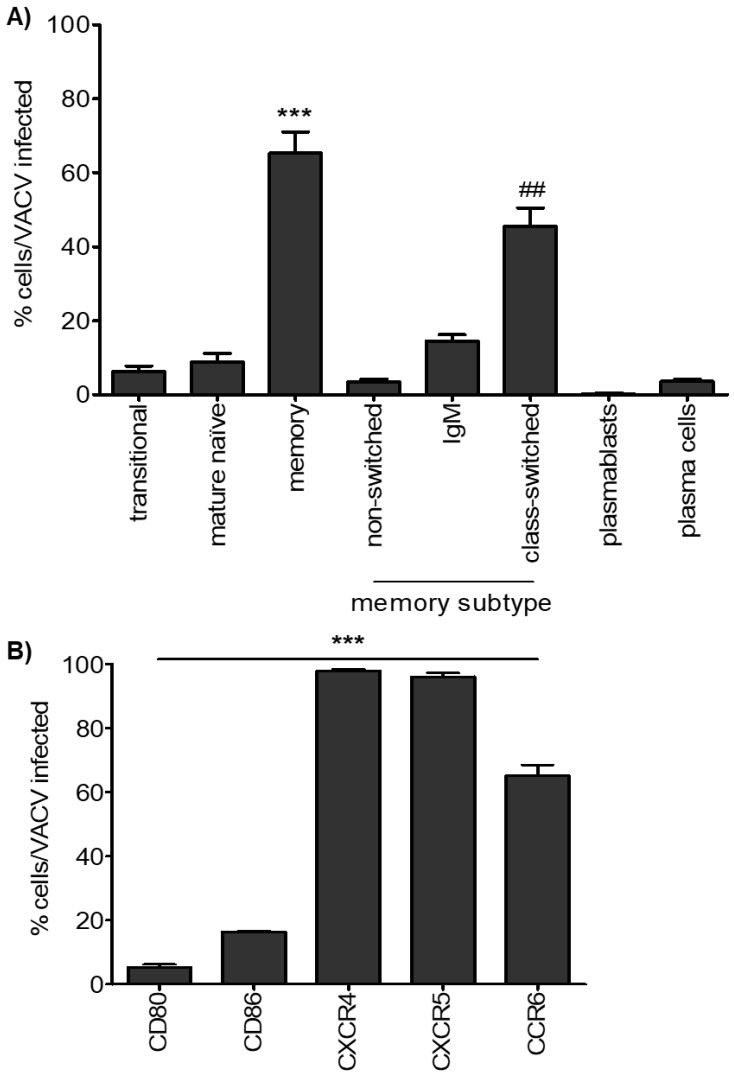

Figure 10. Higher frequency of memory B cells in VACV-infected cells. Evaluation of B cell subsets and surface molecule in the VACV-infected population. A) Percentage of cells displaying specific B cell phenotypes in the VACV-infected cells. B) Percentage of cells displaying specific markers in the VACV- infected cells. Graphs represent mean ± SEM; n=3. ***, $p < 0.001$; ##, $p < 0.01$. *, ANOVA results for the five B cell subsets (transitional, mature naïve, memory, plasmablasts, and plasma cells); #, ANOVA results for the three memory subtypes (non-switched, IgM, and class-switched).

Abortive VACV infection in isolated CD19+ B cells at late stage of VACV gene expression

In addition to examining the B cell subsets preferentially bound or infected by VACV, we sought to better understand VACV infection in primary human B cells. Therefore, we examined the progression of VACV infection in isolated B cells. To our knowledge, the progression of VACV infection has only been examined in B cell lines, and VACV infection in cell lines varies greatly from infection in primary human cells (56, 61, 98). However, one study determined the permissiveness of VACV infection in PBMCs and noted that VACV infection in B cells was abortive (56). To confirm VACV infection in B cells is abortive, plaque assays were completed on B cells infected with VACV at a MOI of 2 to measure the production of new infectious virions. In *ex vivo* VACV-infected B cells, viral titers did not significantly change over a 48-hour period suggesting virions were not produced during the infection (Fig. 11). Although the abortive VACV infection in B cells was confirmed, the stage at which the infection was aborted is still unknown.

To determine at which step in the virus infectious cycle VACV infection was halted, the VACV life cycle was systematically examined. The VACV binding observed via FCM by evaluating the percentage of YFP-positive cells (Fig. 4) was confirmed by confocal microscopy. Confocal microscopy of vA5L-YFP incubated and CTB-stained, stains GM1 in lipid rafts, B cells showed no significant defects in viral binding as seen by the colocalization of the green and red signals in the form of yellow signals (Fig. 12). Additionally, confocal

microscopy of vA5L-YFP incubated and L1-stained, envelope protein of VACV, B cells exhibited no significant defects in viral binding as seen by the colocalization of the green (uncoated virion) and red (virus envelope) signals in the form of yellow signals and entry as green signals signifying uncoated virions (Fig. 13a). Moreover, VACV entry was evaluated via confocal microscopy by incubating B cells with VACV-EGFP virions for several hours and staining with CTB to detect lipid rafts. Detection of VACV entry and early gene expression was indicted by detecting green signals from the reporter virus with EGFP under an early/late promotor. Confocal microscopy assessing viral entry showed no defects in viral entry and early gene expression as green signals in the absence of red signals were present (Fig. 13b). VACV entry and early VACV gene expression was confirmed by using VACV-EGFP but assessing via FCM to also allow for quantification (Fig. 4).

As viral binding and entry were not the limiting steps and VACV replication strictly follows a sequential life cycle (Fig. 1), viral gene expression is the subsequent step after entry and thus was measured by RT-PCR. Two genes of early, intermediate, and late viral gene expression temporal stages were assessed. C11R and A23R were the early genes assessed. C11R encodes a growth factor homologous to epidermal growth factor (100), while A23R encodes one of two subunits of vaccinia virus intermediate transcription factor (VITF) (37). Intermediate genes A2L and G8R both encode vaccinia virus late transcription factors (VLTF) VLTF-3 and VLTF-1, respectively (53, 54). Additionally, the late genes A17L, encoding an IMV membrane protein (101), and B7R, encoding a

polypeptide involved in dermal virulence (102), were assessed. Early and intermediate gene expression occurred in *ex vivo* B cells (Fig. 14). Interestingly, VACV late gene expression was minimal despite the expression of VLTF-1 and -3 mRNAs, encoded by G8R and A2L respectively (Fig. 14). Thus, abortive VACV infection in primary B cells was most likely due to the failure to produce the late viral gene products which are important for viral assembly, structure and virulence.

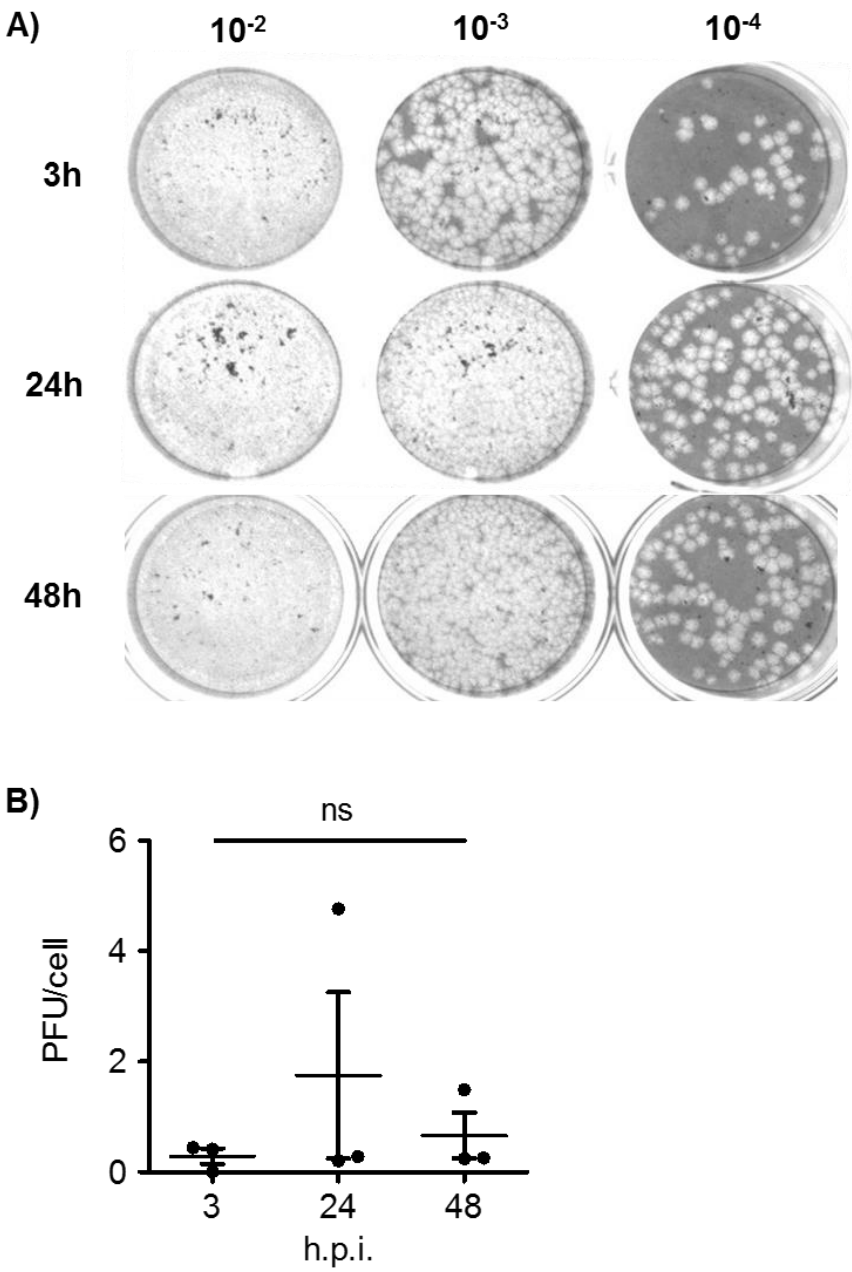

Figure 11. VACV infection in isolated B cells was abortive. Examination of VACV replication in unstimulated B cells. A) Representative plaque assay using unstimulated B cells infected with VACV-WR at a MOI of 2 for various time points after incubation under binding conditions. B) Pooled data from virus plaque assays. Each dot in a group represents data from a single individual; n=3. ns, not

significant; PFU, plaque forming unit; h.p.i., hours post-infection. Lines represent mean ± SEM.

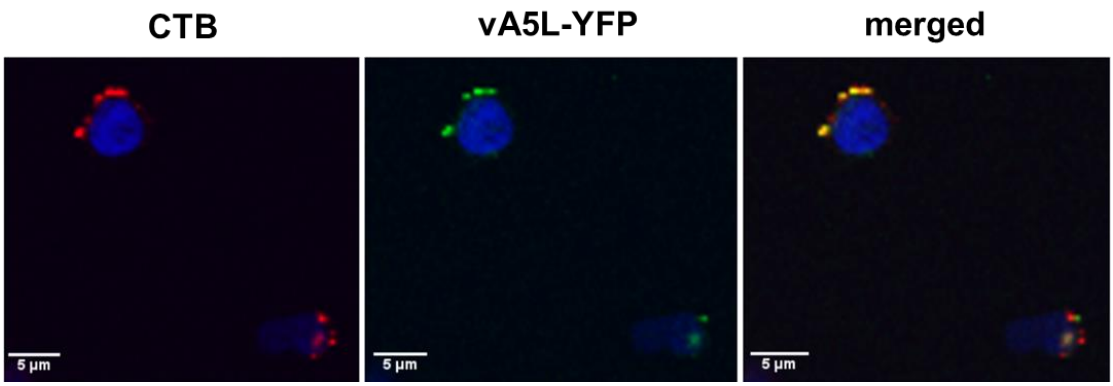

Figure 12. VACV binding to lipid rafts. Evaluation of VACV binding to unstimulated B cells. CTB stained lipid rafts, and vA5L-YFP (MOI 10) was the VACV reporter virus. Blue signal was DAPI for staining the nuclei. n=2. Scale bars represent 5 µM.

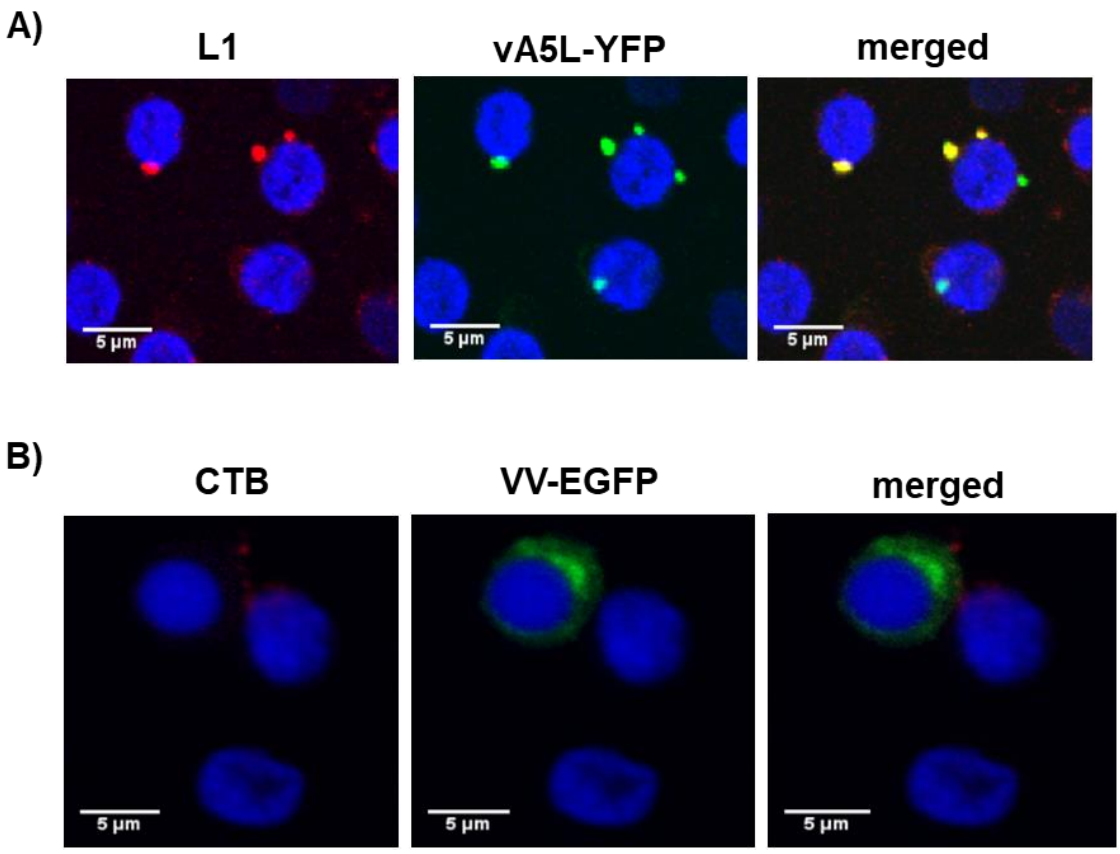

Figure 13. VACV entry into *ex vivo* B cells. Evaluation of VACV entry into unstimulated B cells. A) Confocal microscopy of VACV binding and entry. L1 was the IMV envelope protein, and vA5L-YFP (MOI 10) was the VACV reporter virus. n=3. B) Confocal microscopy of VACV entry and early gene expression in B cells. CTB stained lipid rafts, VV-EGRP (MOI 10) was the VACV reporter virus, and DAPI stained nuclei. n=2. Scale bars represent 5 µM.

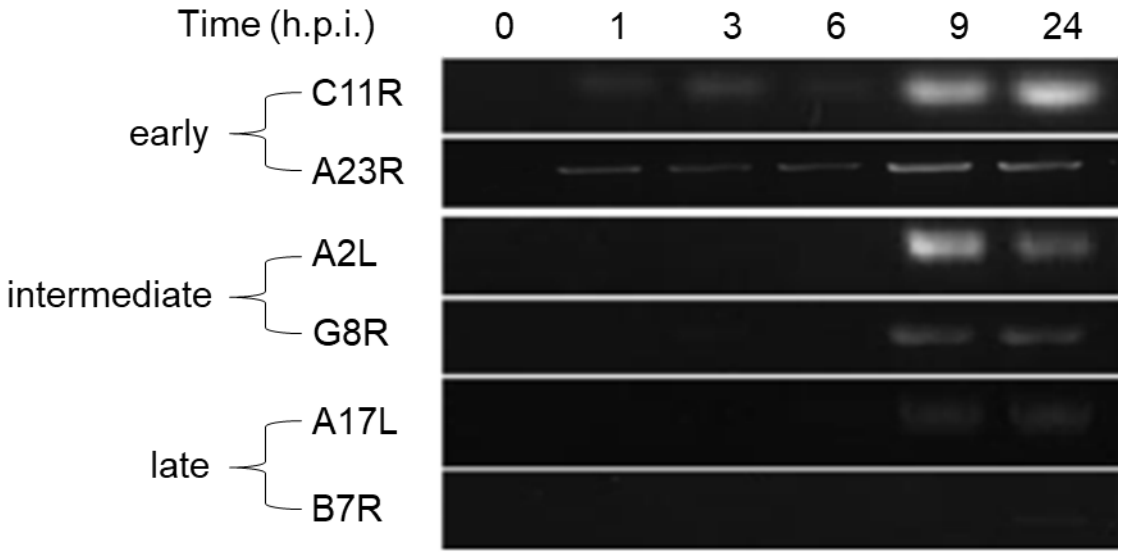

Figure 14. VACV infection in isolated B cells was aborted at late gene expression stage. Examination of VACV-WR gene expression by RT-PCR. VACV early (C11R and A23R), intermediate (A2L and G8R), and late (A17L and B7R) gene expression in unstimulated B cells was assessed by RT-PCR. 0 h was cells incubated with VACV under binding conditions, washed in cold PBS, and subjected to immediate RNA isolation. n=2.

Cytokine production was unaffected by VACV infection in *ex vivo* B cells

To more completely understand VACV infection in primary B cells, we assessed B cell function during VACV infection by examining cytokines, chemokines, and growth factors production via multiplex analysis. Due to the low sample size and high variation between donors, there was no significant differences between mock- and VACV-infected B cells during the various time points (Table 3). Comparing uninfected and infected cells from 6 donors revealed the trend of decreased and increased cytokine production in 8 h.p.i. and 24 h.p.i. infected cells, respectively (Table 3). Additionally, using 3 h.p.i. as the baseline and examining the trends, infected cells exhibited predominately a decrease or no change in cytokine production at 8 h.p.i. with a subsequent rise to baseline levels or above at 24 h.p.i. for many secreted factors (Table 3). Increased expression of IL-6, IL-1β, IL-10, and IL-18 was noted by 24 h.p.i., which is in agreement with other studies in various cell types (103-105). Of note, the majority of the cytokines and chemokines were minimally expressed by *ex vivo* B cells. Surprisingly, VACV infection did not significantly affect B cell function, measured by cytokine and chemokine production, or activation, suggesting VACV was still able to regulate the host response during an abortive infection. However, the host cell still responded to the viral infection.

Table 3. Functional analysis of *ex vivo* B cells.

Analytes	3 Hours		8 Hours		24 Hours	
	uninfected	infected	uninfected	infected	uninfected	infected
IFNα	2.3 ± 3.3	2.7 ± 3.6	1.9 ± 1.7	0.7 ± 1.2	2.2 ± 2.6	5.2 ± 5.4
IFNγ	2.4 ± 1.7	2.4 ± 1.7	2.2 ± 1.5	1.3 ± 0.8	1.8 ± 1.3	2.2 ± 1.1
IL-1α	1.0 ± 1.1	0.7 ± 0.8	0.5 ± 0.7	0.5 ± 0.5	0.9 ± 0.8	1.5 ± 1.7
IL-1β	1.5 ± 1.7	3.1 ± 4.5	1.1 ± 1.0	0.9 ± 1.4	1.0 ± 1.8	3.2 ± 3.6
IL-1RA	108.1 ± 122.8	153.5 ± 296.5	165.1 ± 183.5	49.7 ± 77.8	235.8 ± 331.4	1267 ± 2563
IL-2	13.8 ± 8.7	11.7 ± 6.5	15.6 ± 11.7	10.6 ± 4.0	10.7 ± 5.2	14.1 ± 9.9
IL-4	10.5 ± 6.5	10.6 ± 8.5	9.7 ± 6.5	5.9 ± 3.3	9.8 ± 4.3	11.9 ± 6.4
IL-5	5.6 ± 1.8	6.1 ± 4.8	4.7 ± 2.6	4.3 ± 3.3	4.6 ± 3.3	6.7 ± 4.3
IL-6	19.5 ± 30.9	35.3 ± 46.7	27.4 ± 31.6	5.9 ± 13.5	16.0 ± 25.6	77.2 ± 109.4
IL-7	1.7 ± 1.4	2.2 ± 1.3	2.0 ± 1.8	1.0 ± 1.0	1.9 ± 1.4	3.5 ± 2.0
IL-9	18.4 ± 35.7	10.6 ± 20.2	15.6 ± 25.0	2.0 ± 3.7	8.1 ± 18.6	19.2 ± 24.6
IL-10	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.3	1.1 ± 0.5	1.0 ± 0.2	2.6 ± 1.8
IL-12p70	0.04 ± 0.0	0.04 ± 0.0	0.04 ± 0.0	0.04 ± 0.0	0.04 ± 0.0	0.3 ± 0.4
IL-13	5.2 ± 5.6	4.2 ± 4.7	4.3 ± 5.0	1.5 ± 1.6	2.8 ± 3.3	3.6 ± 3.0
IL-15	8.6 ± 5.6	13.1 ± 14.2	6.9 ± 6.4	5.0 ± 4.2	12.1 ± 5.5	13.7 ± 12.4
IL-17A	3.4 ± 3.1	4.4 ± 5.0	3.9 ± 2.5	1.7 ± 1.9	3.1 ± 1.8	5.5 ± 4.1
IL-18	10.5 ± 6.2	15.0 ± 12.6	12.2 ± 10.8	8.5 ± 4.9	11.4 ± 6.5	15.1 ± 10.6
IL-21	11.5 ± 9.4	23.6 ± 21.5	10.7 ± 10.1	6.4 ± 6.2	17.3 ± 11.2	25.5 ± 27.1
IL-22	86.4 ± 96.2	50.0 ± 38.8	53.9 ± 37.0	38.2 ± 18.7	42.0 ± 33.5	63.8 ± 49.6
IL-23	289.9 ± 351.4	235.5 ± 305.3	138.3 ± 129.6	175.9 ± 155.8	439.7 ± 418.6	478.5 ± 470.0
IL-27	59.4 ± 85.7	73.4 ± 116.3	48.6 ± 67.6	5.1 ± 0.0	28.0 ± 56.1	56.9 ± 70.5
IL-31	54.6 ± 119.1	27.0 ± 58.0	39.5 ± 63.5	7.5 ± 10.3	3.3 ± 0.0	12.7 ± 23.1
LIF	0.6 ± 0.4	1.0 ± 0.3	0.7 ± 0.4	0.7 ± 0.4	0.7 ± 0.6	0.9 ± 0.5
TNFα	7.9 ± 2.9	8.2 ± 3.0	8.0 ± 4.3	6.9 ± 3.8	7.0 ± 2.7	26.0 ± 42.0
TNFβ	1.6 ± 0.0	1.6 ± 0.0	1.7 ± 0.2	1.6 ± 0.0	1.6 ± 0.0	1.6 ± 0.0
Eotaxin	1.5 ± 0.2	1.9 ± 0.9	1.6 ± 0.3	1.3 ± 0.3	1.7 ± 0.6	2.2 ± 1.5
GROα	1.4 ± 1.6	1.0 ± 1.1	0.8 ± 0.6	3.3 ± 3.8	1.3 ± 1.3	16.3 ± 33.4
IL-8	193.5 ± 344.9	132.1 ± 233.7	356.8 ± 745.8	276.6 ± 327.7	392.1 ± 786.7	988.9 ± 1095
IP-10	6.6 ± 4.1	7.4 ± 4.1	7.2 ± 4.3	4.6 ± 3.6	5.9 ± 6.5	12.2 ± 12.8
MCP-1	338.4 ± 424.5	294.7 ± 409.0	307.8 ± 356.2	269.6 ± 357.7	1317± 1469	1262 ± 1533
MIP-1α	3.6 ± 3.0	3.7 ± 2.3	5.2 ± 6.0	8.8 ± 9.8	5.8 ± 2.9	115.1 ± 258.3
MIP-1β	23.7 ± 32.4	28.2 ± 29.9	18.8 ± 23.2	35.8 ± 34.0	52.7 ± 17.6	119.9 ± 125.0
RANTES	6.2 ± 8.9	6.0 ± 7.4	4.9 ± 4.8	3.2 ± 2.7	3.7 ± 3.6	12.1 ± 15.5
SDF-1α	394.0 ± 528.0	342.8 ± 576.6	197.1 ± 212.8	296.1 ± 392.9	806.0 ± 915.1	809.5 ± 824.5
BDNF	2.3 ± 2.5	2.5 ± 2.6	2.0 ± 2.0	1.3 ± 0.7	2.1 ± 2.3	4.0 ± 3.7
EGF	3.6 ± 4.0	3.7 ± 3.9	2.6 ± 1.5	2.1 ± 1.4	3.9 ± 4.3	4.2 ± 3.7
FGF-2	6.1 ± 8.2	13.7 ± 20.9	7.1 ± 7.2	44.0 ± 87.1	5.3 ± 4.3	26.4 ± 37.0
GM-CSF	13.1 ± 13.4	16.7 ± 12.3	20.2 ± 22.9	8.6 ± 8.9	9.3 ± 10.2	22.0 ± 12.6
HGF	8.0 ± 5.7	7.6 ± 5.4	7.0 ± 4.4	10.1 ± 11.0	9.1 ± 4.4	12.6 ± 11.0
NGFβ	2.5 ± 1.5	4.7 ± 4.8	4.2 ± 2.1	3.2 ± 2.4	2.9 ± 2.1	3.8 ± 3.3
PDGF-BB	8.4 ± 6.0	10.7 ± 6.7	5.8 ± 1.8	7.6 ± 4.1	7.1 ± 3.7	11.0 ± 4.9
PIGF-1	3.4 ± 4.4	4.8 ± 7.0	2.2 ± 3.1	2.2 ± 2.5	3.5 ± 3.5	16.1 ± 21.5
SCF	3.7 ± 3.1	3.2 ± 3.4	2.5 ± 2.9	1.2 ± 0.9	2.5 ± 2.1	3.2 ± 3.2
VEGF-A	11.9 ± 15.9	15.3 ± 17.6	8.0 ± 10.8	6.8 ± 8.8	10.0 ± 11.5	35.4 ± 38.3
VEGF-D	2.3 ± 2.4	3.0 ± 2.9	1.6 ± 0.8	2.1 ± 1.7	3.4 ± 3.9	3.9 ± 4.2

Complete multiplex analysis of 45 cytokines, chemokines, and growth factors from supernatants of unstimulated B cells either incubated with or without VACV-WR at a MOI of 2. Pooled data from 6 donors.

Detection of two B cell populations differing in size and complexity via FCM

While evaluating VACV binding and infection in *ex vivo* isolated B cells, two populations differing in size and complexity were observed by FCM (Fig. 15). Two populations of B cells have been noted previously, but were found by density gradient rather than FCM (106). To confirm that both populations were indeed live cells, the populations in isolated B cells were assessed by calcein AM, which fluoresces in live cells with functional esterases to cleave the small molecule. Greater than 95% of both populations, deemed B_{small} (left) and B_{large} (right), was live and viable cells (Fig. 16). Additionally, the cell cycle phase and proliferation were tested. B_{large} displayed an increased frequency of cells in the S or M phase as compared to B_{small} (Fig. 17a). Moreover, B_{large} exhibited an increased frequency of Ki-67-positive cells, denoting proliferating cells, as compared to B_{small} (Fig. 17b). These data suggest that both B_{small} and B_{large} populations were comprised of viable cells and similar to that observed previously by density gradient (106).

Since both populations were viable and there was a difference in the proliferative ability, phenotypic analysis of B_{small} and B_{large}, both *ex vivo* and mock infected cells, was subsequently completed. When comparing B_{small} and B_{large}, B_{large} displayed an increased frequency of memory B cells, while the remaining B cell subsets were consistent between the two populations (Fig. 18a). No difference in the prevalence of cells displaying activation markers was observed between both populations (Fig. 18b). B_{large} exhibited a decrease in the percentage of CXCR4-positive cells and an increase in CCR6-positive B cells

compared to B_{small} (Fig. 18b). However, the frequency of CXCR5-positive cells was equally high in both populations (Fig. 18b). Phenotypic analysis was also completed in 12 h.p.i. mock-infected B cells because of the differences in subset frequencies in cultured *ex vivo* total B cells. In 12 h.p.i. mock-infected cells, the difference in memory B cell prevalence was still noted, nevertheless there was also increased plasma cell percentages in B_{small} (Fig.19a). Additionally, great variation in the frequency of chemokine receptor or activation marker positive B cells was observed between the two populations. CXCR5 expressing cells remained at the same frequency in B_{small}, whereas the frequency increased in B_{large} (Fig. 19b). An increase in CXCR4-positive cells was noted in both populations, but to a greater extent in B_{large} (Fig. 19b). These data suggest possible activation in both B_{small} and B_{large} post-incubation. Additionally, the increased percentage of CCR6-positive cells in B_{large} remained consistent even as the percentage of CCR6-positive cells decreased (Fig. 19b). CXCR5 expressing cells decreased slightly in B_{small} compared to B_{large} (Fig. 19b). B_{small} displayed higher CD80-positive cells, while B_{large} displayed higher CD86-positive cells (Fig. 19b). These data suggest that culturing B cells, even in the absence of virus, leads to alterations in cell surface molecule and cell death.

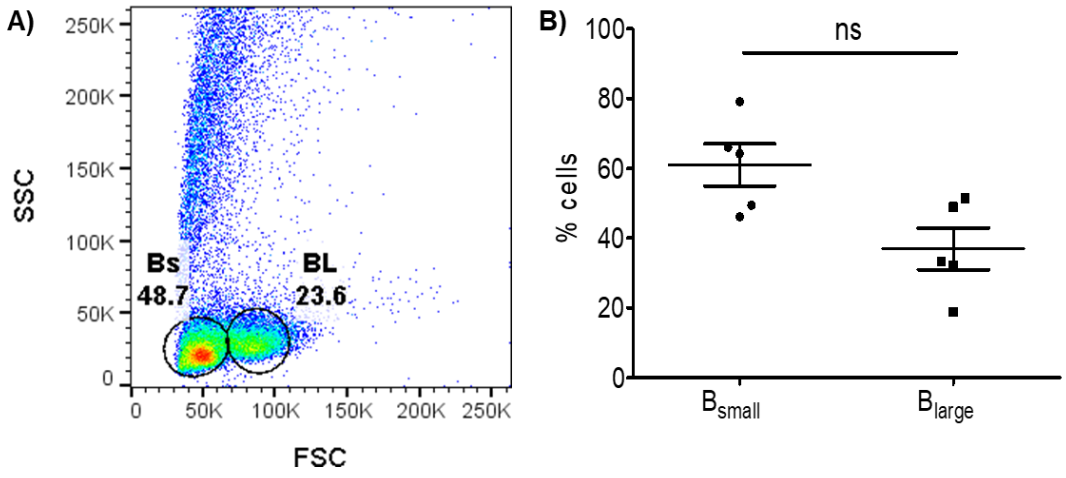

Figure 15. Two B cell populations observed in isolated B cells. A) Representative FCM depicting two populations in total B cells. B) Pooled data for B cell populations. Each dot in a group represents data from a single individual; n=5. ns, not significant; Bs, B_{small} B cell population; BL, B_{large} B cell population. Lines represent mean ± SEM.

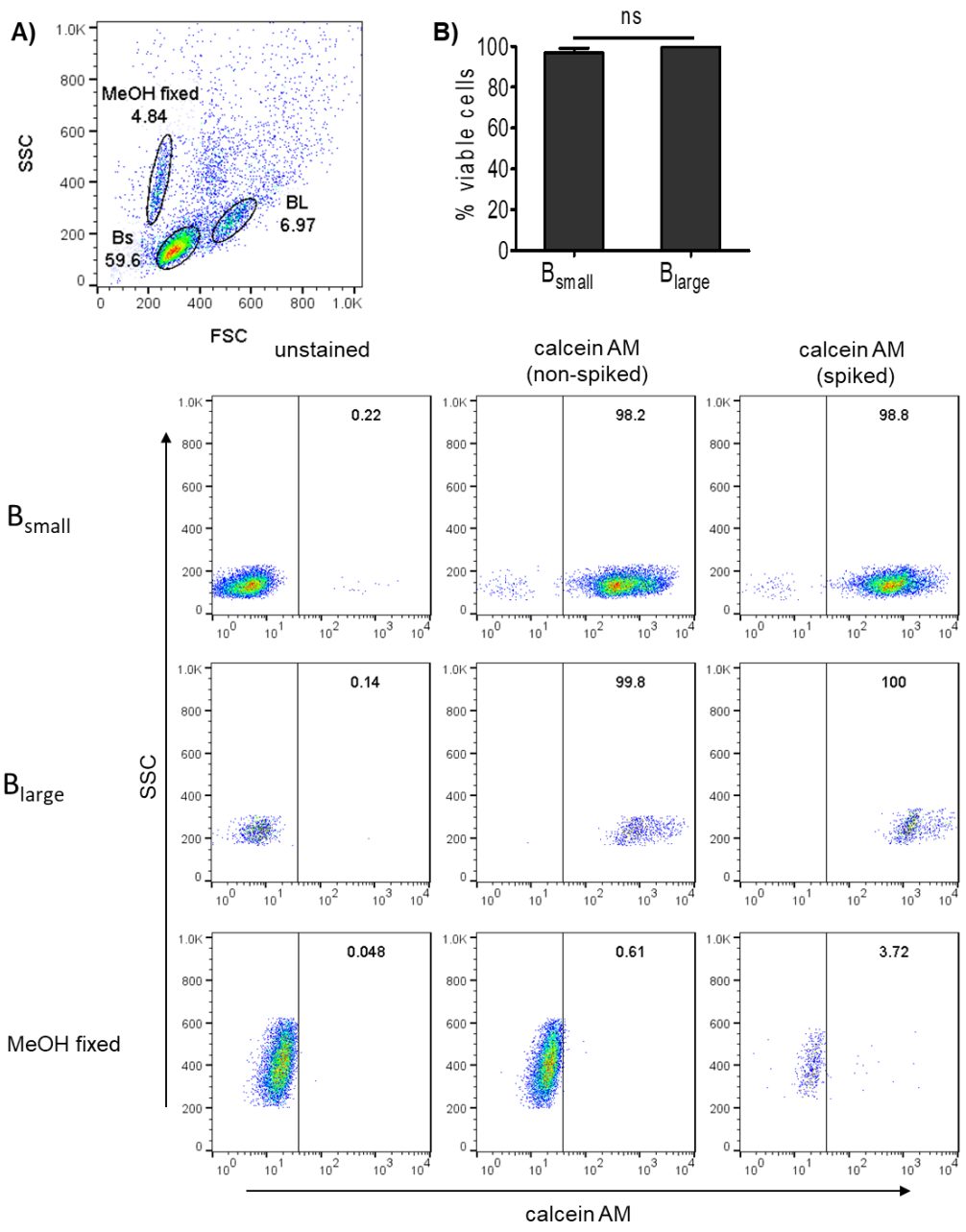

Figure 16. B_{small} and B_{large} populations were both viable. A) Representative FCM depicting cell viability staining using calcein AM. B) Pooled data for the two B cell population viability. Graphs represent mean ± SEM; n=3. ns, not significant; Bs, B_{small} B cell population; BL, B_{large} B cell population; MeOH fixed, methanol fixed dead cells.

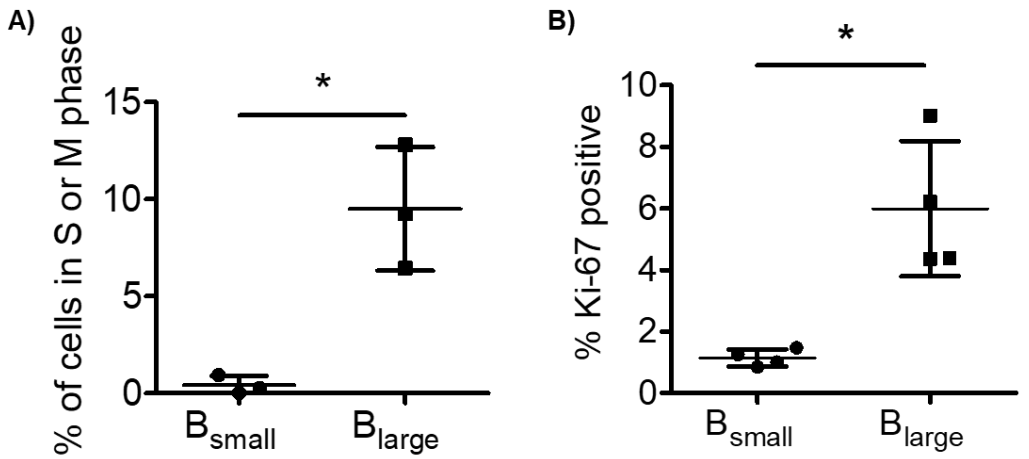

Figure 17. B_{large} displayed increased proliferation. A) Analysis of cell cycle phase by propidium iodide (PI) staining. Each dot in a group represents data from a single individual; n=3. B) Evaluation of proliferating cells via Ki-67 staining. Each dot in a group represents data from a single individual; n=4. Lines represent mean ± SEM. *, $p < 0.05$.

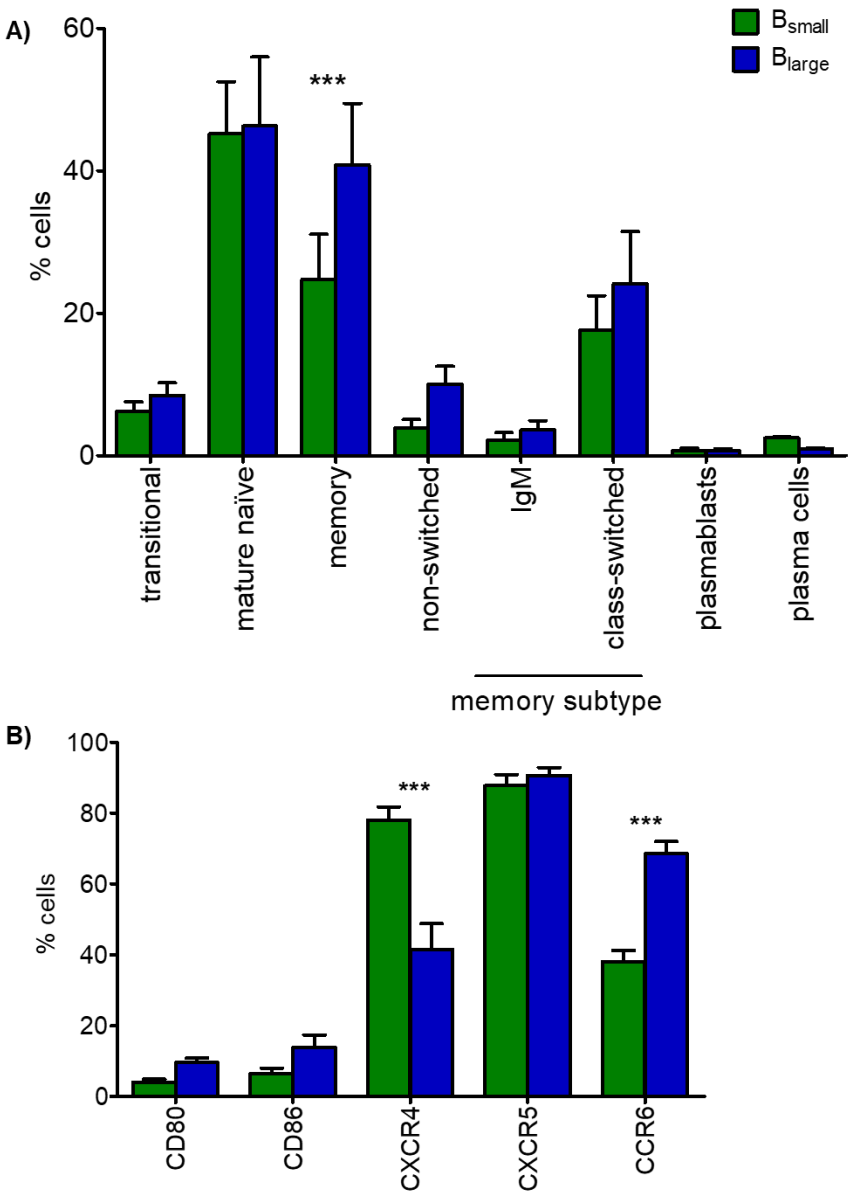

Figure 18. B_{large} contained more memory B cells in *ex vivo* B cells. A) Evaluation of specific B cells populations in B_{small} and B_{large} using the four B cell phenotyping antibody panels from Table 1. Distribution of B cell subsets in *ex vivo* B cell populations. B) Percentage of cells expressing specific surface molecules in B cell populations. Graphs represent mean ± SEM; n= 5. ***, $p < 0.001$.

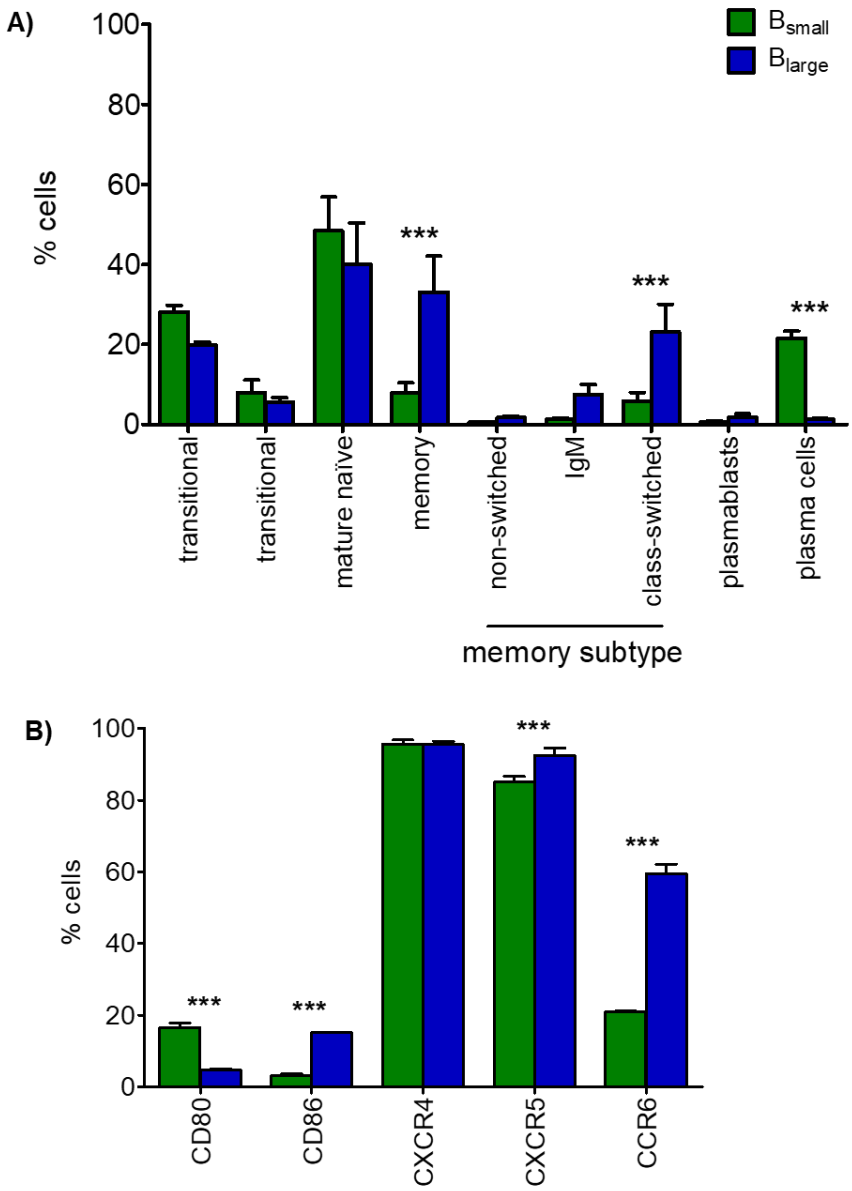

Figure 19. B_{small} contained more plasma cells in incubated B cells. A) Evaluation of specific B cells populations in B_{small} and B_{large} incubated for 12 h using the four B cell phenotyping antibody panels from Table 1. Distribution of B cell subsets in 12 h-incubated B cell populations. B) Percentage of cells expressing specific surface molecules in B cell populations. Graphs represent mean ± SEM; n= 5. ***, $p < 0.001$.

Differential VACV binding to B_{small} and B_{large}

To assess if the differences between the two populations affected VACV infection, VACV binding to B_{small} and B_{large} was first examined via FCM. Although B_{large} was the minor population in *ex vivo* B cells, VACV bound to a greater percentage of cells in the B_{large} population as compared to B_{small} (Fig.20a, 20b). However, B_{small} cells did display VACV binding in greater than 40% of the population (Fig. 20a, 20b). Since a disparity was observed between B_{small} and B_{large} VACV binding, we sought to assess VACV binding in specific B cell subsets in these populations by FCM. Of note, in both populations, VACV binding displayed relatively low binding to plasmablasts, once more suggesting that plasmablasts are resistant to VACV binding (Fig. 21a). The remaining B cell subsets exhibited differences between the two populations, with B_{large} having a higher prevalence of VACV-bound cells (Fig. 21a). Additionally, B_{large} exhibited increased VACV binding to cells expressing activation markers and chemokine receptors when compared to B_{small} (Fig. 21b). These data suggest that the cell surface molecules on B_{large} cells inherently differ from B_{small} cells, which allow for increased VACV binding. Additionally, the increase in virus binding in the B_{large} cells could be due to increased cell surface.

As expected, examination of B cell populations in VACV-infected cells revealed the distribution of the B cell subsets in the VACV-bound cells (Fig. 22a) was similar to the distribution seen in the mock-infected B_{small} and B_{large} cells (Fig. 18a). Evaluation of specific activation markers and possible poxvirus receptors in

VACV-bound cells revealed results resembling the distribution noted in mock-infected B cells (Fig. 22b, 18b).

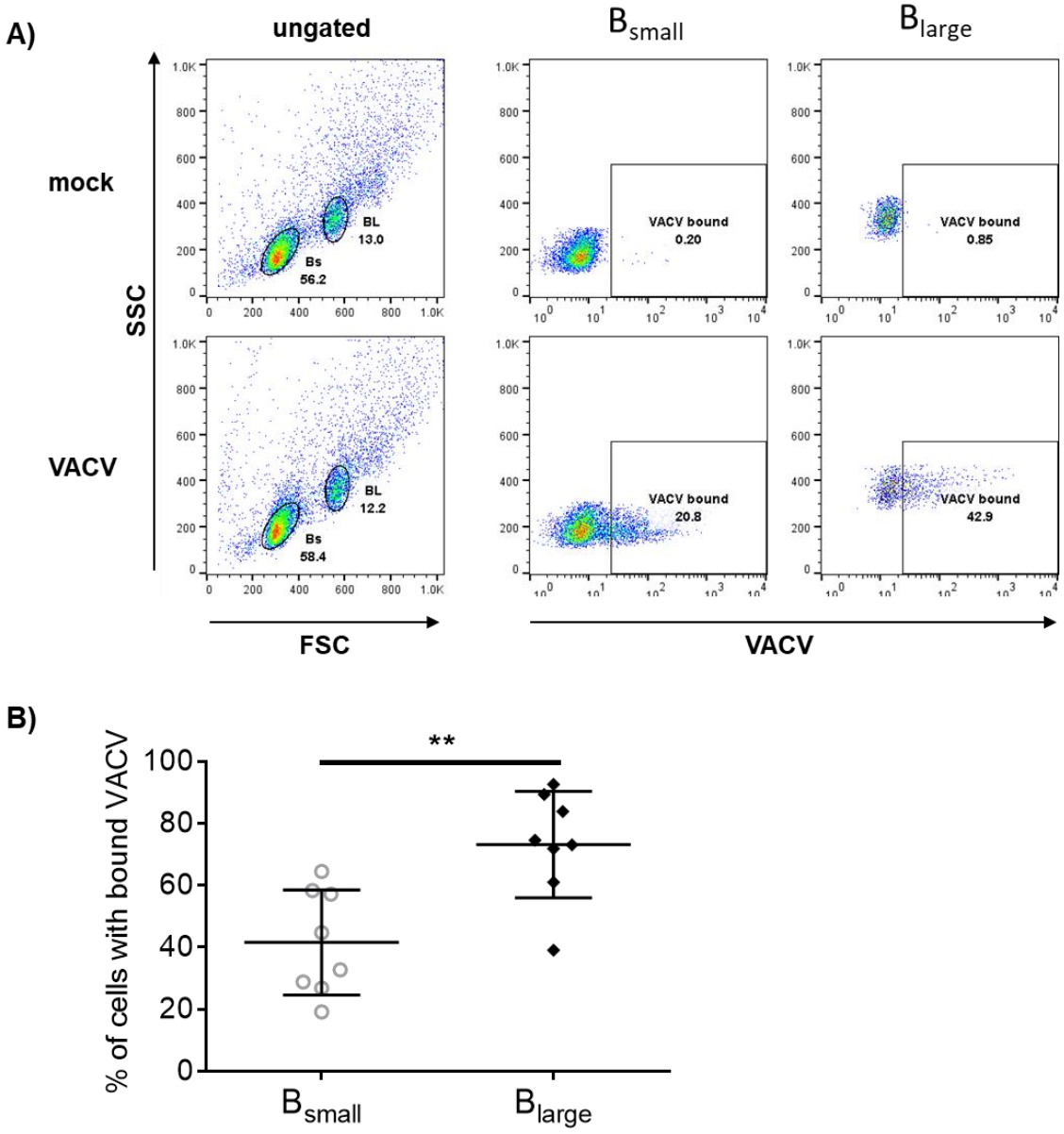

Figure 20. Differential VACV binding in B_{small} and B_{large}. Analysis of VACV binding at a MOI of 0.5 in both B_{small} and B_{large} populations. A) Representative FCM plots for VACV binding in B_{small} and B_{large}. B) Pooled data from eight healthy donors. Each dot in a group represents data from a single individual. **, $p < 0.01$. Bs, B_{small} B cell population; BL, B_{large} B cell population.

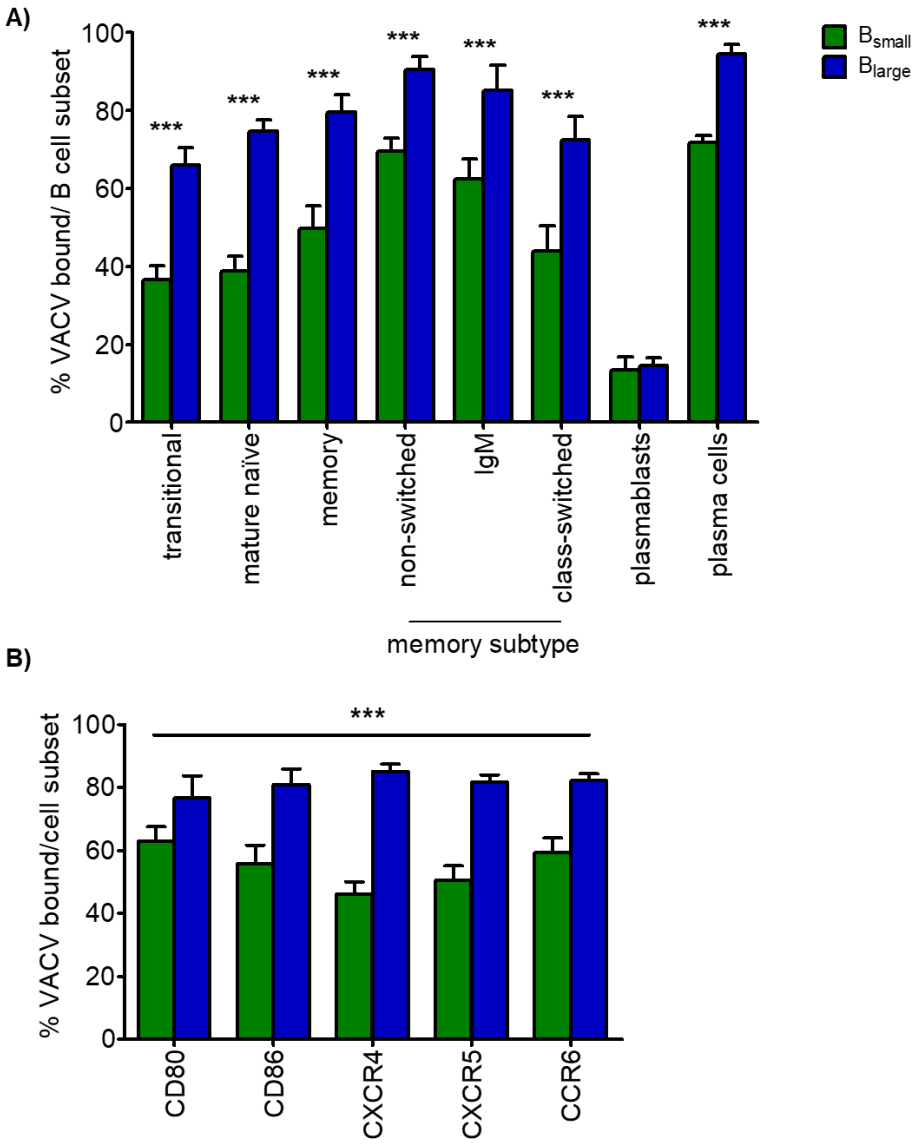

Figure 21. Plasmablasts were resistant to VACV binding in both B_{small} and B_{large} populations. Analysis of VACV binding (vA5L-YFP) at a MOI of 0.5 in both B cell populations. A) In the two B cell populations, VACV binding in five major B cell subsets found in peripheral blood and three subtypes of memory B cells. B) Analysis of VACV binding to cells expressing common activation markers and chemokine receptors in both populations. Graphs represent mean ± SEM; n= 5. ***, $p < 0.001$.

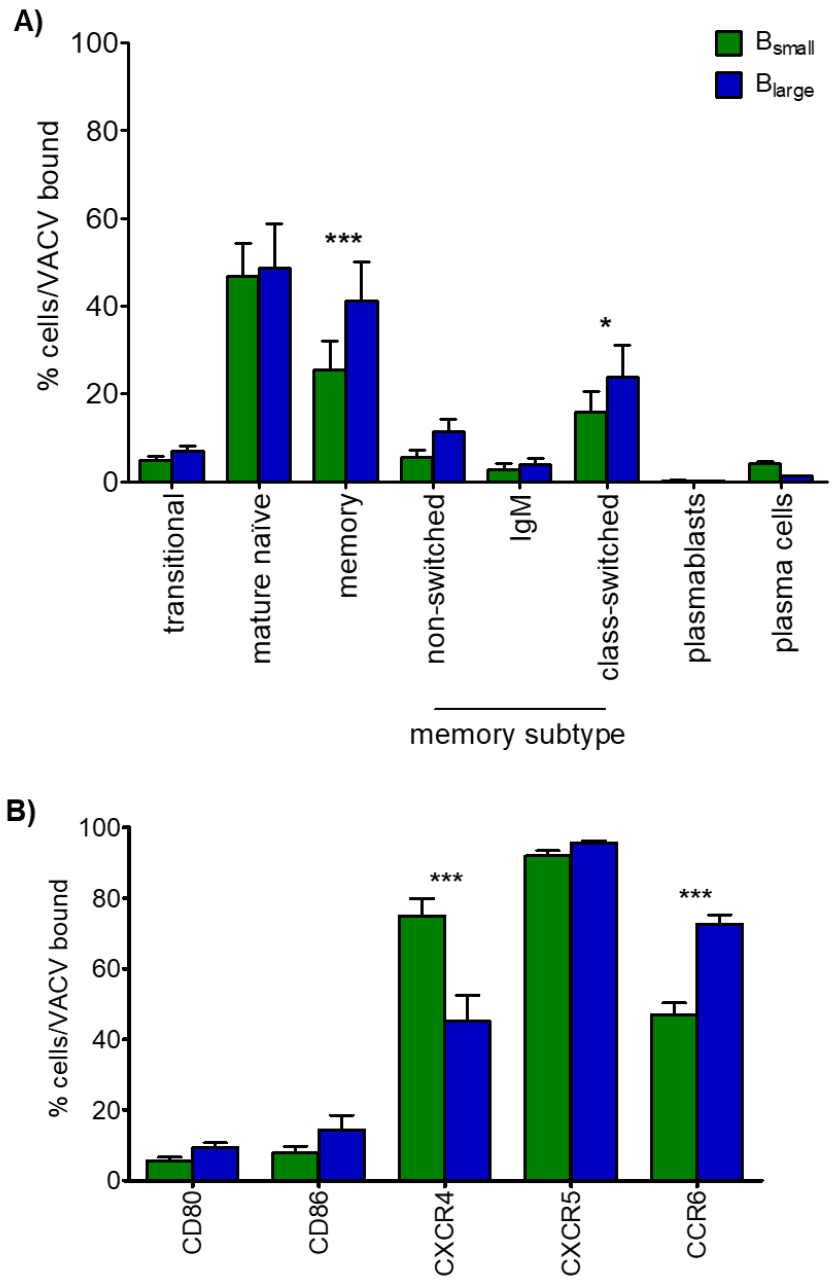

Figure 22. B cell subsets in VACV bound B cells. A) Percentage of cells displaying specific B cell phenotypes in the VACV-bound (vA5L-YFP) cells within both B cell populations. B) Percentage of cells displaying specific markers in the VACV- bound (vA5L-YFP) cells within both populations. Graphs represent mean ± SEM; n= 5. *, $p < 0.05$; ***, $p < 0.001$.

Differential VACV infection to B_{small} and B_{large}

As mentioned previously, B_{large} exhibited increased memory B cell frequency, and memory B cells were preferentially entered and infected by VACV in total B cells (Fig.18a, 9b). This suggests that VACV entry and infection in B_{large} would be increased compared to B_{small}. Assessment of VACV infection in both populations demonstrated an increase in VACV-infected cells in B_{large} as compared to B_{small} (Fig. 23a, 23b). Since VACV displayed an infection disparity between B_{small} and B_{large} cells, we sought to assess VACV infection in specific B cell subsets by FCM. The majority of the B cell subsets exhibited similar percentages of VACV-infected cells between B_{small} and B_{large} cells, except for transitional B cells which had a higher frequency of VACV-infected B_{large} cells (Fig. 24a). Of the activation markers and chemokine receptors examined, B_{large} cells displayed higher frequencies of VACV-infected cells when compared to B_{small} (Fig. 24b). However, VACV infection was similar between the two populations in CD86 expressing cells (Fig. 24b) despite B_{large} cells displaying a larger percentage of VACV-bound cells (Fig. 21b).

Examination of B cell populations in VACV-infected cells revealed memory B cells in the B_{large} population, specifically IgM only and class-switched memory, displayed an increased frequency of VACV-infected cells, whereas plasma cells in the B_{small} population revealed an increased frequency of VACV-infected cells (Fig. 25a). While B_{small} cells had less CD86 and more CD80 expressing cells, no difference in the percentage of VACV-infected cells was noted between the two populations (Fig. 25b). Evaluation of specific chemokine

receptors in VACV-infected cells in B_{small} and B_{large} cells revealed results resembling the distribution noted in mock infected B cells (Fig. 25b).

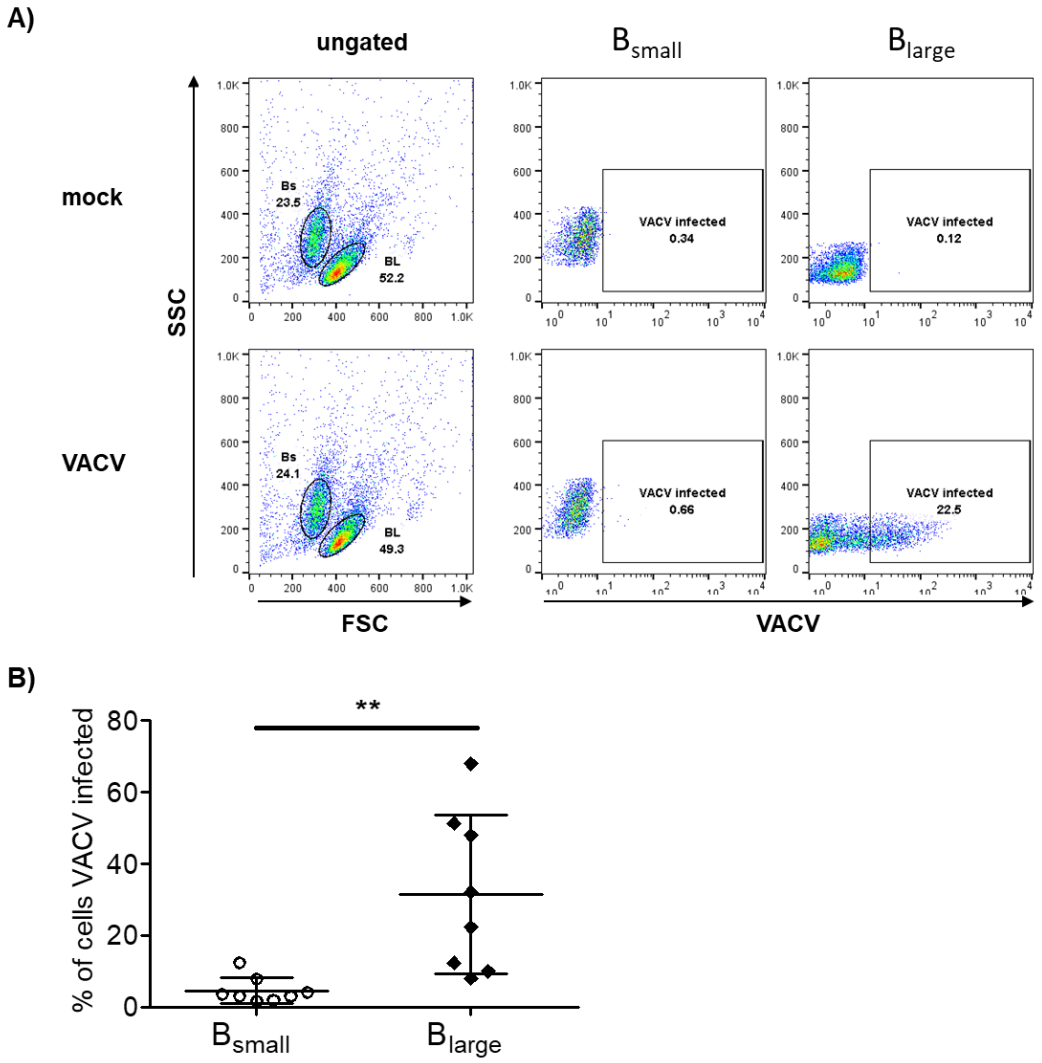

Figure 23. Differential VACV infection in B_{small} and B_{large}. Analysis of VACV infection (12 h) at a MOI of 0.5 (VV-EGFP) in both B cell populations. A) Representative FCM plots for VACV infection in B_{small} and B_{large}. B) Pooled data from eight healthy donors. Each dot in a group represents data from a single individual. **, $p < 0.01$. Bs, B_{small} B cell population; BL, B_{large} B cell population.

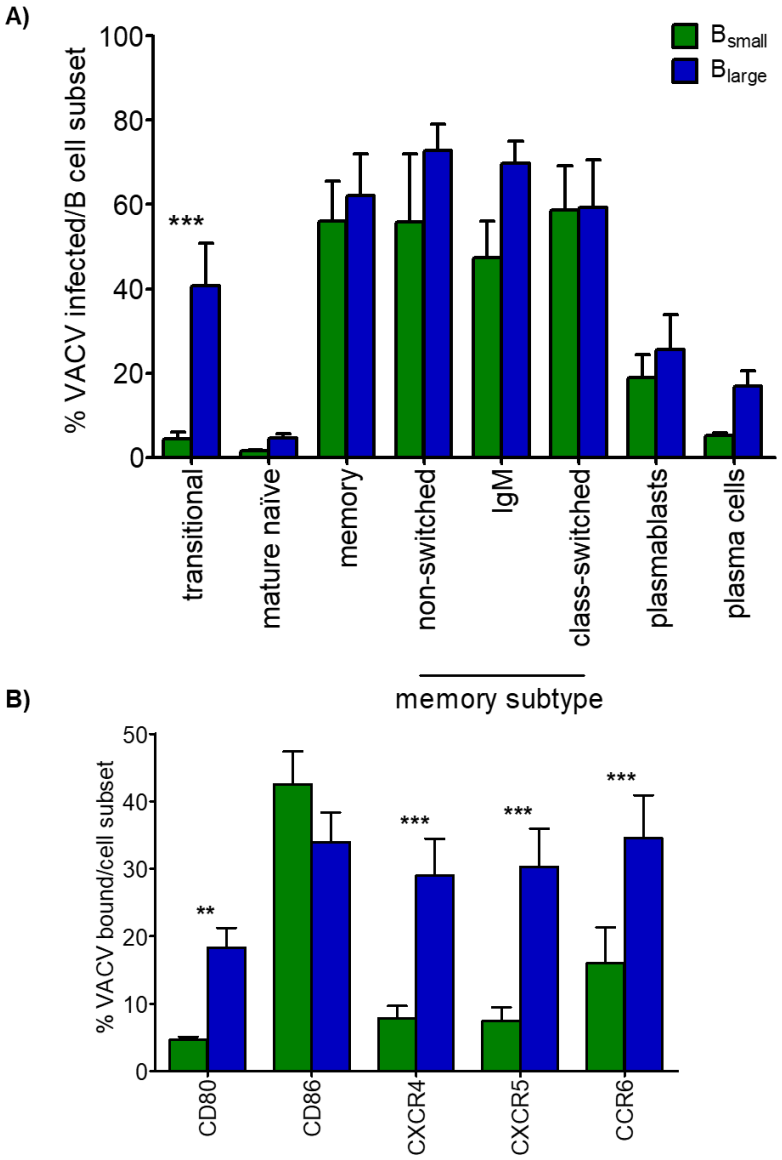

Figure 24. Transitional B cells displayed increased infection in B_{large} cells. Analysis of 12 h VACV infection (VV-EGFP) at a MOI of 0.5 in both B cell populations. A) In the two B cell populations, VACV infection in five major B cell subsets found in peripheral blood and three subtypes of memory B cells. B) Analysis of VACV infection in cells expressing common activation markers and chemokine receptors in both populations. Graphs represent mean ± SEM; n= 3. **, $p < 0.01$; ***, $p < 0.001$.

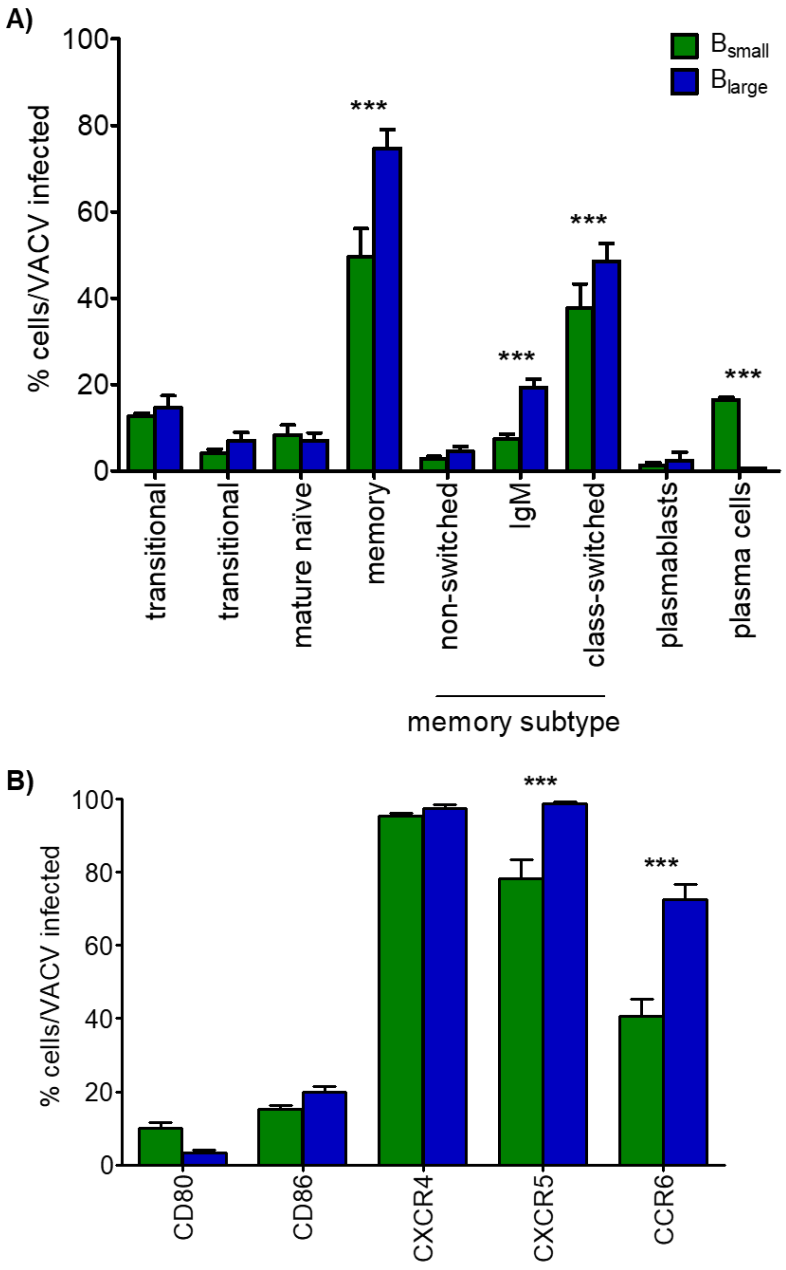

Figure 25. B cell subsets in VACV infected B cells. A) Percentage of cells displaying specific B cell phenotypes in the VACV-infected cells within both B cell populations. B) Percentage of cells displaying specific markers in the VACV-infected cells within both populations. Graphs represent mean ± SEM; n= 3. *, $p < 0.05$; ***, $p < 0.001$.

Stimulated B cells displayed productive VACV infection

Previous studies have shown that activation of naïve T cells leads to susceptibility to VACV binding and infection contrary to VACV binding resistant resting T cells (56, 98). Additionally, autoimmune skin disorders are a contraindication for receiving the smallpox vaccine and these disorders result in overactive B cells (92, 107). Thus, we examined VACV infection in stimulated B cells to assess the effect of cell activation on VACV infection in B cells. Cell surface marker expression and cytokine production were assessed to first confirm B cell activation. Stimulated B cells displayed increased surface expression of CD69, a common early lymphocyte activation marker, and CD86, a common lymphocyte activation marker, suggesting successful activation (Fig. 26a). CD83, a B cell activation marker, expression was also increased in the stimulated B cells compared to the unstimulated B cells (Fig. 26a). Expression of CD80 was slightly upregulated, but to a lesser degree when compared to CD69, CD83, and CD86 (Fig. 26a). In addition to cell surface activation markers, the cytokines IL-6 and IL-10, which are usually upregulated during activation of B cells, were assessed. IL-6 expression was increased in stimulated when compared to unstimulated B cells which confirmed the activation of the B cells (Fig. 26b). Furthermore, IL-10 expression was also increased in the stimulated B cells but was not significant due to the immense variation between samples (Fig. 26c). Evaluation of multiple other cytokines via multiplex confirmed the drastic alterations in cytokine production expected after stimulation (Table 4).

To assess the effect of stimulation on VACV infection in B cells, both non-stimulated and stimulated B cells were infected with VACV. By 24 h.p.i., stimulated B cells exhibited greater VACV infection as compared to unstimulated cells (Fig. 27). To further examine the effect of stimulation on VACV infection in B cells, VACV gene expression was examined by RT-PCR. The viral gene expression pattern observed in HeLa cells is early viral gene expression occurring within 2 h.p.i., intermediate within 3 to 6 h.p.i., and late genes by 6 to 8 h.p.i. Assessment of VACV gene expression in infected stimulated B cells showed expression of early, intermediate, and late viral gene expression (Fig. 28). The pattern of viral gene expression in stimulated B cells was similar to that observed in the HeLa cell line. Since the viral gene expression was similar to that of a cell line with productive VACV infection, we next examined viral replication in stimulated B cells. Plaque assays were completed on stimulated B cells infected with VACV WR at a MOI of 2 and their supernatants. In stimulated VACV-infected B cells, viral titers displayed a trend of increasing in the cells over the 48 hour period (Fig. 289a, 29b). However, viral titers reduced over time in the supernatants suggesting new virions were generated but did not undergo egress (Fig. 29c, 29d).

To better understand the effect of VACV infection in stimulated B cells, we evaluated B cell function during VACV infection by examining cytokine production via multiplex analysis. When infected cells were compared to uninfected, the 8 h.p.i. and 24 h.p.i. infected cells displayed a trend of decreased cytokine levels (Table 5). Additionally, using 3 h.p.i. as the baseline, there was a trend of steadily

increasing cytokine levels in the infected, stimulated B cells in the later time points (Table 5). When the cytokine production was narrowed down to several cytokines and chemokines involved in viral infection and examined on a single donor level, IL-10 and IL-21 displayed patterns amongst the six donors. For the majority of the donors, IL-10 levels stayed level at 3 h.p.i. and decreased at 8 h.p.i. and 24 h.p.i. in infected B cells when compared to uninfected cells (Table 6). IL-21 displayed a different pattern with increased expression at 3 h.p.i. followed by decreased and subsequently increased expression at 8 h.p.i. and 24 h.p.i., respectively (Table 6). Of note, IFNα levels were decreased or stayed relative level over the 24 h of infection, suggesting that VACV infection was regulating the immune response as seen previously in other studies (Table 6) (108).

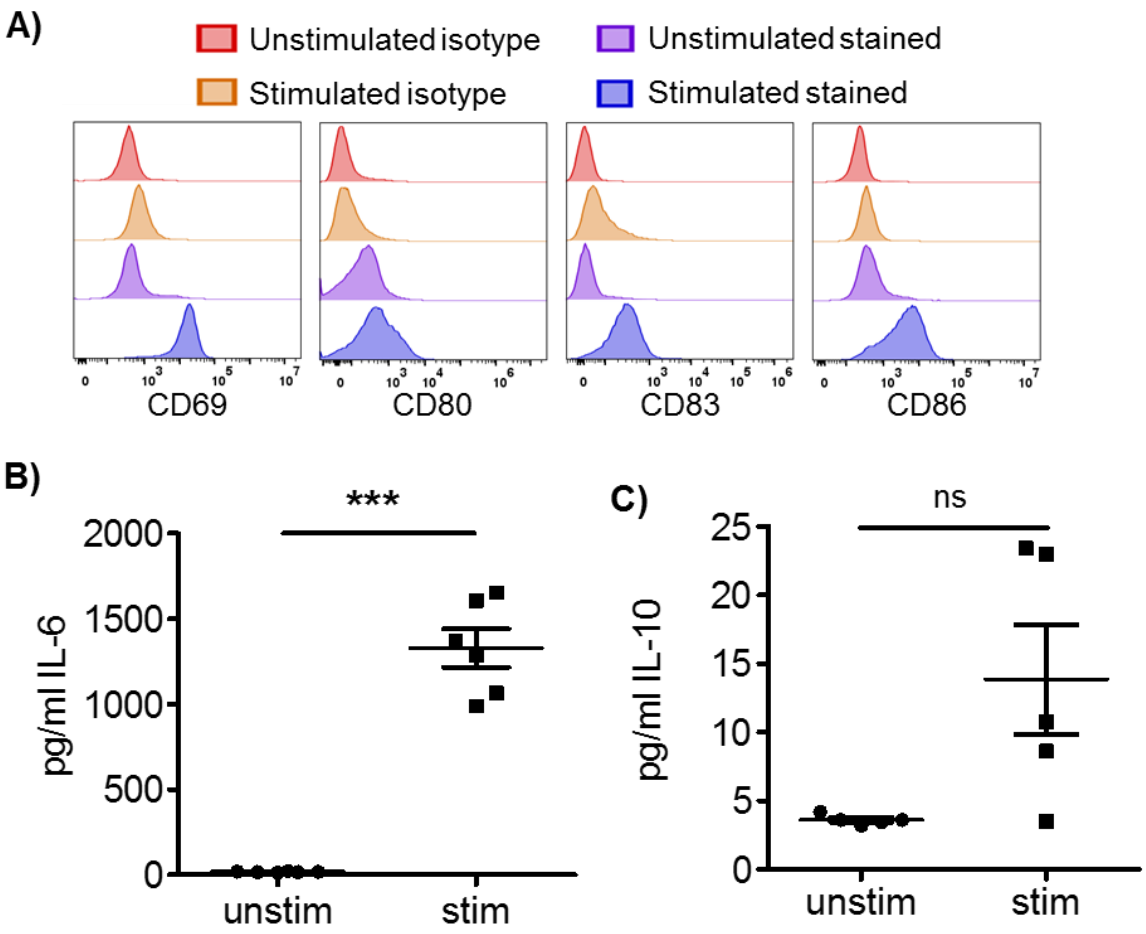

Figure 26. B cell activation analysis. Evaluation of stimulation in B cells. Purified B cells were cultured in the presence of 20 ug/ml AffiniPure F(ab')$_2$ Goat Anti-Human IgG + IgM (H+L) and 50 nM TLR9 agonist CpG ODN 2006 for 24 h. A) Analysis of B cell activation by assessing common B cell activation markers via FCM. B) IL-6 levels in the supernatant of unstimulated and stimulated B cells. n=6. C) IL-10 levels in unstimulated and stimulated B cells. n=5. unstim, unstimulated B cells; stim, stimulated B cells; ns, not significant; h.p.i., hours post-infection. Lines represent mean ± SEM. ***, $p < 0.001$. ns, not significant.

Table 4. Cytokine, chemokine, and growth factor production in unstimulated and stimulated B cells.

Analytes	3 Hours		8 Hours		24 Hours	
	unstimulated	stimulated	unstimulated	stimulated	unstimulated	stimulated
IFNα	2.3 ± 3.3	19.9 ± 13.7	1.9 ± 1.7	35.8 ± 21.1	2.2 ± 2.6	47.0 ± 54.8
IFNγ	2.4 ± 1.7	44.0 ± 40.0	2.2 ± 1.5	83.9 ± 71.4	1.8 ± 1.3	79.0 ± 113.4
IL-1α	1.0 ± 1.1	8.4 ± 5.5	0.5 ± 0.7	11.8 ± 8.4	0.9 ± 0.8	14.2 ± 15.8
IL-1β	1.5 ± 1.7	38.3 ± 27.2	1.1 ± 1.0	76.4 ± 65.4	1.0 ± 1.8	125.2 ± 169.2
IL-1RA	108.1 ± 122.8	1761 ± 1369	165.1 ± 183.5	3661 ± 2915	235.8 ± 331.4	36391 ± 82493
IL-2	13.8 ± 8.7	132.8 ± 86.8	15.6 ± 11.7	230.6 ± 192.5	10.7 ± 5.2	200.8 ± 212.3
IL-4	10.5 ± 6.5	75.1 ± 45.7	9.7 ± 6.5	142.9 ± 99.1	9.8 ± 4.3	147.0 ± 140.5
IL-5	5.6 ± 1.8	33.1 ± 19.1	4.7 ± 2.6	47.7 ± 27.7	4.6 ± 3.3	33.9 ± 22.6
IL-6	19.5 ± 30.9	748 ± 399	27.4 ± 31.6	1738 ± 1261	16.0 ± 25.6	2921 ± 3939
IL-7	1.7 ± 1.4	12.1 ± 5.9	2.0 ± 1.8	15.7 ± 7.7	1.9 ± 1.4	18.6 ± 8.5
IL-9	18.4 ± 35.7	18.1 ± 27.4	15.6 ± 25.0	36.8 ± 42.3	8.1 ± 18.6	83.3 ± 121.6
IL-10	1.0 ± 0.4	32.0 ± 26.2	1.0 ± 0.3	127.8 ± 144.5	1.0 ± 0.2	215.0 ± 146.0
IL-12p70	0.04 ± 0.0	26.7 ± 18.5	0.04 ± 0.0	38.2 ± 21.5	0.04 ± 0.0	42.3 ± 36.0
IL-13	5.2 ± 5.6	35.5 ± 19.1	4.3 ± 5.0	53.7 ± 33.1	2.8 ± 3.3	61.8 ± 52.3
IL-15	8.6 ± 5.6	87.4 ± 37.3	6.9 ± 6.4	136.0 ± 68.9	12.1 ± 5.5	146.6 ± 124.1
IL-17A	3.4 ± 3.1	50.6 ± 29.8	3.9 ± 2.5	67.4 ± 44.6	3.1 ± 1.8	67.1 ± 61.9
IL-18	10.5 ± 6.2	54.3 ± 25.5	12.2 ± 10.8	105.5 ± 69.2	11.4 ± 6.5	138.7 ± 183.2
IL-21	11.5 ± 9.4	458.9 ± 338.7	10.7 ± 10.1	566.2 ± 356.9	17.3 ± 11.2	411.4 ± 340.5
IL-22	86.4 ± 96.2	95.9 ± 29.4	53.9 ± 37.0	130.6 ± 63.7	42.0 ± 33.5	138.6 ± 51.2
IL-23	289.9 ± 351.4	334.2 ± 266.4	138.3 ± 129.6	557.2 ± 360.0	439.7 ± 418.6	701.7 ± 529.6
IL-27	59.4 ± 85.7	545.3 ± 294.9	48.6 ± 67.6	736.1 ± 497.4	28.0 ± 56.1	909.1 ± 909.4
IL-31	54.6 ± 119.1	57.4 ± 64.3	39.5 ± 63.5	105.8 ± 142.5	3.3 ± 0.0	125.4 ± 138.8
LIF	0.6 ± 0.4	7.1 ± 3.5	0.7 ± 0.4	9.2 ± 4.4	0.7 ± 0.6	8.5 ± 5.6
TNFα	7.9 ± 2.9	36.1 ± 22.0	8.0 ± 4.3	73.0 ± 57.6	7.0 ± 2.7	82.0 ± 83.3
TNFβ	1.6 ± 0.0	1.7 ± 0.2	1.7 ± 0.2	10.4 ± 17.7	1.6 ± 0.0	130.3 ± 226.7
Eotaxin	1.5 ± 0.2	2.9 ± 1.3	1.6 ± 0.3	5.3 ± 2.7	1.7 ± 0.6	6.4 ± 5.0
GROα	1.4 ± 1.6	8.3 ± 11.0	0.8 ± 0.6	28.7 ± 39.0	1.3 ± 1.3	67.1 ± 129.2
IL-8	193.5 ± 344.9	523.8 ± 580.7	356.8 ± 745.8	1339 ± 1058	392.1 ± 786.7	2762 ± 3667
IP-10	6.6 ± 4.1	8.4 ± 3.2	7.2 ± 4.3	12.0 ± 5.3	5.9 ± 6.5	18.0 ± 9.1
MCP-1	338.4 ± 424.5	719.3 ± 799.6	307.8 ± 356.2	1409 ± 1415	1317 ± 1469	2191 ± 2807
MIP-1α	3.6 ± 3.0	65.7 ± 35.5	5.2 ± 6.0	123.1 ± 72.3	5.8 ± 2.9	274.3 ± 414.8
MIP-1β	23.7 ± 32.4	174.4 ± 54.3	18.8 ± 23.2	393.1 ± 125.4	52.7 ± 17.6	841.3 ± 620.6
RANTES	6.2 ± 8.9	4.7 ± 3.5	4.9 ± 4.8	16.8 ± 26.0	3.7 ± 3.6	19.7 ± 19.1
SDF-1α	394.0 ± 528.0	1777 ± 1581	197.1 ± 212.8	3377 ± 2579	806.0 ± 915.1	6279 ± 9814
BDNF	2.3 ± 2.5	3.5 ± 1.9	2.0 ± 2.0	5.3 ± 2.8	2.1 ± 2.3	8.2 ± 10.8
EGF	3.6 ± 4.0	8.1 ± 2.6	2.6 ± 1.5	10.7 ± 4.1	3.9 ± 4.3	13.2 ± 9.1
FGF-2	6.1 ± 8.2	8.7 ± 8.7	7.1 ± 7.2	12.5 ± 14.3	5.3 ± 4.3	14.0 ± 16.4
GM-CSF	13.1 ± 13.4	168.8 ± 78.1	20.2 ± 22.9	197.3 ± 111.0	9.3 ± 10.2	180.3 ± 126.2
HGF	8.0 ± 5.7	86.1 ± 46.7	7.0 ± 4.4	127.0 ± 79.8	9.1 ± 4.4	162.3 ± 139.8
NGFβ	2.5 ± 1.5	20.9 ± 12.7	4.2 ± 2.1	42.8 ± 30.7	2.9 ± 2.1	64.6 ± 88.3
PDGF-BB	8.4 ± 6.0	13.9 ± 5.4	5.8 ± 1.8	15.9 ± 6.4	7.1 ± 3.7	13.8 ± 5.7
PIGF-1	3.4 ± 4.4	58.0 ± 35.3	2.2 ± 3.1	93.1 ± 60.1	3.5 ± 3.5	81.2 ± 70.8
SCF	3.7 ± 3.1	32.6 ± 22.6	2.5 ± 2.9	56.0 ± 41.4	2.5 ± 2.1	62.9 ± 69.4
VEGF-A	11.9 ± 15.9	470.5 ± 324.9	8.0 ± 10.8	607.0 ± 328.1	10.0 ± 11.5	454.0 ± 266.5
VEGF-D	2.3 ± 2.4	42.3 ± 20.3	1.6 ± 0.8	47.7 ± 32.1	3.4 ± 3.9	41.2 ± 31.8

Cytokine, chemokine, and growth factor multiplex analysis of supernatants from unstimulated and stimulated (anti-Human IgG + IgM and CpG ODN 2006 for 24 h) B cells of six healthy donors.

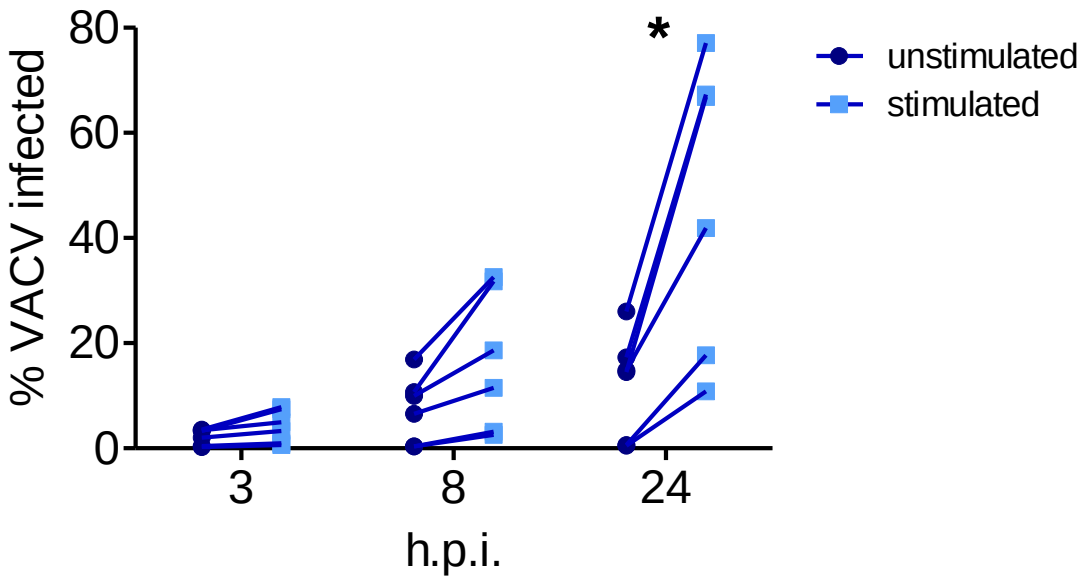

Figure 27. Increased VACV-infected cells in stimulated B cells. Evaluation of VACV infection in stimulated B cells using VV-EGFP at a MOI of 2. Each dot in a group represents data from a single individual; n=5. *, $p < 0.05$. h.p.i., hours post-infection.

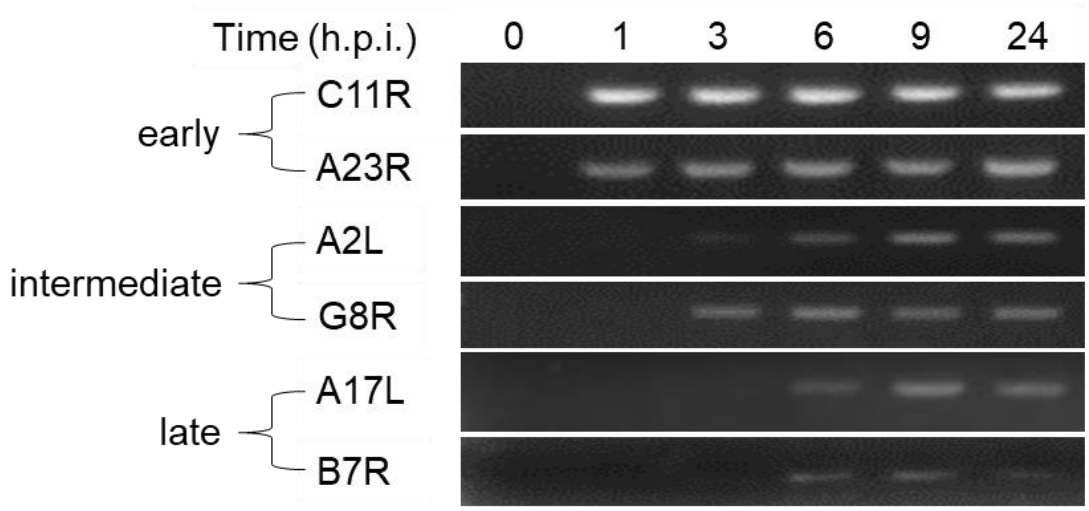

Figure 28. VACV gene expression occurred in stimulated B cells. VACV early (C11R and A23R), intermediate (A2L and G8R), and late (A17L and B7R) gene expression in stimulated B cells (anti-Human IgG + IgM and CpG ODN 2006 for 24 h) assessed by RT-PCR. n=2.

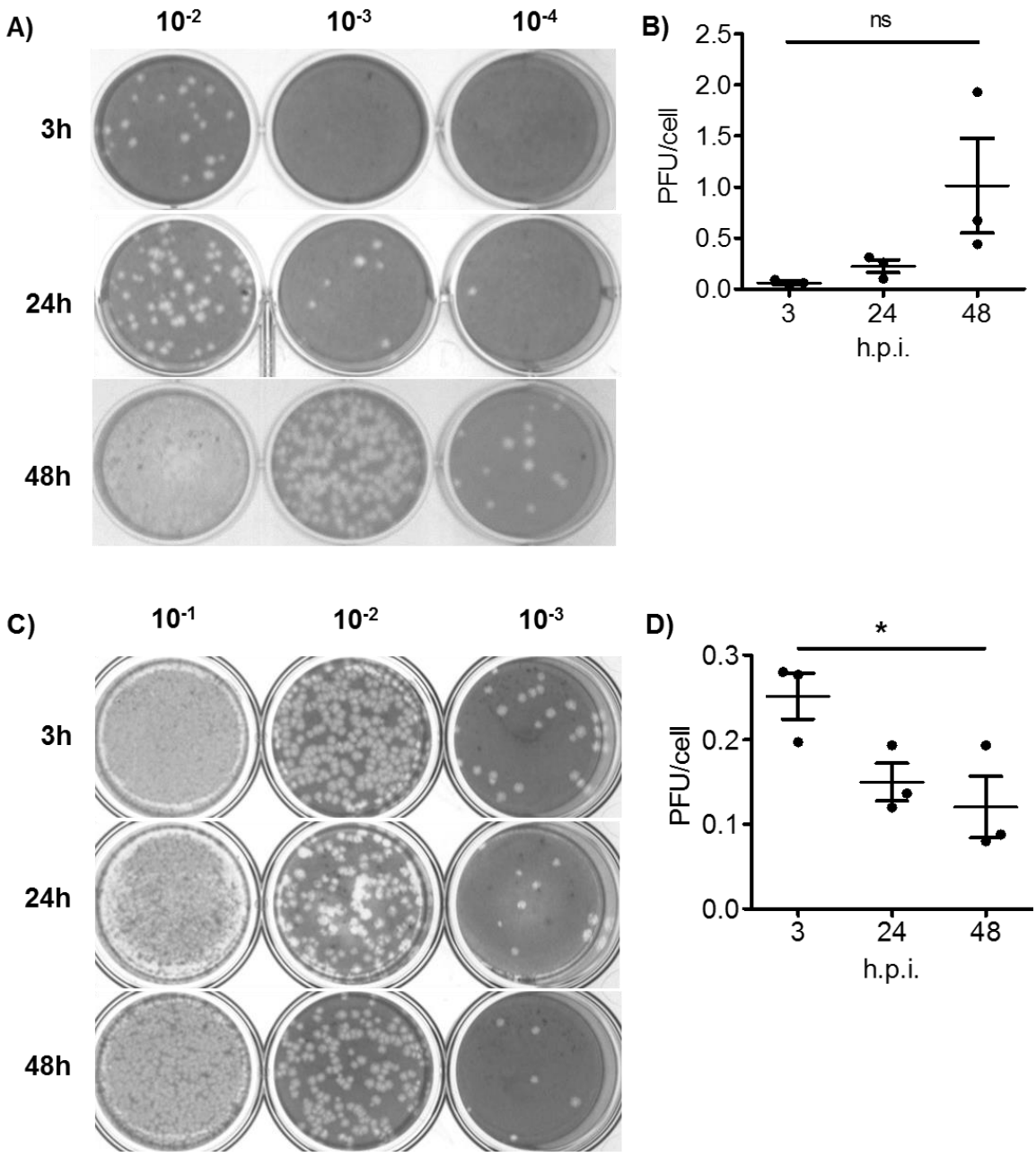

Figure 29. VACV infection in stimulated B cells was permissive. A) Representative virus plaque assay using stimulated B cells infected with VACV-WR at a MOI of 2 for various time points after incubation under binding conditions. B) Pooled data from plaque assays. Each dot in a group represents data from a single individual; n=3. C) Representative plaque assay using

stimulated B cell supernatant from B cells infected with VACV-WR at a MOI of 2 for various time points after incubation under binding conditions. D) Pooled data for plaque assays from stimulated B cell supernatants. Each dot in a group represents data from a single individual; n=3. Graphs represent mean ± SEM. *, $p < 0.05$. ns, not significant; h.p.i., hours post-infection; PFU, plaque forming unit.

Table 5. Functional analysis of stimulated B cells.

Analytes	3 Hours		8 Hours		24 Hours	
	uninfected	infected	uninfected	infected	uninfected	infected
IFNα	19.9 ± 13.7	19.2 ± 12.3	35.8 ± 21.1	26.2 ± 17.4	47.0 ± 54.8	34.3 ± 22.8
IFNγ	44.0 ± 40.0	42.1 ± 33.8	83.9 ± 71.4	47.3 ± 42.6	79.0 ± 113.4	55.7 ± 65.9
IL-1α	8.4 ± 5.5	8.5 ± 5.3	11.8 ± 8.4	7.8 ± 7.2	14.2 ± 15.8	13.5 ± 12.2
IL-1β	38.3 ± 27.2	40.1 ± 32.2	76.4 ± 65.4	54.9 ± 42.7	125.2 ± 169.2	78.2 ± 60.7
IL-1RA	1761 ± 1369	1609 ± 1064	3661 ± 2915	2286 ± 1984	36391 ± 82493	3407 ± 2218
IL-2	132.8 ± 86.8	144.4 ± 115.3	230.6 ± 192.5	163.2 ± 120.5	200.8 ± 212.3	201.8 ± 255.1
IL-4	75.1 ± 45.7	81.3 ± 49.9	142.9 ± 99.1	98.6 ± 64.8	147.0 ± 140.5	122.9 ± 97.3
IL-5	33.1 ± 19.1	34.4 ± 20.4	47.7 ± 27.7	37.2 ± 22.3	33.9 ± 22.6	49.4 ± 50.9
IL-6	748.5 ± 399.1	748.9 ± 285.3	1738 ± 1261	1238.0 ± 801.9	2921 ± 3939	3380 ± 2153
IL-7	12.1 ± 5.9	12.3 ± 6.7	15.7 ± 7.7	13.4 ± 6.1	18.6 ± 8.5	18.6 ± 12.4
IL-9	18.1 ± 27.4	14.5 ± 21.8	36.8 ± 42.3	15.4 ± 18.5	83.3 ± 121.6	45.6 ± 25.4
IL-10	32.0 ± 26.2	26.3 ± 15.4	127.8 ± 144.5	77.1 ± 89.3	215.0 ± 146.0	177.0 ± 138.5
IL-12p70	26.7 ± 18.5	22.6 ± 17.8	38.2 ± 21.5	24.7 ± 18.9	42.3 ± 36.0	34.0 ± 18.6
IL-13	35.5 ± 19.1	34.8 ± 21.6	53.7 ± 33.1	41.3 ± 29.5	61.8 ± 52.3	53.3 ± 41.3
IL-15	87.4 ± 37.3	96.8 ± 50.2	136.0 ± 68.9	99.6 ± 57.7	146.6 ± 124.1	124.8 ± 57.0
IL-17A	50.6 ± 29.8	51.6 ± 30.0	67.4 ± 44.6	50.5 ± 34.7	67.1 ± 61.9	51.6 ± 35.5
IL-18	54.3 ± 25.5	70.1 ± 42.9	105.5 ± 69.2	66.5 ± 44.5	138.7 ± 183.2	83.7 ± 62.5
IL-21	458.9 ± 338.7	458.0 ± 279.7	566.2 ± 356.9	404.6 ± 308.0	411.4 ± 340.5	580.8 ± 539.3
IL-22	95.9 ± 29.4	99.5 ± 33.9	130.6 ± 63.7	99.2 ± 53.8	138.6 ± 51.2	144.9 ± 48.1
IL-23	334.2 ± 266.4	342.2 ± 312.2	557.2 ± 360.0	414.3 ± 241.5	701.7 ± 529.6	556.9 ± 265.9
IL-27	545.3 ± 294.9	510.9 ± 299.3	736.1 ± 497.4	586.2 ± 424.2	909.1 ± 909.4	717.5 ± 478.4
IL-31	57.4 ± 64.3	57.8 ± 72.3	105.8 ± 142.5	52.1 ± 76.5	125.4 ± 138.8	98.8 ± 111.9
LIF	7.1 ± 3.5	6.9 ± 2.8	9.2 ± 4.4	6.9 ± 3.9	8.5 ± 5.6	7.4 ± 3.5
TNFα	36.1 ± 22.0	37.4 ± 22.7	73.0 ± 57.6	56.3 ± 36.5	82.0 ± 83.3	73.7 ± 71.6
TNFβ	1.7 ± 0.2	1.6 ± 0.0	10.4 ± 17.7	6.6 ± 8.3	130.3 ± 226.7	40.8 ± 50.6
Eotaxin	2.9 ± 1.3	4.0 ± 2.1	5.3 ± 2.7	4.0 ± 2.3	6.4 ± 5.0	4.8 ± 2.1
GROα	8.3 ± 11.0	8.0 ± 10.2	28.7 ± 39.0	15.8 ± 21.0	67.1 ± 129.2	20.5 ± 22.0
IL-8	523.8 ± 580.7	474.7 ± 523.7	1339 ± 1058	958.9 ± 728.6	2762 ± 3667	1477 ± 983.0
IP-10	8.4 ± 3.2	7.9 ± 5.6	12.0 ± 5.3	9.0 ± 3.2	18.0 ± 9.1	14.5 ± 5.4
MCP-1	719.3 ± 799.6	611.6 ± 749.7	1409 ± 1415	796.5 ± 793.8	2191 ± 2807	1244 ± 940.7
MIP-1α	65.7 ± 35.5	49.3 ± 30.3	123.1 ± 72.3	95.4 ± 44.8	274.3 ± 414.8	157.3 ± 53.5
MIP-1β	174.4 ± 54.3	154.0 ± 55.8	393.1 ± 125.4	298.7 ± 183.6	841.3 ± 620.6	648.8 ± 274.8
RANTES	4.7 ± 3.5	5.7 ± 5.1	16.8 ± 26.0	8.8 ± 7.7	19.7 ± 19.1	14.9 ± 17.2
SDF-1α	1777 ± 1581	1617 ± 1041	3377 ± 2579	1617 ± 1328	6279 ± 9814	2758 ± 2049
BDNF	3.5 ± 1.9	4.3 ± 4.0	5.3 ± 2.8	3.1 ± 1.5	8.2 ± 10.8	5.0 ± 2.2
EGF	8.1 ± 2.6	8.5 ± 3.9	10.7 ± 4.1	8.6 ± 3.8	13.2 ± 9.1	10.2 ± 2.7
FGF-2	8.7 ± 8.7	15.1 ± 14.7	12.5 ± 14.3	12.4 ± 13.7	14.0 ± 16.4	17.7 ± 13.9
GM-CSF	168.8 ± 78.1	170.7 ± 93.7	197.3 ± 111.0	176.7 ± 86.2	180.3 ± 126.2	168.4 ± 129.9
HGF	86.1 ± 46.7	96.8 ± 57.1	127.0 ± 79.8	99.6 ± 69.5	162.3 ± 139.8	118.8 ± 67.9
NGFβ	20.9 ± 12.7	25.5 ± 16.0	42.8 ± 30.7	33.9 ± 23.4	64.6 ± 88.3	34.6 ± 31.3
PDGF-BB	13.9 ± 5.4	14.4 ± 4.8	15.9 ± 6.4	12.9 ± 6.0	13.8 ± 5.7	15.5 ± 6.0
PIGF-1	58.0 ± 35.3	65.4 ± 34.9	93.1 ± 60.1	59.2 ± 47.5	81.2 ± 70.8	88.0 ± 80.7
SCF	32.6 ± 22.6	30.3 ± 21.2	56.0 ± 41.4	38.2 ± 30.6	62.9 ± 69.4	45.0 ± 40.6
VEGF-A	470.5 ± 324.9	467.5 ± 227.0	607.0 ± 328.1	424.5 ± 341.0	454.0 ± 266.5	584.0 ± 507.4
VEGF-D	42.3 ± 20.3	45.2 ± 24.1	47.7 ± 32.1	40.4 ± 31.0	41.2 ± 31.8	33.9 ± 15.1

Complete multiplex analysis of cytokines, chemokines, and growth factors in the supernatants of stimulated B cells (anti-Human IgG + IgM and CpG ODN 2006 for 24 h) either incubated with or without VACV. n=6.

Table 6. Individualized cytokine production in stimulated B cells.

		GM-CSF					
		D1	D2	D3	D4	D5	D6
3h	uninfected	266.98	109.36	67.87	168.32	149.09	251.12
	infected	241.42	120.32	67.87	266.98	73.22	254.32
8h	uninfected	380.73	153.01	111.58	83.46	183.1	271.67
	infected	303.66	75.83	88.39	181.28	231.56	179.45
24h	uninfected	383.47	128.8	288.59	62.35	111.58	107.11
	infected	156.9	158.82	111.58	50.63	111.58	421.1
		IFNa					
3h	uninfected	26.89	9.47	4.63	16.54	18.9	42.92
	infected	33.2	14.52	4.5	6.75	30.25	26.25
8h	uninfected	70.2	28	14.81	39.27	15.67	46.66
	infected	51.95	7.29	10.02	37.05	33.03	17.57
24h	uninfected	157.07	15.96	36.37	15.09	20.1	37.56
	infected	47.92	20.85	18.31	18.31	25.31	74.92
		IL-4					
3h	uninfected	105.43	44.35	14.89	72.18	68.29	145.21
	infected	108.79	50.9	35.86	139.01	25.03	128.49
8h	uninfected	279.14	63.67	48.7	62.9	234.21	168.72
	infected	177.94	16.12	35.16	144.32	137.24	80.87
24h	uninfected	426.33	45.79	106.27	76.11	85.69	141.66
	infected	100.42	56.1	87.3	109.63	66.74	317.27
		IL-5					
3h	uninfected	59.8	20.9	11.82	24.11	28.51	53.47
	infected	50.98	23.03	11.82	54.73	13.75	52.22
8h	uninfected	89.12	29.63	21.96	21.96	67.57	55.99
	infected	64.96	15.74	12.78	31.89	62.37	35.34
24h	uninfected	59.8	25.19	64.96	13.75	24.11	15.74
	infected	35.34	38.85	21.96	9.93	40.04	150.57
		IL-6					
3h	uninfected	1358.4	578.34	233.69	689.86	572.2	1058.48
	infected	1238.71	717.04	391.38	808.01	564.02	773.96
8h	uninfected	2649.5	1324.55	611.18	640.04	3824.94	1377.87
	infected	1791.81	509.02	484.66	1375.43	2493.15	773.96
24h	uninfected	*10934.86	968.94	1298.14	1267.13	1122.29	1938.6
	infected	2090.06	4656.07	913.91	2395.44	3265.17	6961.8
		IL-10					
3h	uninfected	32.04	10.1	5.41	28.37	79.02	36.84
	infected	37.59	10.7	6.23	37.77	42.63	22.58
8h	uninfected	79.8	28.56	25.1	66.32	404.71	162.33
	infected	60.84	7.65	12.68	71.63	251.05	58.82
24h	uninfected	231.2	41.88	76.78	227.74	263.71	448.76
	infected	52.82	50.54	60.55	316.05	238.22	344.09
		IL-21					
3h	uninfected	526.18	371.71	96.36	179.1	532.92	1047.3
	infected	873.41	416.37	128.73	564.56	175.4	589.66
8h	uninfected	730.03	880.84	232.09	98.02	981.02	475.08
	infected	640.46	175.4	141.05	267.37	918.15	285.33
24h	uninfected	578.22	459.72	991.16	104.72	175.4	158.97
	infected	352.86	861.07	201.51	139.28	378.03	1552.18

Individualized donor data for several cytokines and chemokines from the multiplex analysis of stimulated B cells (anti-Human IgG + IgM and CpG ODN

2006 for 24 h) either incubated with or without VACV. *, value above maximum detection limit.

Chapter IV – Discussion

Profile of VACV binding and infection in B cells

Currently, various viral vaccines and oncolytic cancer vaccines based on live poxvirus vectors, such as MVA and ALVAC, are being used in veterinary medicine and tested in clinical trials for use in humans (109-112). These poxvirus vectors typically display a reduced induction of the immune response compared to wildtype strains. Many clinical trials are evaluating the use of VACV vectors, such as MVA, as preventative and therapeutic vaccines to target various diseases, such as HIV-1, hepatitis B, influenza, malaria, tuberculosis, and cancers (113-121). The RV144 clinical trial demonstrated a limited although promising protective effect against HIV-1 by using a poxvirus-based HIV-1 vaccine prime in combination with a HIV-1 envelope boost (110). Additionally, clinical trials using a poxvirus-based oncolytic therapy have demonstrated promising safety and efficacy in patients with various cancers (111, 112). The interest in poxvirus biology has been renewed due to these successes in poxvirus-based vaccines. Discovering VACV receptors and characterizing VACV binding and infection tropism will aid in better understanding the distinctive VACV-based efficacy, development of more specific and potent oncolytic poxvirus-based therapies, and development of more efficacious and safer VACV-based vaccines against smallpox and other infectious viruses.

In the present study, we systematically studied VACV binding to and infection of unstimulated *ex vivo* and stimulated primary human B cells. We

observed that VACV bound to primary *ex vivo* B cells robustly but weakly infected these cells (Fig. 4), which is in agreement with previous reports using PBMCs (56, 60, 98). Further examination into the binding and infection in these B cells showed three patterns of virus binding and infections in the various B cell subsets (Fig. 5b, 9b). Our study revealed that only one B cell subset, plasmablasts, displayed resistance to VACV binding, while the other subsets displayed similar viral binding to each other and other APCs (Fig. 5b). Additionally, the majority of the memory B cells with VACV bound displayed viral entry and infection, whereas the other subsets were more resistant to viral entry and infection despite the high rates of binding (Fig. 5b, 9b). These results suggest that most B cell subsets express the receptor(s) necessary for either non-specific or specific binding of VACV. However, the greater part of B cells appears to lack the cellular signaling pathways necessary for viral uptake and entry, and memory B cells may possess these essential viral entry signaling pathways and other cellular pathways crucial for other downstream viral events. Additionally, these results may indicate that plasmablasts might lack high expression of glycoprotein attachment factors necessary for the initial encounter between host cell and VACV, as well as the specific receptor(s) necessary for viral entry. Although many viruses such as influenza A or hepatitis A use a single molecular species as their receptors (25), VACV may require multiple molecular species as receptors and only certain subsets of B cells, such as memory B cells, express the obligatory receptors for both binding and entry. The drastic difference in VACV binding between plasmablasts and the other B cells subsets

along with the three binding and infection patterns observed among the B cell subsets indicate the various B cell subsets could be an invaluable tool to examine the molecular mechanisms involved in VACV binding, penetration, and entry potentially, leading to a better understanding of poxvirus tropism and binding receptor(s) as well as efficacy of VACV-based smallpox vaccines.

Differences in permissivity of infection between *ex vivo* and stimulated B cells were observed in this study. Of note, stimulated B cells displayed higher levels of infection when compared to unstimulated cells (Fig. 27). Stimulated B cells also exhibited a trend of increasing viral titers over a two-day period, suggesting viral replication (Fig. 29a). These data imply stimulation of B cells might lead to synthesis of the receptor(s) necessary for viral binding and entry and essential changes in signaling pathways to permit viral entry signaling and viral replication. Stimulation or activation of B cells leads to changes in endocytosis. Activated B cells endocytose various lipid rafts to internalize B cell receptors that translocate to lipid rafts (122). Additionally, VACV has been shown to localize to receptors in lipid rafts (61).Thus, the higher rate of VACV infection noted in stimulated B cells (Fig. 27) may be related to the changes in endocytosis upon activation of B cells leading to increased endocytosis of VACV. The use of unstimulated and stimulated B cells may also prove to be a valuable model to assess the downstream viral events possibly affecting poxvirus tropism.

Permissivity of VACV infection in *ex vivo* and stimulated B cells

In the past, orthopoxvirus infection in humans focused on keratinocytes, fibroblasts, epithelial cells, and macrophages depending on the route of infection, such as respiratory or dermal. However, animal studies have shown that other cell types, including T and B cells, could be important to orthopoxvirus infection. A tail scarification mouse study using VACV showed T and B cells were required for the anti-viral immune response to provide inoculation site infection control and prevention of viral dissemination, respectively (123). Additionally, an assessment of immune cell infiltration in rabbits with VACV keratitis revealed infiltration of T and B cells into the eye after a corneal VACV inoculation (124). VACV infection is permissive in dermal cells and macrophages, and resting T cells are resistant to infection (56, 61). Also, activated T cells were shown to be permissive to VACV infection (56, 61). However, VACV infection in B cells has not been definitively shown. B cell were shown to express low levels of the VACV late gene A56R via a reporter virus, implying infection was abortive (56). We have validated abortive infection in B cells by showing no virus production in primary B cells using virus plaque assays and minimal viral late gene expression (Fig. 11, 14). In contrast, we found that upon stimulation, B cells displayed increased VACV infection in stimulated compared to unstimulated (Fig. 27) and VACV infection was no longer abortive (Fig. 29).

VACV infection in memory B cells

Memory B cells are crucial for the Ab response to be recalled upon recognition of a previously encountered specific Ag and replenishing the plasma cell pool. These memory cells are essential for long-term immunity against pathogens and developed by vaccination. The smallpox vaccine demonstrates a respectable safety profile and leads to the generation of memory B cells to provide a long-term immunity (86). VACV displayed robust binding, 65.4%, to memory B cells (Fig. 5b). Interestingly, VACV showed a bias towards infecting memory B cells, infecting over 50% of memory B cells, when compared to the other subsets, which less than 20% were infected (Fig. 9b). Furthermore, class-switched memory B cells were the predominate memory B cells subtype observed in the VACV-infected cells (Fig. 10a). Additionally, B_{large} B cells showed increased VACV infection and percentage of memory B cells (Fig. 18a, 19a, 21a). These data suggest that memory B cells are more likely to be infected by VACV, when compared to other B cell subsets. An implication of VACV infecting memory B cells is the possibility of VACV infection affecting memory B cell function and even immunity. Thus, the smallpox vaccine could affect immunity to other antigens or pathogens depending on the reaction to the vaccination, such as generalized vaccinia or progressive.

VACV infection in relation to proliferation

Viral infection of a cell usually triggers cell death, but viruses have numerous mechanisms to inhibit the viral-induced apoptosis (125). These cellular

death subversion mechanisms include producing homologues of anti-apoptotic factors (126) or inhibitors of pro-apoptotic factors (127). By inhibiting apoptosis, the survival of the cell usually permits viral replication. However, these factors are not produced shortly after VACV infection (128). To subvert the induced apoptosis early in infection, VACV produces an epidermal growth factor (EGF)-like growth factor to activate the EGF receptor (EGFR)-Ras pathway required for viral replication (129) and act as a mitogen to prime nearby cells for infection (130). Additionally, VACV is thought to infect proliferating cells.

In total B cells, we observed two populations differing in size and complexity, and B_{large} displayed an increased percentage of VACV-infected cells (Fig. 23). Interestingly, these populations varied in the cell cycle phase and proliferative state, with B_{large} cells having a higher percentage of proliferating cells (Fig. 17). These data suggest B_{large} cells may express increased levels of EGFR to support VACV infection. Another possibility for the increased VACV infection is that B_{large} cells are primed, due to their proliferative state, for infection. Additionally, the alterations associated with B cell stimulated permitted VACV infection and replication when compared to non-permissive *ex vivo* B cells (Fig. 29), suggesting that stimulation of B cells support VACV infection possibly through EGFR signaling or production of viral factors necessary for evasion of apoptosis.

Chapter V – Future Directions

Detection of potential VACV receptor(s)

No specific poxvirus receptor has been discovered despite the well documented preference of the virus for infection of specific primary cells and the VACV binding tropism. Identification of the poxvirus binding receptor(s) would lead to better engineering strategies for poxvirus-based infectious disease and cancer therapies. Additionally, treatments for VACV infection include vaccinia immune globulin (VIG) and anti-viral drugs like cidofovir, but anti-viral drugs are the only treatment available for orthopoxvirus infections. The development of better poxvirus specific anti-viral therapies to treat infections in both the general public and vulnerable groups, such as immunocompromised individuals, would be assisted by the discovery of the poxvirus receptor(s) for viral binding.

This study revealed B cell subsets could be used to assess VACV receptors due to the differential binding to specific subsets. Furthermore, our lab has demonstrated enrichment of putative VACV receptors in detergent-resistant membranes (DRMs) from primary human leukocytes (61). The results from these studies from our group (61) reveal that analyzing the DRM fractions from plasmablasts compared to plasma or memory B cells would be a method for identifying potential VACV binding receptor(s). These B cell subsets provide a comparison between a more binding-resistant subset and binding-sensitive subsets. Additionally, comparing the non-bound and virus bound plasmablasts would permit further narrowing of potential receptor molecules.

DRMs are typically isolated via detergent solutions, either non-ionic or ionic. Non-ionic detergent solutions allow for DRM isolation with potential loss of some associated proteins, whereas ionic detergents permit isolation and preserves the associated proteins. However, detergent-free isolation of DRMs is best in preparation for mass spectrometry to prevent anomalies or false-positives and support optimal lipid raft characterization. Thus, a detergent-free method would need to be employed (131). Proteome analysis of the isolated DRMs would require delipidation followed by liquid chromatography mass spectrometry (132). These new findings and developed protocols may support the discovering of the previously elusive VACV binding receptors.

Analysis of VACV entry and infection in primary human B cell subsets

Viral entry is one of the main events restricting VACV infection in primary cells. In addition to identifying the VACV binding receptor(s), understanding viral entry will support the development of a safer smallpox vaccine and improved infectious disease and cancer vaccines. As memory B cells and to a lesser extent transitional B cells displayed significant percentages of VACV infected cells along with strong binding, viral entry appears to be affected. VACV entry and uncoating between the B cell subsets can be assessed to examine defects in uncoating. The same confocal assessment used to visualize VACV binding and entry can be used to examine any defects in entry and uncoating in various sorted B cell subsets. Using the vA5L-YFP reporter virus with staining for an IMV membrane protein at different times post-infection, visualization of single-color

events would represent virus entry and potential uncoating. Double color signals would indicate extracellular virions because the cell membrane would not have been permeabilized. To further confirm uncoating, another condition would require membrane permeabilization and viral membrane staining to show a lack of membrane on the internalized virions.

Early and intermediate gene expression was demonstrated to have occurred in total B cells. With the variable viral infection among the subsets, viral expression would also be assessed by RT-PCR in the subsets to better define to subsets contributing to the results seen in total B cells. Since memory B cells were the main B cell type noted in the VACV-infected population, VACV infection in these cells mostly likely halts before viral late gene transcription. However, this does not rule out a minor population contributing to the minimal late gene expression seen. Isolated memory B cells would most likely be the B cell subset to use for examining the exact mechanism behind the lack of late gene expression. This could be assessed by Western blotting for the subunits of the vaccinia late transcription factor (VLTF). Additionally, ChIP-seq could be implemented to examine the association of this viral transcription factor with the viral DNA if the Western blotting shows the presence of VLTF.

Analysis of the antiviral response and cell survival

The antiviral response is a key defense against viral infections, and the interferon (IFN) response is one of these antiviral responses. The production of IFN is initiated by pattern recognition receptors (PRRs) sensing pathogen-

associated molecular patterns (PAMPs). One PRR of note is cyclic GMP-AMP Synthase (cGAS), which senses cytosolic DNA. cGAS activates stimulator of interferon genes (STING) which translocates perinuclear to induce interferon regulatory factor 3 (IRF-3) (133, 134). Activation of IRF-3 induces IFNβ production, a Type I IFN, to combat the viral infection. The Western Reserve strain of VACV has been shown to inhibit the IFN response by preventing dimerization of STING and subsequent activation of IRF-3 (134). VACV infection in unstimulated cells resulted in an abortive infection (Fig. 14) and a slight increase in IFNα production, another Type I IFN (Table 3). These data suggest that VACV may not be inhibiting the IFN response and thus leading to an abortive infection. To assess if the antiviral response is activated, IFNβ levels would be measured via ELISA. Additionally, phosphorylated STING and IRF-3 would be evaluated by western blot.

Fowlpox virus and VACV have been shown to either encode a Bcl-2 homologue, an anti-apoptotic factor or increase the expression of Bcl-2, respectively, in human cells (135, 136). This increase in Bcl-2 leads to an inhibition of apoptosis and promotes cell survival. To examine whether apoptosis or cell survival are crucial to permissive VACV infection, Bcl-2 expression, along with expression of the proapoptotic factor Bax, would be measured via FCM and Western blot at various times of VACV infection in both unstimulated and stimulated B cells.

Further analysis of B cell function during VACV infection

Studies in cell lines have demonstrated altered cell function during VACV infection (63, 105). B cell function was assessed in this study by examining cytokine production; however, cytokine production should be measured in more than six samples as we observed great variation between the samples leading to no significant differences. In addition to cytokine production, B cells have various other functions, such as antibody production and antigen presentation. The effect of VACV infection on antibody production can be examined in couple of ways. One method would be to infect B cells and shortly after stimulation with a known Ag, such UV inactivated hepatitis B, to induce antibody production and measure antibody levels via ELISA. Another method would be to infect B cells and stimulate with CpG or IgM crosslinking to activate the cells, and then measure antibody levels. Antigen presentation is easily studied *in vitro* by using cell lines; however, using primary cells for antigen presentation is quite difficult. Thus, studying cytokine production and antibody response should be sufficient to assess B cell function.

To further define the effect of VACV infection on B cell cytokine and chemokine production, VACV-infected B cells would first be sorted by phenotype. The sorted cells would then undergo single-cell multiplexed cytokine profiling. These data would provide a detailed representation of the cytokines produced by specific B cell phenotypes infected with the virus and the changes brought on by viral infection.

Stimulated B cells and eczema vaccinatum

While the smallpox vaccination can lead to various adverse events, one of the more severe reactions is eczema vaccinatum (EV) which occurs when individuals with eczema or atopic dermatitis receive the vaccine. EV is a potentially lethal widespread rash with skin lesions caused by viremia. This reaction can be successfully treated with VIG or cidofovir. Although having eczema is a contraindication to receiving the vaccine, greater than 15% percent of the US population has eczema or eczema-like symptoms (91), and individuals may not be aware their symptoms are related to eczema. Additionally, individuals with eczema could be exposed by a recently vaccinated person with viral shedding, which has occurred in the past (137). Despite EV being treatable, the underlying mechanisms of this widespread viral dissemination is unknown. The skin of individuals with atopic dermatitis have been shown to have defects in the epidermal barrier (138) and anti-microbial peptide production (139). These skin defects support increased VACV replication which may contribute to the widespread lesions. Additionally, immune cells, particularly macrophages, have been implicated in contributing to EV (140).

Macrophages are not the only immune cell type affected by atopic dermatitis or eczema. Among other alterations in the immune system, these individuals have significantly higher frequencies of chronically activated memory B cells (92). These activated memory B cells could be generating more virions along with possibly disseminating the virus to other sites. B cells isolated from individuals with these autoimmune skin disorders could be used to examine the

role of B cells in EV. First, VACV infection in disease-affected B cells should be assessed by flow cytometry and then plaque assays to observe the rate of infection as well as the amount of virus production. Transmission of virus could be analyzed by co-culturing VACV infected B cells with PBMCs from the same donor or a VACV permissive cell line for various amounts of time up to six hours, to prevent new virion generation. Additionally, VACV infection could be examined in B cells from individuals with EV. EV-patient B cells could be lysed and used for plaque assays to assess viral replication or PCR or intracellular VACV core staining to analyze the VACV burden in these cells.

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Curriculum Vitae

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Education

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Presentations

1. Yang K.*, Lan J.*, Hu N., Byrd D., Amet T., Desai M., **Shepherd N.**, Gao J., Yu Q. “Abrogation of human CD59 function regains activities of neutralizing and non-neutralizing antibodies in triggering antibody-dependent complement-mediated lysis of HIV-1 virions and latently infected cells after provirus activation”. American Association of Immunologists Annual Meeting, New Orleans, LA, May 2015. *Co-first author. (*poster*)
2. Lan J., **Shepherd N.**, Xing Y., Yang K., Amet T., Yu Q. “Blockage of HIV-1 cell-to-cell transmission from provirus-activated latently infected cells to uninfected cells”. 35th American Society for Virology Annual meeting, Blacksburg, Virginia, June 2016 (*oral*)
3. **Shepherd N.**, Lan J., Li W., Xing Y., Yu Q. “Characterization of vaccinia virus binding and infection of primary human B cells”. 45th Autumn Immunology Conference, Chicago, IL, November 2016. (*oral and poster*)

4. **Shepherd N.**, Lan J., Li W., Xing Y., Yu Q. “Vaccinia virus preferentially infects primary human memory B cells”. American Association of Immunologists Annual Meeting, Washington, D.C., May 2017. (*poster*)
5. Lan J., Li W., **Shepherd N.**, Xing Y., Yu Q. “Dissemination of HIV-1 from latently infected cells to resting CD4+ T cells requires CD4 and actin remodeling”. American Association of Immunologists Annual Meeting, Washington, D.C., May 2017. (*poster*)
6. **Shepherd N.** and Yu Q. “Characterization of vaccinia virus binding to and infection of primary human B cells”. 46th Autumn Immunology Conference, Chicago, IL, November 2017. (*oral and poster*)
7. **Shepherd N.**, Lan J., Li W., Xing Y., Rane S., Yu Q. “Primary human B cells and plasma cells exhibit different sensitivities to vaccinia virus binding and infection”. American Association of Immunologists Annual Meeting, Austin, TX, May 2018. (*poster*)

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1. Byrd D., **Shepherd N.**, Lan J., Hu N., Amet T., Yang K., Desai M., Yu Q. Primary human macrophages serve as vehicles for vaccinia virus replication and dissemination. *The Journal of Virology*, 2014 June 15; 88(12):6819-6831. PMID: 24696488.
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8. Stanley M., **Shepherd N**., Duvall N., Jenkinson S., Steele G., Davis C., Bull M., Watkins D., Roper R. Dysphagia in young infants with Down syndrome. *Under revision*
9. Lan J., Li W., **Shepherd N.**, Xing Y., Rane S., Yu Q. Kinetic and Mechanistic Analysis of HIV-1 Cell-to-Cell Transmission from Provirus-Activated Latently Infected Cells to Uninfected Cells. *Under revision*
10. **Shepherd N.**, Li W., Lan J., Xing Y., Rane S., Yu Q. Primary human B cells and plasma cells exhibit different sensitivities to vaccinia virus binding and infection. *Under revision*