CHARACTERIZING NEW MODELS FOR STUDYING ANTIVIRALS AGAINST VARIOLA VIRUS

by

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(Under the Direction of Robert J Hogan)

ABSTRACT

The threat of *Variola virus* (VARV), the causative agent of smallpox, remains today although the disease was declared eradicated in 1980. The development of medical counter measures (MCMs) for smallpox are vital to biopreparedness should smallpox re-emerge. To determine the efficacy of MCMs, surrogate orthopoxviruses (OPXV), such as *Monkeypox virus* (MPXV), are required. VARV is a solely human pathogen and previous attempts to identify an animal model with a route of infection and disease progression that mimics human smallpox post VARV challenge have been unsuccessful. The goal of this work is to characterize new models for studying antivirals against VARV by utilizing the prairie dog MPXV surrogate model to determine if a potential MCM, a monoclonal antibody (mAb) cocktail called Mix4, is efficacious and to determine if humanized mice could serve as a VARV animal model. Our longevity study demonstrated that prairie dogs had no side effects to intraperitoneal administration of human mAbs (48 mg/kg). A plaque reduction neutralization assay (PRNT) determined 50% virus neutralization was seen beginning 1 day post injection until >7 days. In an efficacy study where animals were treated 1 day pre-challenge, challenged on day 0 and treated again 6 days post infection, Mix4 treatment resulted in 80% survival compared to 40% for Vaccinia Immune Globulin and 25% for a non-specific mAb. Mix4 did not provide protection

from morbidity and PRNTs assessing the two main forms of OPXV progeny identified that Mix4 only strongly neutralized one form of viral progeny. PRNTs against the main forms of viral progeny have identified different mAbs combinations (Universal Pox Mix) that could serve as a MCM against MPXV and VARV. While surrogate models are useful, MCMs testing against VARV directly would be beneficial. Humanized (hu-) mice, hu-BLT, hu-$CD34^{+}$ and hu-PBMC were all susceptible to intranasal VARV challenge. Hu-mice developed high pain scores, requiring euthanasia prior to study end. Molecular analysis, including viral titers as high as $1.66x10^{11}$ pfu/gram of tissue, and histopathology supported VARV as the cause death. Due to delayed mortality in the hu-PBMC mice, hu-BLT and hu-$CD34^{+}$ are the best candidates for further characterization.

INDEX WORDS: Variola virus, Monkeypox virus, Animal model, Monoclonal antibody, Monoclonal antibody cocktail, Antiviral

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BA, Washington and Jefferson College, 2011

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2020

Characterizing New Models for Studying Antivirals Against Variola Virus

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December 2020

DEDICATION

I'd like to thank my parents for their emotional and financial support in helping me achieve a PhD. I'd like to thank my grandparents for their weekly check-ins and support. To the pineapple crew, Ginny, Wendy and Christy, thank you for the encouraging words, being there during difficult times and forcing me to take a break from work! Lastly, thank you to my colleagues Zach, Kelsey, Aggi and Matt for your encouragement and support throughout the last five years.

ACKNOWLEDGMENTS

I'd like to thank Dr. Christina Hutson and Dr. Victoria Olson for your support, encouragement and assistance throughout my PhD process. From quick turn-around reviews, after hour hypothesizing and battles with analysis programs, I truly could not have done this without your support. You have both been a tremendous support system and pushed me to continue with the PhD journey when I did not think I could make it through. I'd also like to thank Dr. Hogan for guidance on thesis and non-thesis related projects and career advice. Thank you to my committee for your contributions, comments, guidance and pushing me to think past the research constraints. I'd also like to thank the co-authors on the three research chapters for their hard work and contributions.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Smallpox, caused by *Variola virus* (VARV), has been considered one of the deadliest infectious diseases humankind has faced with an estimated 300 million deaths in the 20th century alone. The disease was declared eradicated in 1980 by the World Health Organization (WHO) after an intense vaccination campaign. Prior to eradiation, smallpox was found worldwide with a 1%-30% case fatality rate (CFR) depending on the strain and outbreak (1). The earliest confirmed historic records in India and China mention a smallpox-like disease in 300 A.D. (2-4). The most commonly discussed potential record is from 1157 B.C. highlighting the mummified corpse of Ramses which exhibited skin lesions suggestive of smallpox and contained a smallpox virion identified by electron microscopy (EM) but viral genomic DNA was not isolated (2, 5, 6). The oldest VARV draft genome was collected from the Viking Age ~1000 year ago (7). Smallpox is the only human pathogen eradicated to date. In large part, eradication was successful due to VARV being a solely human pathogen, the development of a heat-stable freeze-dried vaccine and the determination of the project staff. Historically, VARV was categorized into VARV *major,* VARV *minor* and alastrim. More recently, phylogenic analysis of the 63 strains in GenBank categorized the virus strains into two separate clades called primary clade I and primary clade II (1). Primary clade I contains strains with higher CFRs which ranged from <1%-35% (8). Primary clade II is comprised of less virulent West African strains and alastrim strains with CFRs of <1%-12% (9). The last naturally occurring case of smallpox occurred in Merca, Somalia in 1977. The last victim of smallpox, however, was Janet Parker in 1978. It is hypothesized the photographer become infected using a telephone booth that shared an air vent with a smallpox laboratory (10). Prior to isolation, she unknowing infected her mother who survived the disease and her father who's cause of death was a heart attack (11). After this

incident, many called for the destruction of the virus, but a WHO Committee of Experts recommended to preserve viral stocks in a few WHO collaborating centers with strict biosafety and biosecurity measures. Since 1984, only two WHO collaborating centers have been allowed to possess viral stocks in laboratories with the highest biosafety precautions (Biosafety Level 4 [BSL-4] laboratories): the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia and The State Research Center of Virology and Biotechnology (VECTOR Institute) in Koltsovo, Russia.

VARV was transmitted from person-to-person by inhalation of respiratory droplets or by contact with fomites. Infection by conjunctiva and mother-to-child transmission was possible but less common. The route of infection resulted in different disease manifestations with inhalation resulting in a more severe form. There are five main types of smallpox commonly descripted in the literature: ordinary, modified, Variola sine eruption, flat and haemorrhagic. As these, along with VARV *minor* and alastrim, have been described in detail elsewhere (11), I will focus on describing ordinary and haemorrhagic smallpox as these are most relevant for new VARV antiviral models. Ordinary smallpox was further subclassified as confluent, semiconfluent and discrete. Haemorrhagic was further subclassified as early or late-type. Ordinary smallpox had an approximate twelve-day incubation period (ranged from 7-19 days; mean 12 days) prior to the development of a febrile prodrome and at least one other flu-like symptom such as prostration, headache, backache, chills, several abdominal pain and/or vomiting. As the disease progresses, the patient will become “toxic” or moribund. Approximately 1-4 days post prodrome, the rash appears as small reddish macules which were commonly confused with measles and chickenpox. The rash first appears on the oral mucosa/palate, face or forearms before becoming centrifugal in distribution. The rash also appeared on the individual’s palms and/or soles of the feet. The rash evolved from macules to papules to pustules with each stage lasting 1-2 days. Lesions in one part of the body were at the same stage of development so it was possible to have macules on the left arm but papules on the right arm. The pustules were deep

seated, well-circumscribed and firm to the touch and lasted approximately five to eight days (11). They could become confluent and/or become umbilicated. It was common to see a second fever at this point in the disease course as many of the smallpox patients developed secondary bacterial infections or bronchopneumonia (11, 12). The pustules scabbed and remained infectious until the scabs detached and were completely resolved. The scabs themselves could be infectious for years (13, 14). Many of those that did survive infection were left with lifetime scars commonly referred to as pocks. Blindness and arthritis were common in survivors. The exact reason why patients succumbed to smallpox is unknown. Historic literature refers toxemia as the cause of death, but a cytokine storm and septic shock are the more likely culprits (15).

Haemorrhagic-type smallpox is a rare form of smallpox that was primarily seen in adults and was almost always fatal. It is divided into two subtypes, purpura variolosa and variola pustulosa haemorrhagica, commonly referred to today as early and late haemorrhagic smallpox. Early haemorrhagic smallpox was more common than late and was more likely to develop in pregnant women. The disease onset was sudden with high fever, toxic symptoms and viremia that remained until death or disease resolution (11, 16, 17). Early haemorrhagic smallpox is characterized by hemorrhages in skin and mucous membranes early in disease progression. Subconjunctival hemorrhages were common and bleeding from multiple orifices suggest multiple organ involvement (11). Unlike classic smallpox, the skin developed a “finely textured matted surface” and was velvety to touch prior to turning dark purple (11). A typical smallpox rash did not develop with this form of the disease. Severe toxemia, chest pains and respiratory issues developed after the febrile prodrome and death usually occurred within six days post development (11). If the individual survived six days post febrile prodrome, sloughing of the skin occurred (11). Surprisingly the cause of death was not massive hemorrhages but heart failure and/or edema in lungs (11). Late haemorrhagic -type smallpox differs from early in several ways, but the disease progression is similar. Men and women were equally affected with this form. With the late hemorrhagic-type, the rash is slow to develop, lesions have hemorrhagic

bases and bleeding at various mucous membranes is common although less than the early-type (11). The lesions can present as flat and turn black in color (11). This form of the disease is commonly confused with flat-type or ordinary smallpox with complications due to similar disease presentation. The case fatality rates were high and occurred 8-10 days post febrile prodrome (11). As seen with flat-type smallpox, this manifestation of smallpox was likely due to deficiencies in the host's immune system and not related to a specific viral strain.

Historically, VARV was diagnosed based on clinical symptoms alone since it had a rash characterized by deep-seeded lesion. Some stages of the rash and types of smallpox could be confused with other diseases such as severe chickenpox and measles. As science evolved, diagnosis of orthopoxviruses, including VARV, became a combination of clinical symptoms and laboratory tests. Diagnosis based on clinical symptoms alone can be challenging as VARV and human monkeypox, caused by another *Orthopoxvirus* (OPXV), *Monkeypox virus* (MPXV), present almost identically. Historically, OPXV infection could be diagnosed by EM with the presence of the characteristic brick shape virions, growth on chorioallantoic membranes (CAMs) or polymerase chain reaction (11, 18). While EM does not allow for speciation, scientists began to rely on CAM growth at different temperatures (19) and specific real-time PCRs assays to speciate. Today, we still heavily rely on a combination of clinical symptoms and laboratory tests. Since smallpox is eradicated, speciation between OPXVs is critical if smallpox did somehow re-emerge. The CDC has developed the "acute, generalized vesicular or pustular rash illness testing protocol in the United States" for determining if a patient is highly suspected to have smallpox. Of note, both smallpox and monkeypox patients would rate as high risk due to their similar clinical signs. The CDC has published several extensively validated real-time PCR assays for VARV, MPXV and other OPXVs which can be used for clinical specimens (20-23). It is important to remain vigilant in re-confirming specificity of assays each time a novel OPXV is discovered. This was illustrated when the specificity of VARV specific assays was invalidated with the discovery of new *Cowpox virus* (CPXV) isolates (20). While serology and propagation

of clinical material in immortalized cell lines can be used to determine if you have been exposed to an OPXV, it also cannot distinguish between species in the genus.

Since smallpox was eradicated prior to the discovery of many of the current immunology techniques, much remains to be known about VARV disease progression. Analysis of human specimens (serum, blood, nasal and oral washes) (24-26) collected from smallpox patients and data generated in animal models using surrogate OPXVs have been utilized to attempt to generate a picture of the disease progression. Most of the surrogate model data focuses on the ectromelia (mouse pox) studies (27) as VARV and ectromelia are genetically similar. It is hypothesized that respiratory droplets were inhaled and seeded the respiratory and/or oropharyngeal tissues (15). The virus then spread to the lower and upper respiratory tract but was cleared by antigen presenting cells which migrated to the draining lymph node (27) for presentation to T and B cells. The virus replicates in the draining lymph node and spreads to other lymphoid tissues during the first viremia (11). The first viremia was difficult to catch in ordinary smallpox patient samples suggesting a short period and it is unknown exactly which lymphoid and tissues were infected (11). This was not the case with haemorragic smallpox where viremia occurred from febrile prodrome to death (16, 17). A second viremia occurred at febrile prodrome onset which likely seeded the rest of the internal organs and the dermis (11). One to four days later, the classic smallpox rash would appear, and the host immune system would continue to combat infection with both cell-mediated and antibody responses. It was reported that at rash onset, it was possible to collect viable virus from throat swabs (24, 25). As stated earlier, for those that succumbed to disease, it was likely due to a cytokine storm and septic shock rather than massive hemorrhage or organ failure (11).

Numerous efforts have occurred to attempt to find an animal model for studying VARV. Almost all of these attempts have been unsuccessful including adult mice, rats, Syrian hamsters, guinea pigs, prairie dogs, rabbits, cocks, chickens, goats, sheep, pigs, cows and camels (28-30). Suckling and young mice (<5 days old) are susceptible (28, 29), but the

usefulness of these models is extremely limited. CAST Ei/J, ICR and SCID mice experience minimal disease signs and low levels of viral replication in the nasal passages and/or lungs post VARV challenge (31, 32) which might be useful for certain studies. The study of non-human primates (NHPs) with VARV began in the 1800's. Shchelkunov, S.N. *et al.* (29) summarizes these experiments which illustrated that lower monkeys and anthropoid apes were capable of being infected with VARV with disease severity depending on infectious dose and animal age. Early studies suggested that cynomolgus macaques might be the most promising model (33, 34).

As the deadline for destruction for the known VARV stocks loomed in the early 2000s, the United States Army Research Institute of Infectious Diseases (USAMRIID) and CDC conducted studies using cynomolgus macaques to exposure their utility as an animal model. Initial studies with VARV strains JAP46_yam and KOR47_lee (primary clade I) did not produce significant disease when challenged by a fine-particle aerosol (35). A subsequent study utilized Jap51_hrpr and IND64_vel4 (primary clade I) resulting in disease resembling human haemorrhagic smallpox when VARV was administered intravenously (I.V.) or I.V and aerosol at 10^9 plaque forming units (pfu) (35). Infectious doses at 10^8 pfu produced a smallpox-like disease with a lower morality rate (35). While this animal model is useful, it is not without its challenges. NHPs are expensive and difficult to work with in BSL-4 laboratory conditions. The I.V route of infection is an unnatural route of infection and bypasses the incubation and primary viremia stages of human smallpox. Animals administered with the highest dose (10^9 pfu) succumbed to disease within 4 days post infection (35) which makes it a challenging model for studying post-exposure therapeutics. Animals challenged with 10^8 pfu experienced disease which resembled ordinary smallpox with low morality levels (n=1/8) (35), not sufficient to comply with the Food and Drug Administration (FDA) Animal Rule guidelines for a smallpox animal model, which left the door open to exploring other potential models. An ideal animal model would be a small

animal model (i.e. mouse or rat) that would better mimic ordinary smallpox with high mortality rates.

Since the eradication of smallpox, monkeypox has the highest CFR of OPXVs (up to ~11%) and is commonly used as a surrogate virus for VARV. Monkeypox was first described in 1958 in cynomolgus monkey colonies in Denmark (36). The first case of human monkeypox was described in 1971 in the Basankusu Territory in the Republic of Zaire, now called the Democratic Republic of Congo (DRC) (37). The disease circulated in the region prior to 1971 but was commonly mistaken for smallpox since the clinical manifestations of monkeypox are almost identical to discrete ordinary-type or modified-type smallpox with the addition of lymphadenopathy (49). MPXVs are divided into two clades, the Congo Basin and West African clades, based on genetic, geographic and phenotypic differences (38). As the names indicate, the Congo Basin clade circulates in Central African while the West African clade circulates in West Africa. With the cessation of the smallpox vaccination, the global population has become more susceptible to MPXV.

Since 2010, the number of MPXV outbreaks are on the rise for both clades. Most of the outbreaks have been associated with the Congo Basin clade which may be due to higher person-to-person transmission rates that have been reported as high as >6 (39). In DRC, the number of reported cases from 2010-2014 were over 2,000 reported cases per year (40, 41). The high number of reported cases in DRC compared to other countries may be due to the ongoing surveillance projects in the Tshuapa district and that MPXV is a reportable disease in DRC. Since 1970, fifteen countries have reported human MPXV cases: DRC, Central African Republic, Republic of Congo, Camera, Liberia, Sierra Leone, Cote d'Ivoire, Gabon, South Sudan, the United States of America, the United Kingdom, Israel, Singapore, and Nigeria (40, 42-45). Within the last ten years, MPXV appears to be expanding its range with Nigeria, Sierra Leone and Liberia reporting at least one MPXV case which is concerning as these countries had not seen a case of MPXV in over 35 years (40, 46). The ongoing outbreak in Nigeria is the

largest West African MPXV outbreak ever recorded. The outbreak is revealing new things about West African MPXV including that human-to-human transmission is possible with this clade and higher CFR than historically reported (44, 47). The outbreak has been linked to several imported cases in the United Kingdom, Singapore and Israel (43-45) highlighting the threat that MPXV could pose globally. In addition, human-to-human transmission did occur in the United Kingdom from a patient to nurse (44). In 2003, West African MPXV was also imported into the United States from Ghana when infected African rodents were imported and housed with prairie dogs. The prairie dogs were sold as pets and have been linked to 72 confirmed or suspected human cases (42).

Unlike VARV, MPXV is primarily a zoonotic pathogen (scratches, bites, or fomites) and can be transmitted less efficiently person-to-person (inhalation or fomites). While humans and NHPs are capable of being infected with the virus, the reservoir of MPXV is unknown. Based on sero-survey data, there are likely several African rodents that play a role in viral circulation (48, 49). The virus has only been isolated twice from wild animals, a rope squirrel (*Funisciurus anerythrus*) in the DRC (48) and more recently in a deceased sooty mangabey (*Cercocebus atys*) in Cote d'Ivoire (50). Although MPXV is zoonotic in nature, identifying an ideal animal model was not simple. Most adult laboratory rodents do not display significant morbidity when infected with MPXV including laboratory mice, guinea pigs, golden hamsters and white rats (53, 61-63). Non-traditional laboratory rodent species have been shown to be highly susceptible to MPXV such as cotton rats, multimammate rats, dormice and several species of squirrels (53, 61, 65), however, these animals have challenges being outbred, wild caught and limited commercially available reagents. More recently, researchers have showed that CAST Ei/J mice are susceptible to MPXV infection (51, 52) which are derived from wild-caught mice but commercially available. When infected intranasally, death occurred between 6-8 days post inoculation (52). This model has several advantages however, animals did not develop skin lesions and the 6-8 day window before mortality makes studying antivirals challenging.

During the 2003 U.S. MPXV outbreak, prairie dogs were identified as being able to harbor the disease. Laboratory studies revealed that prairie dogs are susceptible to both West African and Congo Basin clades of MPXV when infected intranasally, intraperitoneal or through scarification (53-55). Post challenge, animals have a 9-12 day incubation period prior to the development of systemic rash and symptom onset which is similar to human monkey disease presentation and progression (55). Unfortunately, commercial reagents do not exist for these animals. Although prairie dogs are not an animal model under The Animal Rule, several MCMs have been tested in this model including, ACAM 2000® (Sanofi Paseur Biologics Co.), JYNNEOIS® (Bavarian Nordic) and CMX001® (brincidofovir; Chimerix) and TPOXX® (tecovirimat; SIGA) (56-59). The animal models currently under The Animal Rule are the *Ectromelia virus* mouse model, the *Rabbitpox virus* rabbit model and the NHP MPXV model.

Cynomolgus macaques are more commonly used in laboratory settings and inoculation routes include I.V., aerosol and intratracheal (15). I.V. challenge in these animals results in a rash that more closely resembles human smallpox with the rash first appearing on the head or extremities prior to spreading to the rest of the body with death occurring within 13 days (60). The aerosol inoculation model has been fully characterized with the rash presenting similarly to the IV model and death occurring 9-17 days post exposure due to fibrinonecrotic bronchopneumonia (61). The I.V. inoculation route in rhesus macaques results in a systemic disease which presents similarly to human smallpox including a vesiculopustular rash and morality within 7-15 days (62).

While VARV and MPXV are the most concerning human pathogenic OPXVs, most of what we know about OPXV replication and proteins is based on studies with *Vaccinia virus* (VACV) (63). OPXVs are large double-stranded DNA viruses that vary from 170 to 240 kb with over 200 genes. The large genome allows the virus to contain almost all the machinery required for viral replication. OPXVs are ancient viruses that have evolved alongside their hosts; they are capable of acquiring host DNA which has improved viral fitness (64). These viruses have a

complex life cycle with two main virion progeny forms called the intracellular mature virion (IMV) and the extracellular enveloped virion (EV). IMV are produced in high numbers during infection while EV is believed to be important for dissemination throughout the host (65, 66). IMVs are comprised of a dumbbell-shaped nucleosome core and lateral bodies surrounded by a single lipid membrane bilayer. The EV form is comprised of the IMV with an additional membrane. The receptor used by the virus for cell entry is controversial but it is recognized that IMV and EV use different receptors (67). As the virus enters the cell, it sheds its membrane(s) and the viral core is transported on microtubules to the cytoplasm where early viral transcription begins. Within a few hours of entry, the virus shuts down the host machinery. At this time, cytoplasmic factories, which consist of virus protein and host-derived lipid, are constructed (68). Viral replication and intermediate gene transcription follow next. In the late stages of the life cycle, immature virions assembled in cytoplasmic factories mature into IMVs by proteolytic cleavage of some capsid proteins and condensation (68). IMVs are transported out of the cytoplasmic factories on microtubules. Some will remain as IMVs in the cytoplasm and will go on to infect other cells post cell lysis. The rest will undergo modifications to become EV and infect neighboring cells. For those destined to become EV, the IMV is wrapped by intracellular membranes becoming an intracellular enveloped virion and transported on microtubules to the cell surface. The virion fuses with the cell membrane becoming a cell-associated enveloped virus (CEV). CEVs can induce actin tail formation which pushes the now EV virus into an adjacent cell to continue the infection.

Although the life cycle of OPXVs is understood, prevention and treatment options remain limited. Inoculation, or variolation, was the primary method for prevention of infection until the mid-1800s. It is unknown whom discovered the technique, but it was reported in China, India and the Middle East prior to the 1700’s (4, 10, 69). Inoculation involved taking material from a smallpox patient and pricking the skin of a healthy individual with the material. It was known that individuals who survived smallpox infection had lifelong immunity (11) and inoculation provided

protection as well. Inoculated individuals generally experienced a mild form of ordinary smallpox resulting in the development of typically less than 100 lesions. Inoculation was not without risk as it could result in death and spread other diseases such as syphilis.

Vaccination with a surrogate OPXV began in the late 1700's. While most credit Edward Jenner with discovering vaccination, it was actually Benjamin Jetsy who noticed that milkmaids infected with CPXV did not contract smallpox and vaccinated his family (4). Edward Jenner performed the same experiments in 1796 and pushed for vaccination to replace inoculation. This method of vaccination did not have the same risks as human cowpox is generally a localized lesion, mortality did not occur (or was rare) and other human infections were not transmitted during the process as the material for vaccination was obtained from cows. Due to the success of vaccination, inoculation was outlawed in England in 1840 (70). In the 20th century, the vaccination strain changed to VACV (11), although the change in vaccine strain as well as the origin of VACV remains unknown.

Today, there are several types of medical counter measures (MCMs) for OPXVs including vaccines and antivirals. Three types of smallpox vaccines are recognized: first generation vaccines are those used during the vaccination campaign, second generation vaccines are strains or clones of those used in the first-generation vaccines but manufactured by cell culture, and third generation vaccines are attenuated strains. ACAM2000® (Sanofi Pasteur Biologics Co.), a second-generation vaccine, is licensed by the Food and Drug Administration (FDA) for the prevention of smallpox. JYNNEOS®, a third-generation vaccine (Bavarian Nordic), is licensed by the FDA for prevention of both smallpox and monkeypox. There are also two therapeutics approved for treatment of OPXVs. Vaccinia Immune Globulin (VIG) is FDA licensed for treatment of vaccine-related adverse events. VIG was used towards the end of the smallpox vaccination campaign and limited studies of VIG with vaccination showed additional benefit to prevent smallpox disease, in comparison to vaccination alone, amongst contacts of smallpox cases "treated" post exposure (71-73). Today, it is in limited

supply and not licensed by the FDA for smallpox treatment. TPOXX® (tecovirimat; SIGA) is the only small molecular compound FDA approved for treatment of smallpox. It targets the F13L gene and prevents viral egress which is important for EV formation.

The repertoire of MCMs for the treatment of smallpox should include more than one antiviral. OPXVs contain large genomes and are capable of becoming resistant to a single antiviral therapy (74). This suggests that a multi-therapeutic approach would be beneficial. The goal of this work is to characterize new models for studying antiviral agents against VARV including both *in vitro* and in *vivo* models. Here we sought to use the previously characterize prairie dog MPXV model to determine the efficacy for a monoclonal antibody (mAb) cocktail as a therapeutic for smallpox, confirm the neutralization activity of the cocktail and individual mAbs against VARV *in vitro.* and explore the humanized mouse model as a small animal model for VARV.

Specific Aim 1. Mix4, developed by The Vanderbilt Vaccine Center, has shown promise as an antiviral against multiple OPXVs including the IMV form of VARV *in vitro*. Since a small animal model for smallpox has yet to be characterized, we used a surrogate animal model to determine if Mix4 is capable of neutralizing the next most concerning human pathogenic OPXV, MPXV. Mix4 was administrated as prophylactic treatment in the prairie dog MPXV model which mimics human monkeypox disease progression. Morbidity, mortality and molecular assays were used to compare treatment with Mix4 to VIG to determine if it is a viable candidate as a smallpox MCM.

Specific Aim 2. While surrogate animal models are useful for studying smallpox MCMs, testing against the authentic agent, VARV, is still needed to confirm neutralization as not all OPXVs have the same proteins. Mix4 and the individual mAbs that make up Mix4 were screened against both the IMV and EV forms of VARV and MPXV. The results prompted the exploration to create an Universal Pox Mix instead of Mix4 as a therapeutic for smallpox.

Specific Aim 3. Three different humanized (hu) mice strains, hu-CD34^{+}, hu-BLT and hu-PBMC, were evaluated as a potential small animal model for VARV using an intranasal route of challenge. Daily clinical signs were taken to determine if the virus was affecting the mice. Upon euthanasia, either at the 21-day end point or if mice reached a 10 on a pre-determined pain scale, tissues, whole blood and serum were taken for downstream analyses. Viral DNA and viable virus levels were determined as well as histopathology through standard molecular techniques.

References

1. Li Y, Carroll DS, Gardner SN, Walsh MC, Vitalis EA, Damon IK. 2007. On the origin of smallpox: correlating variola phylogenics with historical smallpox records. Proc Natl Acad Sci U S A 104:15787-92.
2. Voigt EA, Kennedy RB, Poland GA. 2016. Defending against smallpox: a focus on vaccines. Expert Rev Vaccines 15:1197-211.
3. Babkin IV, Babkina IN. 2015. The origin of the variola virus. Viruses 7:1100-12.
4. Gross CP, Sepkowitz KA. 1998. The myth of the medical breakthrough: smallpox, vaccination, and Jenner reconsidered. Int J Infect Dis 3:54-60.
5. Strouhal E. 1996. Traces of a smallpox epidemic in the family of Ramesses V of the Egyptian 20th Dynasty. Anthropologie 34:315-319.
6. Lewin PK. 1984. "Mummy" riddles unravelled. Bulletin - Microscopical Society of Canada:3-8.
7. Muhlemann B, Vinner L, Margaryan A, Wilhelmson H, de la Fuente Castro C, Allentoft ME, de Barros Damgaard P, Hansen AJ, Holtsmark Nielsen S, Strand LM, Bill J, Buzhilova A, Pushkina T, Falys C, Khartanovich V, Moiseyev V, Jorkov MLS, Ostergaard Sorensen P, Magnusson Y, Gustin I, Schroeder H, Sutter G, Smith GL, Drosten C, Fouchier RAM, Smith

DJ, Willerslev E, Jones TC, Sikora M. 2020. Diverse variola virus (smallpox) strains were widespread in northern Europe in the Viking Age. Science 369.

8. Olson VA, Karem KL, Smith SK, Hughes CM, Damon IK. 2009. Smallpox virus plaque phenotypes: genetic, geographical and case fatality relationships. J Gen Virol 90:792-8.

9. Gilchuk I, Gilchuk P, Sapparapu G, Lampley R, Singh V, Kose N, Blum DL, Hughes LJ, Satheshkumar PS, Townsend MB, Kondas AV, Reed Z, Weiner Z, Olson VA, Hammarlund E, Raue HP, Slifka MK, Slaughter JC, Graham BS, Edwards KM, Eisenberg RJ, Cohen GH, Joyce S, Crowe JE, Jr. 2016. Cross-Neutralizing and Protective Human Antibody Specificities to Poxvirus Infections. Cell 167:684-694 e9.

10. Henderson DA, Preston R. 2009. Smallpox: The Death of a Disease : the Inside Story of Eradicating a Worldwide Killer. Prometheus Books.

11. Fenner F, Henderson, D.A., Arita, I., Jezek, Z., Ladnyi, I.D. 1988. Smallpox and its eradication. World Health Organization.

12. Martin DB. 2002. The cause of death in smallpox: an examination of the pathology record. Mil Med 167:546-51.

13. Wolff HL, Croon JJ. 1968. The survival of smallpox virus (variola minor) in natural circumstances. Bull World Health Organ 38:492-3.

14. Downie AW, Dumbell KR. 1947. Survival of variola virus in dried exudate and crusts from smallpox patients. Lancet 1:550-3.

15. Chapman JL, Nichols DK, Martinez MJ, Raymond JW. 2010. Animal models of orthopoxvirus infection. Vet Pathol 47:852-70.

16. Downie AW, Fedson DS, Saint Vincent L, Rao AR, Kempe CH. 1969. Haemorrhagic smallpox. J Hyg (Lond) 67:619-29.

17. Downie AW, McCarthy K, Macdonald A, Maccallum FO, Macrae AE. 1953. Virus and virus antigen in the blood of smallpox patients; their significance in early diagnosis and prognosis. Lancet 265:164-6.

18. Ropp SL, Jin Q, Knight JC, Massung RF, Esposito JJ. 1995. PCR strategy for identification and differentiation of small pox and other orthopoxviruses. J Clin Microbiol 33:2069-76.

19. Bedson HS, Dumbell KR. 1961. The effect of temperature on the growth of pox viruses in the chick embryo. J Hyg (Lond) 59:457-69.

20. Kondas AV, Olson VA, Li Y, Abel J, Laker M, Rose L, Wilkins K, Turner J, Kline R, Damon IK. 2015. Variola virus-specific diagnostic assays: characterization, sensitivity, and specificity. J Clin Microbiol 53:1406-10.

21. Olson VA, Laue T, Laker MT, Babkin IV, Drosten C, Shchelkunov SN, Niedrig M, Damon IK, Meyer H. 2004. Real-time PCR system for detection of orthopoxviruses and simultaneous identification of smallpox virus. J Clin Microbiol 42:1940-6.

22. Li Y, Olson VA, Laue T, Laker MT, Damon IK. 2006. Detection of monkeypox virus with real-time PCR assays. J Clin Virol 36:194-203.

23. Li Y, Zhao H, Wilkins K, Hughes C, Damon IK. 2010. Real-time PCR assays for the specific detection of monkeypox virus West African and Congo Basin strain DNA. J Virol Methods 169:223-7.

24. Sarkar JK, Mitra AC, Mukherjee MK, De SK. 1973. Virus excretion in smallpox. 2. Excretion in the throats of household contacts. Bull World Health Organ 48:523-7.

25. Sarkar JK, Mitra AC, Mukherjee MK, De SK, Mazumdar DG. 1973. Virus excretion in smallpox. 1. Excretion in the throat, urine, and conjunctiva of patients. Bull World Health Organ 48:517-22.

26. Mitra AC, Sarkar JK, Mukherjee MK. 1974. Virus content of smallpox scabs. Bull World Health Organ 51:106-7.

27. Fenner F. 1949. Mouse-pox; infectious ectromelia of mice; a review. J Immunol 63:341-73.

28. Marennikova SS, Kaptsova TI. 1965. Age-Dependence of Susceptibility of White Mice to Variola Virus. Acta Virol 9:230-4.

29. Shchelkunov SN, Marennikova SS, Moyer RW. 2006. Orthopoxviruses Pathogenic for Humans. Springer US.

30. Carroll DS, Olson VA, Smith SK, Braden ZH, Patel N, Abel J, Li Y, Damon IK, Karem KL. 2013. Orthopoxvirus variola infection of Cynomys ludovicianus (North American black tailed prairie dog). Virology 443:358-62.

31. Titova KA, Sergeev AA, Zamedyanskaya AS, Galahova DO, Kabanov AS, Morozova AA, Bulychev LE, Sergeev AA, Glotova TI, Shishkina LN, Taranov OS, Omigov VV, Zavjalov EL, Agafonov AP, Sergeev AN. 2015. Using ICR and SCID mice as animal models for smallpox to assess antiviral drug efficacy. J Gen Virol 96:2832-43.

32. Gallardo N, Hutson, C.L., Carroll, D., Kondas, A.V., Salzer, J. Dietz, S., Smith, S., Hudson, P., Olson, V., Damon, I. 2020. Use of live Variola virus to determine whether CAST/EiJ mice are a suitable surrogate animal model for human smallpox. Virus Res 275:197772.

33. Hahon N, Wilson BJ. 1960. Pathogenesis of variola in Macaca irus monkeys. Am J Hyg 71:69-80.

34. Noble J, Jr., Rich JA. 1969. Transmission of smallpox by contact and by aerosol routes in Macaca irus. Bull World Health Organ 40:279-86.

35. Jahrling PB, Hensley LE, Martinez MJ, Leduc JW, Rubins KH, Relman DA, Huggins JW. 2004. Exploring the potential of variola virus infection of cynomolgus macaques as a model for human smallpox. Proc Natl Acad Sci U S A 101:15196-200.

36. Magnus PV, Andersen, E.K., Petersen, K.B., Birch-Andersen, A. 1959. A Pox-like Disease in Cynomolgus Monkeys. Acta Pathologica Microbiologica Scandinavica:156-176.

37. Ladnyj ID, Ziegler P, Kima E. 1972. A human infection caused by monkeypox virus in Basankusu Territory, Democratic Republic of the Congo. Bull World Health Organ 46:593-7.

38. Likos AM, Sammons SA, Olson VA, Frace AM, Li Y, Olsen-Rasmussen M, Davidson W, Galloway R, Khristova ML, Reynolds MG, Zhao H, Carroll DS, Curns A, Formenty P, Esposito JJ, Regnery RL, Damon IK. 2005. A tale of two clades: monkeypox viruses. J Gen Virol 86:2661-72.

39. Nolen LD, Osadebe L, Katomba J, Likofata J, Mukadi D, Monroe B, Doty J, Hughes CM, Kabamba J, Malekani J, Bomponda PL, Lokota JI, Balilo MP, Likafi T, Lushima RS, Ilunga BK, Nkawa F, Pukuta E, Karhemere S, Tamfum JJ, Nguete B, Wemakoy EO, McCollum AM, Reynolds MG. 2016. Extended Human-to-Human Transmission during a Monkeypox Outbreak in the Democratic Republic of the Congo. Emerg Infect Dis 22:1014-21.

40. Sklenovska N, Van Ranst M. 2018. Emergence of Monkeypox as the Most Important Orthopoxvirus Infection in Humans. Front Public Health 6:241.

41. Mwamba DK KB, Shongo RL, Pukuta E, Kayembe NJM. 2014. Profil épidemiologique du monkeypox en RDC, 2010-2014. Ann African Med doi:Available online at: https://anafrimed.net/profil-epidemiologique-du-monkeypox-en-rdc-2010-2014-monkeypox-in-drc-epidemiological-profile-2010-2014/:1855-60.

42. Reed KD, Melski JW, Graham MB, Regnery RL, Sotir MJ, Wegner MV, Kazmierczak JJ, Stratman EJ, Li Y, Fairley JA, Swain GR, Olson VA, Sargent EK, Kehl SC, Frace MA, Kline R, Foldy SL, Davis JP, Damon IK. 2004. The detection of monkeypox in humans in the Western Hemisphere. N Engl J Med 350:342-50.

43. Erez N, Achdout H, Milrot E, Schwartz Y, Wiener-Well Y, Paran N, Politi B, Tamir H, Israely T, Weiss S, Beth-Din A, Shifman O, Israeli O, Yitzhaki S, Shapira SC, Melamed S, Schwartz E. 2019. Diagnosis of Imported Monkeypox, Israel, 2018. Emerg Infect Dis 25:980-983.

44. Anonymous. December 5 2018. Monkeypox case in England. https://www.gov.uk/government/news/monkeypox-case-in-england. Accessed September 8 2018.

45. Anonymous. 2019. Monkeypox - Singapore, *on* World Health Organization. https://www.who.int/csr/don/16-may-2019-monkeypox-singapore/en/. Accessed

46. Durski KN, McCollum AM, Nakazawa Y, Petersen BW, Reynolds MG, Briand S, Djingarey MH, Olson V, Damon IK, Khalakdina A. 2018. Emergence of Monkeypox - West and Central Africa, 1970-2017. MMWR Morb Mortal Wkly Rep 67:306-310.

47. Yinka-Ogunleye A, Aruna O, Dalhat M, Ogoina D, McCollum A, Disu Y, Mamadu I, Akinpelu A, Ahmad A, Burga J, Ndoreraho A, Nkunzimana E, Manneh L, Mohammed A, Adeoye O, Tom-Aba D, Silenou B, Ipadeola O, Saleh M, Adeyemo A, Nwadiutor I, Aworabhi N, Uke P, John D, Wakama P, Reynolds M, Mauldin MR, Doty J, Wilkins K, Musa J, Khalakdina A, Adedeji A, Mba N, Ojo O, Krause G, Ihekweazu C, Team CDCMO. 2019. Outbreak of human monkeypox in Nigeria in 2017-18: a clinical and epidemiological report. Lancet Infect Dis 19:872-879.

48. Khodakevich L, Jezek Z, Kinzanzka K. 1986. Isolation of monkeypox virus from wild squirrel infected in nature. Lancet 1:98-9.

49. Parker S, Buller RM. 2013. A review of experimental and natural infections of animals with monkeypox virus between 1958 and 2012. Future Virol 8:129-157.

50. Radonic A, Metzger S, Dabrowski PW, Couacy-Hymann E, Schuenadel L, Kurth A, Matz-Rensing K, Boesch C, Leendertz FH, Nitsche A. 2014. Fatal monkeypox in wild-living sooty mangabey, Cote d'Ivoire, 2012. Emerg Infect Dis 20:1009-11.

51. Americo JL, Moss B, Earl PL. 2010. Identification of wild-derived inbred mouse strains highly susceptible to monkeypox virus infection for use as small animal models. J Virol 84:8172-80.

52. Earl PL, Americo JL, Moss B. 2012. Lethal monkeypox virus infection of CAST/EiJ mice is associated with a deficient gamma interferon response. J Virol 86:9105-12.

53. Xiao SY, Sbrana E, Watts DM, Siirin M, da Rosa AP, Tesh RB. 2005. Experimental infection of prairie dogs with monkeypox virus. Emerg Infect Dis 11:539-45.

54. Hutson CL, Carroll DS, Gallardo-Romero N, Drew C, Zaki SR, Nagy T, Hughes C, Olson VA, Sanders J, Patel N, Smith SK, Keckler MS, Karem K, Damon IK. 2015. Comparison of Monkeypox Virus Clade Kinetics and Pathology within the Prairie Dog Animal Model Using a Serial Sacrifice Study Design. Biomed Res Int 2015:965710.

55. Hutson CL, Olson VA, Carroll DS, Abel JA, Hughes CM, Braden ZH, Weiss S, Self J, Osorio JE, Hudson PN, Dillon M, Karem KL, Damon IK, Regnery RL. 2009. A prairie dog animal model of systemic orthopoxvirus disease using West African and Congo Basin strains of monkeypox virus. J Gen Virol 90:323-33.

56. Hutson CL, Kondas, A.V., Mauldin, M., Doty, J., Sickle, K.V., Morgan, C., Dietz-Ostergaard, S., Nakazawa, Y., Kling, C., Martin, B., Ellison, J.A., Carroll, C., Gallardo-Romero, N., Olson, V. 2019. Pharmacokinetics and Activity of Brincidofovir in a Lethal Prairie Dog Animal Model of Monkeypox. Manuscript in draft.

57. Smith SK, Self J, Weiss S, Carroll D, Braden Z, Regnery RL, Davidson W, Jordan R, Hruby DE, Damon IK. 2011. Effective antiviral treatment of systemic orthopoxvirus disease: ST-246 treatment of prairie dogs infected with monkeypox virus. J Virol 85:9176-87.

58. Keckler MS, Carroll DS, Gallardo-Romero NF, Lash RR, Salzer JS, Weiss SL, Patel N, Clemmons CJ, Smith SK, Hutson CL, Karem KL, Damon IK. 2011. Establishment of the black-tailed prairie dog (Cynomys ludovicianus) as a novel animal model for comparing smallpox vaccines administered preexposure in both high- and low-dose monkeypox virus challenges. J Virol 85:7683-98.

59. Keckler MS, Salzer JS, Patel N, Townsend MB, Nakazawa YJ, Doty JB, Gallardo-Romero NF, Satheshkumar PS, Carroll DS, Karem KL, Damon IK. 2020. IMVAMUNE((R)) and ACAM2000((R)) Provide Different Protection against Disease When Administered Postexposure in an Intranasal Monkeypox Challenge Prairie Dog Model. Vaccines (Basel) 8.

60. Huggins J, Goff A, Hensley L, Mucker E, Shamblin J, Wlazlowski C, Johnson W, Chapman J, Larsen T, Twenhafel N, Karem K, Damon IK, Byrd CM, Bolken TC, Jordan R,

Hruby D. 2009. Nonhuman primates are protected from smallpox virus or monkeypox virus challenges by the antiviral drug ST-246. Antimicrob Agents Chemother 53:2620-5.

61. Zaucha GM, Jahrling PB, Geisbert TW, Swearengen JR, Hensley L. 2001. The pathology of experimental aerosolized monkeypox virus infection in cynomolgus monkeys (Macaca fascicularis). Lab Invest 81:1581-600.

62. Hooper JW, Thompson E, Wilhelmsen C, Zimmerman M, Ichou MA, Steffen SE, Schmaljohn CS, Schmaljohn AL, Jahrling PB. 2004. Smallpox DNA vaccine protects nonhuman primates against lethal monkeypox. J Virol 78:4433-43.

63. Smith GL, Vanderplasschen A, Law M. 2002. The formation and function of extracellular enveloped vaccinia virus. J Gen Virol 83:2915-31.

64. Bugert JJ, Darai G. 2000. Poxvirus homologues of cellular genes. Virus Genes 21:111-33.

65. Chan WM, Ward BM. 2012. The A33-dependent incorporation of B5 into extracellular enveloped vaccinia virions is mediated through an interaction between their lumenal domains. J Virol 86:8210-20.

66. Payne LG. 1980. Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J Gen Virol 50:89-100.

67. Vanderplasschen A, Smith GL. 1997. A novel virus binding assay using confocal microscopy: demonstration that the intracellular and extracellular vaccinia virions bind to different cellular receptors. J Virol 71:4032-41.

68. Smith GL. 2006. Poxviruses. Birkhäuser Basel.

69. Willis NJ. 1997. Edward Jenner and the eradication of smallpox. Scott Med J 42:118-21.

70. Razzell PE. 1977. The Conquest of Smallpox: The Impact of Inoculation on Smallpox Mortality in Eighteenth Century Britain. Caliban Books.

71. Kempe CH, Bowles C, Meiklejohn G, Berge TO, St Vincent L, Babu BV, Govindarajan S, Ratnakannan NR, Downie AW, Murthy VR. 1961. The use of vaccinia hyperimmune gamma-globulin in the prophylaxis of smallpox. Bull World Health Organ 25:41-8.

72. Kempe CH, Berge TO, England B. 1956. Hyperimmune vaccinal gamma globulin; source, evaluation, and use in prophylaxis and therapy. Pediatrics 18:177-88.

73. Marennikova SS. 1962. The use of hyperimmune antivaccinia gamma-globulin for the prevention and treatment of smallpox. Bull World Health Organ 27:325-30.

74. Lederman ER, Davidson W, Groff HL, Smith SK, Warkentien T, Li Y, Wilkins KA, Karem KL, Akondy RS, Ahmed R, Frace M, Shieh WJ, Zaki S, Hruby DE, Painter WP, Bergman KL, Cohen JI, Damon IK. 2012. Progressive vaccinia: case description and laboratory-guided therapy with vaccinia immune globulin, ST-246, and CMX001. J Infect Dis 206:1372-85.

CHAPTER 2

MIX4, A MONOCLONAL ANTIBODY COCKTAIL, PROVIDES PROTECTION IN THE PRIARIE DOG MONKEYPOX VIRUS MODEL[2]

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Abstract

Since the eradication of smallpox (*Variola virus), Monkeypox virus* (MPXV) is the most concerning human pathogen in the genus *Orthopoxvirus* (OPXV). Although smallpox is eradicated, the threat of re-emergence remains. Due to disease progression similarities and animal models available, MPXV serves as a surrogate OPXV for testing smallpox medical counter measures (MCMs). Here we provide further evidence that the prairie dog MPXV model is a valuable tool for studying smallpox MCMs. A longevity study determined no negative side effects were seen post injection of high levels of human monoclonal antibodies (mAb) and a plaque reduction neutralization assay (PRNT) determined that neutralizing antibodies were detected as soon as one day post injection in the model. A subsequent MPXV challenge study comparing the efficacy of a mAb cocktail, Mix4, and Vaccinia immune globulin (VIG) to an irrelevant mAb (2D22) revealed that Mix4 provided better protection against mortality compared to other treatment groups. Additionally, DNAemia was delayed in Mix4 treated animals. PRNTs against two main forms of viral progeny, the intracellular mature virion (IMV) and the external enveloped virion (EV), revealed that Mix4 treatment results in exogenous IMV neutralizing antibodies one day post injection. However, EV PRNTs revealed that both Mix4 and VIG treatment did not result in the ability to neutralize the EV form. While Mix4 was capable of providing protection against mortality, the absence of EV neutralization achieved by Mix4 and morbidity seen in treatment animals suggests a new mAb cocktail comprised of different EV mAbs should be considered.

Introduction

Monkeypox virus (MPXV), the causative agent of monkeypox, is an *Orthopoxvirus* (OPXV) endemic throughout Africa. MPXV can be grouped into two clades, the West African and Congo Basin, with Congo Basin clade causing an estimated case fatality rate of ~11% (1, 2). The virus is zoonotic and can be spread several ways including contact with fomites, person-to-person by aerosol droplets and animal-to-person by scratches and bites. The virus

reservoir(s) are unknown, although evidence points to different rodents such as squirrels (3-5). Human monkeypox presents almost clinically identical to ordinary smallpox, caused by *Variola virus* (VARV) a closely related OPXV. The main difference in clinical symptoms with monkeypox is the addition of lymphadenopathy but otherwise follows a similar disease progression as smallpox (6). With the cessation of the smallpox vaccination, the global population has become susceptible to MPXV as evident by the increased outbreaks for both clades over the last decade. Most of the outbreaks have been associated with the Congo Basin clade which may be due to higher person-to-person transmission rates that have been reported as high as 6 (4). In the Democratic Republic of Congo (DRC), the number of reported cases from 2010-2014 were over 2,000 reported cases per year (7, 8). The higher number of reported cases in DRC compared to other countries may be due in part to the ongoing CDC surveillance project in the Tshuapa district, increased deforestation increasing animal/person interactions, increased consumption of bush meat, and/or that MPXV is a reportable disease in DRC. Within the last ten years, MPXV appears to be on the move within Africa with Nigeria, Sierra Leone and Liberia reporting at least one MPXV case; this is concerning as none of these countries had reported a case of MPXV in over 35 year (7, 9). The ongoing outbreak in Nigeria is the largest West African MPXV outbreak ever recorded. The outbreak is revealing new information about West African MPXV including that human-to-human transmission is possible with this clade (10, 11). The outbreak has been linked to several imported cases in the United Kingdom, Singapore and Israel (11-13) highlighting the threat that MPXV could pose globally. In addition, human-to-human transmission did occur in the United Kingdom from a patient to nurse (11). In 2003, West African MPXV was also imported into the United States from Ghana when infected African rodents were imported and housed with prairie dogs. The prairie dogs were sold as pets and have been linked to 72 confirmed or suspected human cases (14).

Unlike VARV, MPXV is capable of infecting a variety of hosts including humans, non-human primates (NHPs) and rodents (3, 5, 15-19). Since MPXV can infect non-human hosts

and has a similar transmission, disease presentation and progression to smallpox, it can be used as a surrogate model to better understand VARV and to develop medical counter measures (MCMs). The development of an ideal animal model for MPXV has been quite extensive, with all models having different strengths and weaknesses (16, 19). Most laboratory mice and some adult rodents are not susceptible to MPXV. Other models are susceptible but present different challenges such as not being commercially available, outbred models, uncharacterized, no cutaneous rash development, infection route not similar to humans, and lack of laboratory reagents (16, 20-24). The CAST/EiJ mice have been shown to be susceptible to MPXV infection (20, 25, 26) and are commercially available but they do not develop skin lesions and succumb to disease relatively quickly after infection (6-8 days post inoculation [dpi]); this presents a challenge when evaluating post exposure MCMs. A variety of NHP MPXV models have been characterized with the development of skin lesions and disease progression similar to that of smallpox (24, 27-29) and this model falls under the Food and Drug Administration (FDA) Animal Rule. However, NHPs are difficult to work with in a biosafety level 3 (BSL-3) setting and animal numbers remain low in efficacy studies due to housing constrains and ethical concerns. In 2003, a MPXV outbreak occurred in prairie dogs co-housed with imported African rodents resulting in the observation that the disease presentation within these animals was strikingly similar to humans (1, 17). Since then, the prairie dog MPXV model has been fully characterized including the incubation period, systemic rash and antibody development, all of which are similar to human disease progression, making the model valuable for studying MCMs for OPXVs (30-32). Evaluation of several MCMs such as a vaccine (ACAM 2000®; Sanofi Pasteur Biologics Co.) and small molecular antiviral compounds, for instance TPOXX® (tecovirimat; SIGA) and CMX001® (brincidofovir; Chimerix), have occurred in this model (31-33).

In 2018, TPOXX® (tecovirimat; SIGA) become the first antiviral to receive FDA licensure for treatment of smallpox. However, resistance to TPOXX® has been reported (34) suggesting a

multi-therapeutic treatment for OPXVs would be beneficial. Monoclonal antibody (mAb) cocktails could be an option to fill the need for a second therapeutic compound for both smallpox and monkeypox disease. The OPXV life cycle has two main forms of progeny virions that occur during the replication cycle. High levels of intracellular mature virions (IMV) are produce by infected cells and the external enveloped virion (EV) is important for virus dissemination (35). Since these virions have different surface proteins (36, 37), a mAb cocktail comprised of mAbs that target both main forms could be effective as an OPXV MCM. The Vanderbilt Vaccine Center has identified and developed n=48 mAbs that were shown to neutralize the IMV or EV form of an OPXV (*Vaccinia virus* [VACV], MPXV or *Cowpox virus* [CPVX]) (38). Based on the nonvariola OPXV neutralizing data, the Vanderbilt Vaccine Center created a mAb cocktail, Mix4, that had high capacity to neutralize both the IMV and EV form of nonvariola OPXVs *in vitro* and in several VACV mouse models (38). Mix4 is a cocktail comprised of antibodies that target IMV antigens L1, A27 and EV antigens B5 and A33. It was developed to be OPXV generic with the goal that they would neutralize a broad range of OPXVs. We hypothesized that Mix4 would be efficacious in the prairie dog MPXV model based previous *in vitro* and VACV mouse model results (38). The study was conducted in two parts. Part one comprised of a non-challenge longevity study and determined that prairie dogs had no negative side effects following administration of human mAbs and retained ≥50% neutralization in serum samples for ~ 7 days. Part two was a MPXV efficacy study that revealed that Mix4 provided better protection against mortality than vaccinia immune globulin (VIG) in a lethal challenge of MPXV in the prairie dog model. Our results provide additional evidence that mAb cocktails could be used to treat monkeypox disease and could fill the need for another smallpox antiviral.

Materials and Methods

Longevity Study

Animals

Wild caught black-tailed prairie dogs (*Cynomys ludovicianus*) from Texas underwent a veterinary assessment and quarantine in the field, before another quarantine period at the Centers for Disease Control and Prevention (CDC). At the time of the study, all animals were considered to be adults and in good health. A sterile passive integrated transponder (PIT) tag was injected subcutaneously (SQ) at the base of the neck for animal identification. Animals were cared for under CDC IACUC approved protocol 2916HUTPRAC which included daily enrichment. Study groups contained two males and two females. Animals were single housed to monitor appetence for 21 days and then moved back to group housing for the remainder of the study.

Dosing and Animal Sampling

The development and characterization of mAbs used in this study has been described (38). For all treatments, a single dose on day 0 (as described below) was given to each animal. A mAb, anti-L1 referenced as MPXV-26, that is present in Mix4 was dosed at 8 mg/kg. At the time of the longevity, there were discussions about whether Mix4 or another mAb cocktail named Mix6 (38) should be used in these studies. If Mix6 was used in the efficacy study, the amount of each mAb used in the treatment would be 8 mg/kg which was why that dose was selected as opposed to 12 mg/kg per mAb for Mix4. Due to the cost of manufacturing mAbs under an FDA Investigation New Drug (IND) protocol and the limited protective benefit from the two additional mAbs in Mix6, Mix4 was selected. The longevity study was not repeated with 12 mg/kg, although ≥50% neutralization could have been detected longer than 7 days post injection at 12 mg/kg with MPXV-26, because we wanted to give Mix4 the best chance of success in this pilot study and a different dosing regimen would not have been chosen. VIG (lot no. 10703689) was provided by the Strategic National Stockpile (SNS) and dosed at 12,000 IU/kg. While 6,000 IU/kg of VIG is the standard human dose, higher doses, such as 12,000 IU/kg, can be given when treating humans. Since an lethal dose 90 (LD_{90}) of MPXV would be used in the subsequent efficacy study, a higher than standard VIG dose was selected. An irrelevant mAb,

2D22, was dosed at 48 mg/kg which was the maximum dose that would be used in the prairie dogs in an efficacy study (38).

After acclimation, a blood sample (pre-) was collected via the femoral veins (CDC IACUC policy 026). Animals were injected intraperitoneal (I.P.) under 3-5% inhalation (isoflurane) anesthesia on Day 0 with the appropriate treatment. Clinical signs and appetence were recorded daily. When needed, animals were given Lactated Ringer's Solution SQ due to blood loss from sampling. Clinical scores were determined by weight loss, behavior and appearance. Physicals, weights and blood draws occurred every other day on day 1 – 13 under 3-5% inhalation anesthesia. After day 17, animals were moved from single housing to group floor housing and additional blood draws were taken at 20 and 28 days post treatment. For all blood collections, serum was separated (Sarstedt) and stored at 4°C for downstream analysis. The following samples were unable to be collected during the study from VIG and MPXV-26 treated animals respectively: 13068 day 13, 9996 day 1 and 9996 day 3.

Plaque Reduction Neutralization Assays (PRNTs)

IMV PRNTs: The West African strain MPXV-USA-2003-044 (DQ011153 (1)) was selected for PRNTs. Working stocks of the IMV virus were prepared as previously described (39), diluted to $1x10^6$ plaque forming units (pfu)/ml and stored at -80°C. Vero E6 cells were seeded in 96-well, flat-bottom, tissue-culture plates (Costar) with DMEM (Thermo Fisher) containing 10% FBS (Atlanta biologicals; heat inactivated at 56°C for 1 hour) and incubated 20-24 hours at 37°C and 5.0% CO_2 until ≥90% confluent. Prairie dog serum samples and VIG (assay positive control) were heated for 30-60 min at 56-60°C to inactivate complement and serially diluted 2-fold starting with a 1:40 dilution in RPMI (Thermo Fisher) supplemented with 2% FBS (Atlanta biologicals; heat inactivated at 56°C for 1 hour). VIG (lot no. 10703654) provided by SNS, was diluted the same as above starting with a 1:640 dilution.

For 12 wells on each plate no serum was added (virus-only control), and for 6 wells no serum or virus was added (cell-only control). The IMV working stock was thawed, sonicated 2

times on ice in 1 min increments at 40% output with a 1 minute incubation on ice between, and diluted such that 27 – 93 plaques per well was detected in virus only wells and added per well to a 96-well incubation plate containing appropriate serum dilutions. The MPXV and diluted serum was allowed to incubate for ~18-20 hours at 35.5°C and 5% CO_2. After the incubation, the mixture was added to a confluent cell monolayer in duplicate plates. Plates were rocked intermittently for 1 hour, then RMPI supplemented with 2% FBS and 0.5% methylcellulose was added and incubated 48 hours at 35.5°C and 5% CO_2. After incubation, crystal violet stain containing 40% formalin was added to all wells and incubated 15 minutes at room temperature. Plates were washed 3 times with water, air dried, and scanned using a CTL analyzer.

PRNT Analysis: BioSpot Professional (v. 7.0.17, CTL) was used to count and quality control plaques from scanned images. Wells on the external edge of the plate were excluded from analysis. Results from duplicate plates were accepted if the mean number of plaques per well for the virus-only wells fell within a 95% tolerance interval of 27 – 93 plaques per well (actual range 30-55 pfu) and cell only controls had 0 plaques per well The mean pfu per well was calculated from duplicate plates for virus-only and the 1:40 serum dilution. The mean pfu per well for the serum was compared to the mean virus-only from the same plates to determine the percent neutralization. The mean and standard deviation percent neutralization were calculated and plotted using GraphPad Prism (v 6.07).

Efficacy Study

Animals

Wild caught black-tailed prairie dogs (*Cynomys ludovicianus*) procedures were identical to that described above unless listed below. At the time of the study, animals were estimated to be between 8 months and three years of age. Animals used in the longevity study were excluded from the efficacy study. There were n=5 animals per group except for 2D22 which had n=4 animals. Animal sex was split evenly in the 2D22 group and for VIG and Mix4 groups was the same (n=3 males, n=2 females per group). Single housing acclimation occurred for at least

three days (CDC IACUC policy 005) and animals were cared for under CDC IACUC approved protocol 2924KONPRAC.

Challenge Virus, Inoculation and Treatment Dosing

A purified viral preparation of West African MPXV, MPXV-USA_2003-044 (DQ011153 (1)) was selected as the challenge virus. The isolate underwent 4 passages in BSC-40 cells before undergoing purification as previously described (39). Animals were challenged intranasal (IN) under 3-5% inhalation anesthesia (isoflurane) with a LD_{90} (5×10^5 pfu) of MPXV diluted in phosphate buffered saline (PBS), which was confirmed by back-titration immediately following inoculation. Animals were inoculated via the intranasal (IN) route under 3-5% inhalation anesthesia (isoflurane) by instilling 5 µl of inoculate into each of the nares (10 µl total). Treatment dosing occurred one day prior to inoculation and 6 dpi by the I.P. route (while under inhalation anesthesia) with one of the following treatments: 12,000 IU/kg of VIG (lot no. 10703654; SNS), 48 mg/kg of Mix 4 (equates to 12 mg/kg per mAb) or 48 mg/kg of 2D22 (irrelevant mAb used as the control).

Animal Sampling, Observations and Euthanasia

All invasive samples were collected under 3-5% inhalation isoflurane anesthesia. Prior to inoculation, a blood sample (pre-) was collected via the femoral vein (CDC IACUC policy 026). Clinical signs and an appetence log were recorded daily. Physicals, oral swabs, whole blood and serum samples (Starsedt) were taken on the following dpi: 1, 4, 6, 8, 10, 12, 14, 17 and 21. Clinical scores were determined by weight loss, behavior and appearance. Once a score ≥5 was reached, animal checks increased to twice daily and buprenorphine (0.1 mg/kg) was given if warranted based on clinical signs SQ twice a day for no longer than three days total based on previous studies (40). At 21 dpi or a pain score ≥10, euthanasia was performed via exsanguination and beuthanasia solution by intracardiac administration under 5% inhalation anesthesia. A necropsy was performed, and necropsy tools were decontaminated in 5% micro-

chem, scrubbed with a brush and rinsed with water. All tissues, whole blood and serum were frozen at -80°C.

Molecular Assays

Swabs were processed as previously described (30). Tissues and whole blood in EDTA were thawed on ice. Aliquots of 1 mm zirconia/silica beads (BIOSPEC) and PBS for tissue homogenizing were made by mixing 0.5 ml and 1 ml respectively. Each bead/PBS tube was weighed; the tube was then weighed again after the tissue was added to get an approximate tissue weight. Tissues were homogenized using the Precellys 24 (Precellys) under the manufacture setting “cycle 5” which grinds for 20 seconds twice with a one minute break in-between. A 100 µl aliquot of processed tissue sample or eluant (whole blood in EDTA and oral swab) was mixed with 10 µl of Proteinase K (Qiagen) and 100 µl of AL buffer (Qiagen) and heated for 15 minutes ≥56°C to inactivate the sample. Inactivated samples were then transferred to BSL-2 for DNA extraction using the BioRobot EZ-1 Workstation (QIAGEN) and EZ-1 DNA Tissue Kit (QIAGEN). Real-time PCR was performed using Taqman master mix (Thermo Fisher) and primers and probe that target the E9L gene (41). Using the Applied Biosystems® 7500 Fast Dx real-time PCR Instrument (Thermo Fisher), cycling conditions included activation at 95°C for 20 seconds, followed by 40 cycles of denaturation for 3 seconds at 95°C and elongation for 30 seconds at 60°C. A standard curve of purified MPXV DNA was present on every PCR plate (1 ng/µl – 10 fg/µl) in order to quantify DNA levels. The positive cut-off value was 2 fg/rxn and samples were considered negative for DNA if they did not reach this level.

PRNTs

IMV PRNTs: IMV PRNTs were performed as described above except the starting serum dilution was 1:160.

EV PRNTs: EV was produced for each PRNT by infecting Vero E6 cells at a multiplicity of infection (MOI) of 1 with an IMV working stock. After incubating for 1 hour at 35.5°C with 5%

CO_2, the inoculation was removed and 1.2 ml of RPMI-1640 (Thermo Fisher) supplemented with 2% FBS (Atlanta biologicals; heat inactivated at 56°C for 1 hour) and 10 µg/ml of IMV antibody 7D11 (USAMRIID) was added to wells to neutralize any IMV. The cells were incubated for ~48 hours at 35.5°C with 5% CO_2 before the supernatant was collected, centrifuged at 1000 x g for 20 minutes and transferred to a new tube to remove any remaining cellular debris (42). On the day of harvesting the EV, titration was performed by serially diluting EV in RPMI-1640 supplemented with 2% FBS and 10% tissue culture grade baby rabbit complement (Cedar Lane); then adding 0.5ml of each dilution to vero cells (~95% confluence) in a twelve well tissue culture plate (Corning). Plates were incubated for 1 hour at 35.5°C with 5% CO_2 followed by the removal of inoculum, addition of RPMI-1640 with 2% FBS and continued incubation for 48 hours at 35.5°C with 5% CO_2. After incubation, plates were stained with crystal violet as described above. Once the titer was confirmed, PRNTs were performed within 48-72 hours. The EV PRNTs were conducted similarly to the IMV PRNTs with several modifications. The virus was diluted in RPMI-1640 with 10% baby rabbit complement (Cedar Lane) and 2% FBS. The EV and serum diluted 1:40 was thoroughly mixed and incubated 1 hour (opposed to overnight) at 35.5°C and 5% CO_2 prior to adding to a confluent cell monolayer in duplicate plates. Plates were rocked intermittently for 1 hour, then RMPI supplemented with 2% FBS and 0.5% methylcellulose was added and incubated 72 hours at 35.5°C and 5% CO_2. Serum from Animal 17013 6 dpi was not available for testing.

PRNT analysis: Data were collect as described above. The 50% endpoint serum dilution was calculated from the linear regression of mean pfu per well over log-transformed dilutions and 50% of the mean pfu per well from virus-only controls on the same plates. At least three points around the 50% endpoint were used in the linear regression. Results from duplicate plates were accepted if the mean pfu per well for the virus-only wells fell within a 95% tolerance interval of 27-93 plaques per well (actual range 41-75 pfu for IMV and 37-61 for EV). The geometric mean titer for each group was calculated and plotted using GraphPad Prism (v 6.07).

Histopathology

Tissues were fixed in formalin for 48 hours and were then considered to be fully inactivated and transferred to a BSL-2 laboratory. Prior to pathology analysis, samples were changed from formalin to 70% ethanol. Tissues were processed for routine paraffin histology, sectioned at 4 µm, and stained by hematoxylin-eosin for histopathological evaluation.

Statistics

Comparisons of 50% PRNTs for IMV and EV were made between groups for each day of sampling using a Wilcoxon rank-sum test. Correlations between PRNT results (IMV and EV) and mortality were calculated using each individual animal's peak IMV and EV viral load and date of necropsy. Differences in dates of euthanasia were compared using Wilcoxon rank sum test. Survivorship differences between groups was assessed using the Kaplan-Meier method, log rank test. A p value of < = 0.05 was considered statistically significant. Data analysis was performed using SAS (v9.3). Descriptive statistics including mean and median were used to summarize the DNA levels for each group, on each day.

RESULTS

Longevity Study

To the best of our knowledge, human mAbs have never been tested in prairie dogs. Before starting the efficacy study, we wanted to ensure that animals could tolerate human mAbs at the anticipated efficacy study dose (48 mg/kg), determine when and for how long neutralizing mAbs appear in the bloodstream, and when ≥50% neutralization is seen. At the start of the efficacy study, VIG was the only FDA licensed product for OPXV infections and it is approved for VACV vaccination adverse events. In this study and subsequent efficacy study, we used it as a control to compare to the efficacy of Mix4. Animals were injected I.P. with one of the following treatments: MPXV-26 at 8 mg/kg, VIG at 12,000 IU/kg or 2D22 at 48 mg/kg. Animals tolerated the antibodies well in all treatment groups with no negative side effects seen in terms of behavior, appetence and weight loss (data not shown).

Several animals developed health issues that were assessed by the attending veterinarian (AV) and deemed not to be treatment related; as these are wild-caught outbred animals various health issues are not unanticipated. Throughout the study, several animals were treated with chlorhexidine to resolve dermatitis and/or pododermatitis by the AV: animal 13068 (VIG treatment) on Days 1, 3, 5 and 7 and Animal 120916 (MPXV-26) on Day 3. Animal 13068 lost both upper incisors likely while trying to escape the single housed cage on Day 5 and the wound healed without issue. As no other animals in this group experienced tooth loss, it was believed to be unrelated to treatment. One animal from the VIG group (13118) had blood noted in its urine on Day 11 and a full physical was performed by the AV. The animal was deemed fit to remain on the protocol and given 0.1 mg of buprenorphine. A sterile urine sample was collected for bacterial culture, but no infection was identified. On day 13 post injection, blood was again noted in the urine. X-rays, urine and blood were collected for urinalysis, culture, and a CBC/chemistry. The animal received additional 0.1 mg of buprenorphine and remained on the protocol. Urinalysis revealed a urinary tract infection and treatment with Baytril resolved the infection. The animal was monitored for 30 days post study end and remained healthy.

Neutralization capacity of the mAbs in the serum was determined using an IMV PRNT. At day 5 post treatment, the average of both treatment groups was ≥50% neutralization (Figure 2.1). Day 7 post injection was the last sample day for both groups where a majority of animals had ≥50% neutralization (data not shown). As expected, there was great deal of variation in neutralization between animals within the same group as this is an outbred animal. Some level of neutralization was still detected until Day 13 post treatment for VIG animals and until Day 28 post treatment for MPXV-26 animals. Samples from the 2D22 group, irrelevant mAb, were not analyzed by the PRNT. Based on the longevity study results, a two-dose regimen of treatment one day prior to infection and six days post inoculation was selected for the efficacy study.

Efficacy Study

For the efficacy study, animals were treated with 48 mg/kg of Mix4 (n=5; 12 mg/kg per mAb in the Mix), 12,000 IU/kg of VIG (n=5) or 48 mg/kg of 2D22 (n=4). Animals were given treatment one day prior to infection, animals were challenged with an LD_{90} of WA MPXV on day 0, and a second treatment dose was administered 6 dpi based on the longevity serum analysis. Unfortunately, two of the VIG treated animals did not receive the full second dose on day 6 pi due to inadequate VIG volume. Animal 17035 was given 2,500 IU/kg and 17106 was given 9,000 IU/kg. Mix4 provided better protection than VIG and 2D22 in terms of mortality with survival rates of 80%, 40% and 25% respectively (Figure 2.2, Table 2.1). A majority of the Mix4 treated animals had delayed lesion onset, developed <5 lesions and experienced minimal weight loss (<5 %). One Mix4 animal did experience mild weight loss (10-15%) but only at 21 dpi. Several of these animals did experience mild respiratory distress which suggests inflammation in the respiratory tract. One animal treated with Mix4 was euthanized at 17 dpi by a veterinarian; this animal did not reach a pain score of 10, but had other concerning clinical signs requiring euthanasia including severe respiratory distress, hemorrhagic nasal discharge and gastric bloating. This animal, 17122, had gross lesions observed on several of its internal organs including the liver, spleen, small intestine and on mesenteric lymph nodes at necropsy which were likely due to the viral infection. Multiple tissues (heart, skin, tongue, lung, spleen, liver, bladder, testis, intestine) collected from this animal were sent to a pathologist for further analysis and MPXV as a cause of death was not evident (data not shown). This could be potentially due to sampling bias during necropsy as has been shown to be possible with this animal model (43). A strict euthanasia criteria is used for this animal model and it is possible this animal would have recovered from infection. Excluding this animal, minimal findings were observed during the study for the other Mix4 animals (predominantly mild respiratory distress). At necropsy of the other Mix4 treated animals only a pale, friable liver was noted for animal 17002. VIG treated animals also had delayed lesion onset, develop <5 lesions but experienced moderate weight loss (<15%) and moderate-to-severe respiratory distress. While 40% (2/5) of

the animals survived infection, the two animals that did not receive a full second dose of VIG (Animals 17035 and 17106) and Animal 17038 (which did receive two full doses) met criteria to be euthanized prior to the end of the study (21 dpi). These animals experienced moderate to severe respiratory distress and/or weight loss. The VIG group had findings at necropsy that were indicative of a viral infection such as enlarged lymph nodes, splenomegaly with or without splenic lesions, pale and/or mottled kidneys and friable liver. 2D22 treated animals experienced the following clinical signs: moderate weight loss (≤15%), skin lesions, decreased appetence and moderate-to-severe respiratory distress. While a high number of lesions was not noted in 50% (2/4) of the animals, these animals were euthanized prior to lesion onset. Animals that met euthanasia criteria had necropsy findings similar to the VIG group such as splenomegaly, splenic lesions enlarged lymph nodes and pale liver.

Viral DNA isolated from oral swabs throughout the study was similar in regard to viral load when comparing the three different treatment groups (Figure 2.3), however there were subtle differences observed in onset and duration depending on treatment regimen. Viral DNA was detectable on 6 dpi in oral swabs in most Mix4 treated animals (4/5) and had a sharp decrease in viral load on 8 dpi (3/5 animals) suggesting the second treatment dose on 6 dpi was efficacious (Figure 2.3A). However, in all animals the levels continued to rise on 10 dpi and peaked a second time on 12-17 dpi (last sample collected). Levels began to decline in 3/5 animals 12-14 dpi when the prairie dogs mount their own immune response to infection (30). Although two animals (17002 and 17036) still had increasing oral viral DNA levels 17 dpi, these animals survived with minimal clinical signs to the study end suggesting the virus was being cleared by the host immune system and residual DNA remained. Animals treated with VIG had more variable levels in oral swabs compared to Mix4 treated animals (Figure 2.3B). Animal 17035 had similar trends with Mix4 group with a decrease in viral levels post the second treatment dose suggesting 2,000 IU/kg of VIG still neutralized MPXV. Two animals (17038 and 17046) had no viral DNA detected in oral swabs until 6-8 dpi compared to n=1 animal (17116)

that had a consistent low level of viral DNA detected in oral swabs throughout the study. For the remaining animal (17035), the second dose of VIG at 9,000 IU/kg did not appear to have any effect on the virus evident by rising detectable levels through 6-11 dpi. For animals treated with 2D22, viral DNA in oral swabs was first detected 4 dpi and continued to rise in most animals until euthanasia (Figure 2.3C). Animal 17111, which survived until study end, had a gradual increase in viral DNA in oral swabs from 6-14 dpi until levels declined from 14-17 dpi suggesting the development of a strong host immune response.

DNAemia was slightly delayed in the Mix4 group starting at 6 dpi compared to 4 dpi for the VIG and 2D22 groups (Figure 2.4). 2D22 and VIG group had significantly higher DNA level when compared to MIX4 group on day 4 of the study (p=0.018 and 0.008). Group 2D22 and VIG had higher DNA levels throughout the study, but this difference failed to reach significance after day 4, likely due to small sample size. The Mix4 group had the lowest mean levels ($2.78x10^1$ fg/rxn group mean) with the highest individual DNAemia level of $1.54x10^2$ fg/rxn (Table 2.1). Although the VIG group had lower levels than 2D22, the group mean level were ~1 log higher than the Mix4 group with the VIG group mean at $1.26x10^2$ fg/rxn and the highest individual DNAemia level of $1.38x10^3$ fg/rxn. As predicted, the highest levels of DNAemia were seen in 2D22 group with the group mean at $5.76x10^2$ and highest individual value at $5.81x10^3$ fg/rxn (Figure 2.4, Table 2.1).

Viral DNA isolated from tissues throughout the study was similar in regard to viral load when comparing the three different treatment groups (Figure 2.5, Table 2.1). In the Mix4 group, the most frequent tissues positive and those with the highest levels were detected in the skin, tongue and nares (Figure 2.5A). The oral cavity (tongue/nares) was the site of inoculation while the skin is one of the last organs to be infected in this model (30). However, since there were minimal skin lesions in the Mix4 group (<4), this suggests that treatment delayed/prevented lesion formation (Figure 2.5A, Table 2.1). While viral DNA loads were similar between the treatment groups, the number of tissues positive per group greatly varied. The Mix4 group had

69% tissues positive for viral DNA compared to VIG at 82% and 2D22 at 94% (Figure 2.5A-C). Since all three treatment groups had animals that did not survive until study end, tissue loads of internal organs for non-survivors were analyzed (Figure 2.5D). 2D22 had the highest levels overall followed by VIG and Mix4 groups. The Mix4 animal (17122), did have very high levels in the mesenteric lymph node and liver likely due to lesions being present in those tissues.

The 50% endpoint serum dilution was similar within the Mix4 group when looking at IMV neutralization between animals (Figure 2.6A). The titer begins to increase ~12 dpi which is around the time the host has detectable antibodies against the virus in this model (30), and the titer continues to increase for all animals until 21 dpi (study end). The Mix4 treated animal euthanized prior to study end, Animal 17122, had the weakest titer post the first treatment. While the second treatment resulted in a comparable titer to the other Mix4 animals 8 dpi, the titer was once again the lowest in the group at 10 dpi. Similar, but slightly lower, titers against IMV were detected after the first VIG treatment (Figure 2.6B). As seen with Mix4, the VIG non-survivors had the lowest 50% endpoint serum after the first treatment and at 10 dpi. Animal 17116 developed a strong host immune response as evident by increasing titers 14-21 dpi which was similar to Mix4 survivors. Animal 17046 developed a milder host humoral response against IMV compared to 17116. The highest pain score reached between the two animals was similar (pain score of 4 vs. 5). The 2D22 group provided additional insight to host antibodies against IMV appearing at ~10 dpi for a majority of the animals (Figure 2.6C). The survivor, Animal 17111, had the highest measured titer from any treatment group.

IMV titers likely due to exogenous antibody were significantly higher on days 1-6 for Mix 4 and VIG groups when compared to 2D22 (p-values ranging from 0.006–0.02). 2D22 group had higher IMV titers starting on day 10 (likely due to the animals' immune response) and continuing through the end of the study, although these differences did not reach significance. The overall geometric mean titer was the highest for the 2D22 group, followed by Mix4 and then VIG (Wilcoxon rank sum test p=0.0002). In assessing protection, peak IMV titers for each

animal were positively correlated with the date of necropsy, when controlling for group (correlation coefficient=0.697, p=0.0081). In other words, as IMV neutralization capacity increased, the animals survived for longer times post infection. The 50% endpoint serum dilution was also determined for EV neutralization in the presence of complement. Unlike IMV titers, no neutralization was detected post the first treatment for either Mix4 or VIG treatment groups (Figure 2.7A-B). Low titers were detected for some animals in Mix4 and VIG groups beginning at 12 dpi, when host antibodies are detectable in this model (30), suggesting neither Mix4 or VIG were able to neutralize EV. While all Mix4 animals did develop a titer against EV, not all VIG treated animals developed a detectable titer regardless of surviving infection. As seen with IMV neutralizing, the 2D22 survivor had the highest detectable titer. The geometric mean titer was the highest for the 2D22 group, followed by Mix4 and then VIG (Figure 2.7D) (Wilcoxon rank sum test p-value=0.015). The correlate of protection from mortality was calculated using each animal's peak EV result. Peak EV result levels were somewhat positively correlated with date of necropsy, less so than IMV (correlation coefficient=0.59282, p=0.033).

Discussion

The specific immune response required for protection against OPXVs has been studied extensively and studies have shown that neutralizing antibodies are critical for protection against disease. Previous studies during the smallpox eradiation determined that individuals with neutralizing titers (<1:20 or <1:32) were more likely to develop smallpox post exposure than those that had titers at >1:20 or ≥1:32 (44, 45). A study in NHPs with MPXV challenge has supported that humoral responses are necessary for protection compared to cell-mediated responses (46). However, the human and NHP studies only evaluated neutralization of the IMV form of the virus. While this is a traditional method for detecting antibody neutralization, it only tells part of the story. EV is believed to play a critical role in viral dissemination (35), is more difficult to neutralize than IMV (37) and anti-sera of A33 or B5 (EV proteins) alone are protective in animal models (47). Previous research suggests that vaccination against one OPXV protein

(either IMV or EV) or treatment with an exogenous mAb targeting one OPXV protein (either IMV or EV) can provide protection against mortality but not morbidity (27, 47-49). However, animals treated with MCMs targeting both IMV and EV protected protection against mortality and lessened morbidity (27, 37, 48, 49). Our current study supports this notion as prairie dogs treated with Mix4 resulted in 80% survival, and despite being treated with Mix4, were unable to neutralize MPXV EV. Previous *in vitro* results with MPXV suggested that one of the α-EV mAbs in Mix4 (α-33) was capable of neutralization (38), nonetheless these results do not always correlate *in vivo*. Mix4 provided 100% protection in a lethal VACV challenge in C57BL/6 mice and all four mAbs in Mix4 were shown to neutralize their respective protein targets *in vitro* (38). However, our *in vivo* results demonstrate Mix4 was insufficient to neutralize MPXV EV *in vivo* and Mix4 may not be the ideal mAb combination for a MPXV MCM. The Vanderbilt Vaccine Center has developed n=48 mAbs that are capable of neutralizing either IMV or EV of one representative strain of an nonvariola OPXV (38). As MPXV poses a global threat, the replacement of one or both α-EV mAbs within Mix4 should be considered to strength the potential of Mix4 as a MCM against both monkeypox and smallpox. PRNTs against VARV should also be conducted prior to making the decision to alter Mix4 in order to provide a potential MCM for both MPXV and VARV.

It should be noted that there are several limitations in the use of our IMV and EV PRNTs to determine viral neutralization within the serum. For instance, the levels of neutralizing antibodies in these studies could be higher than reported. In order to achieve detectable neutralizing responses in the EV PRNT, complement use in the assay is required giving insight to the mechanism of action for the α-EV mAbs. Without complement, neutralization was not detected against EV for multiple representative strains of OPXV (38). This suggests the mechanism of action could be cell lysis by activating complement membrane attack complex opposed to viral neutralization by binding to viral cell entry receptors. Neutralization by opsonization and phagocytosis are also not assessed in these assays. Future PRNT studies

should be conducted with prairie dog macrophages and a human macrophage cell line to better understand the potential contribution of other mechanism of actions by the mAbs within Mix4. In particular, α-B5 mAbs have been reported to use complement to increase opsonization (42). Additionally, historic literature has suggested that complement in the presence of α-A33 antibodies may allow for viral entry by complement lysing the extracellular membrane forming IMV and allows for “escape” from the antibody and viral entry into the cell (50). Future PRNTs with α-A33 antibodies should also be conducted with and without an IMV neutralizing antibody to aid in identifying the mechanism of action.

Our study presents evidence that Mix4 could be used as a potential candidate for a smallpox and monkeypox MCM and supports the use of testing human mAbs or mAb cocktails in the prairie dog MPXV model. The licensure of TPOXX® (tecovirimat; SIGA) by the FDA for treatment of smallpox was a step forward in smallpox preparedness, however clinical use of this compound has resulted in OPXVs developing resistance (34) suggesting a multi-therapeutic approach would be more advantageous. Additionally, there is still no therapeutic FDA approved for monkeypox even though this disease poses a global threat. While VIG has received licensure by the FDA for adverse reactions to smallpox vaccination (VACV), VIG is in limited supply since it is no longer mass-produced after eradication of smallpox and cessation of routine smallpox vaccinations. Furthermore, several studies in surrogate OPXV animal models have provided evidence administration of mAb cocktails provide better protection than a single mAb or VIG (37, 48, 49, 51). mAb cocktails could be an option to fill the need for additional therapeutics for both smallpox and monkeypox disease. In recent years, mAbs have been FDA licensed for treatment of multiple conditions and pathogens, including cancer, asthma, arthritis, anthrax (obiltoxaximab; raxibacumab), *Clostridium difficile* toxin B (bezlotoxumab) and respiratory syncytial virus (palivizumab). Our results, along with others, indicate that mAbs or mAb cocktails are a possible candidate for a smallpox therapeutic and should be evaluated in addition to small molecule compounds (37, 48, 49, 51). We present further evidence that

although the prairie dog is not a covered animal model under the FDA Animal Rule, it is a valuable small animal model for evaluating OPXV MCMs due to its similarity to human monkeypox. This model can be used to test the efficacy of human mAbs or mAb cocktails, as no negative reactions were seen even at high doses, and longevity studies with PRNT assays can be used to determine when neutralizing mAbs appear post injection and how long they remain in circulation. As the model develops systemic cutaneous lesions following an incubation period similar to human disease (~7-14 days), the model could also be used in future studies to test efficacy of a mAb cocktail at lesion onset. While Mix4 provided protection against disease mortality and delayed DNAemia, one animal in this group was euthanized and several other animals experienced mild respiratory distress. The Vanderbilt Vaccine Center has developed multiple OPXV neutralizing mAbs and a new mix should be considered if the goal of this mAb cocktail MCM is for both smallpox and monkeypox. While improvements can be made, the Mix4 prairie dog MPXV study should be considered a successful pilot study and a step forward in smallpox biopreparedness.

Acknowledgements

The authors would like to thank Will Carson and Zachary Weiner for their technical support with molecular assays, the CDC Comparative Medicine Branch for their animal care and support and the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) for the α-L1 (7D11) monoclonal antibody.

References

1. Likos AM, Sammons SA, Olson VA, Frace AM, Li Y, Olsen-Rasmussen M, Davidson W, Galloway R, Khristova ML, Reynolds MG, Zhao H, Carroll DS, Curns A, Formenty P, Esposito JJ, Regnery RL, Damon IK. 2005. A tale of two clades: monkeypox viruses. J Gen Virol 86:2661-72.

2. Chen N, Li G, Liszewski MK, Atkinson JP, Jahrling PB, Feng Z, Schriewer J, Buck C, Wang C, Lefkowitz EJ, Esposito JJ, Harms T, Damon IK, Roper RL, Upton C, Buller RM. 2005. Virulence differences between monkeypox virus isolates from West Africa and the Congo basin. Virology 340:46-63.

3. Khodakevich L, Jezek Z, Kinzanzka K. 1986. Isolation of monkeypox virus from wild squirrel infected in nature. Lancet 1:98-9.

4. Nolen LD, Osadebe L, Katomba J, Likofata J, Mukadi D, Monroe B, Doty J, Hughes CM, Kabamba J, Malekani J, Bomponda PL, Lokota JI, Balilo MP, Likafi T, Lushima RS, Ilunga BK, Nkawa F, Pukuta E, Karhemere S, Tamfum JJ, Nguete B, Wemakoy EO, McCollum AM, Reynolds MG. 2016. Extended Human-to-Human Transmission during a Monkeypox Outbreak in the Democratic Republic of the Congo. Emerg Infect Dis 22:1014-21.

5. Hutson CL, Nakazawa YJ, Self J, Olson VA, Regnery RL, Braden Z, Weiss S, Malekani J, Jackson E, Tate M, Karem KL, Rocke TE, Osorio JE, Damon IK, Carroll DS. 2015. Laboratory Investigations of African Pouched Rats (Cricetomys gambianus) as a Potential Reservoir Host Species for Monkeypox Virus. PLoS Negl Trop Dis 9:e0004013.

6. Damon IK. 2011. Status of human monkeypox: clinical disease, epidemiology and research. Vaccine 29 Suppl 4:D54-9.

7. Sklenovska N, Van Ranst M. 2018. Emergence of Monkeypox as the Most Important Orthopoxvirus Infection in Humans. Front Public Health 6:241.

8. Mwamba DK KB, Shongo RL, Pukuta E, Kayembe NJM. 2014. Profil épidemiologique du monkeypox en RDC, 2010-2014. Ann African Med doi:Available online at: https://anafrimed.net/profil-epidemiologique-du-monkeypox-en-rdc-2010-2014-monkeypox-in-drc-epidemiological-profile-2010-2014/:1855-60.

9. Durski KN, McCollum AM, Nakazawa Y, Petersen BW, Reynolds MG, Briand S, Djingarey MH, Olson V, Damon IK, Khalakdina A. 2018. Emergence of Monkeypox - West and Central Africa, 1970-2017. MMWR Morb Mortal Wkly Rep 67:306-310.

10. Yinka-Ogunleye A, Aruna O, Dalhat M, Ogoina D, McCollum A, Disu Y, Mamadu I, Akinpelu A, Ahmad A, Burga J, Ndoreraho A, Nkunzimana E, Manneh L, Mohammed A, Adeoye O, Tom-Aba D, Silenou B, Ipadeola O, Saleh M, Adeyemo A, Nwadiutor I, Aworabhi N, Uke P, John D, Wakama P, Reynolds M, Mauldin MR, Doty J, Wilkins K, Musa J, Khalakdina A, Adedeji A, Mba N, Ojo O, Krause G, Ihekweazu C, Team CDCMO. 2019. Outbreak of human monkeypox in Nigeria in 2017-18: a clinical and epidemiological report. Lancet Infect Dis 19:872-879.

11. Anonymous. December 5 2018. Monkeypox case in England. https://www.gov.uk/government/news/monkeypox-case-in-england. Accessed September 8 2018.

12. Erez N, Achdout H, Milrot E, Schwartz Y, Wiener-Well Y, Paran N, Politi B, Tamir H, Israely T, Weiss S, Beth-Din A, Shifman O, Israeli O, Yitzhaki S, Shapira SC, Melamed S, Schwartz E. 2019. Diagnosis of Imported Monkeypox, Israel, 2018. Emerg Infect Dis 25:980-983.

13. Anonymous. 2019. Monkeypox - Singapore, *on* World Health Organization. https://www.who.int/csr/don/16-may-2019-monkeypox-singapore/en/. Accessed

14. Reed KD, Melski JW, Graham MB, Regnery RL, Sotir MJ, Wegner MV, Kazmierczak JJ, Stratman EJ, Li Y, Fairley JA, Swain GR, Olson VA, Sargent EK, Kehl SC, Frace MA, Kline R, Foldy SL, Davis JP, Damon IK. 2004. The detection of monkeypox in humans in the Western Hemisphere. N Engl J Med 350:342-50.

15. Radonic A, Metzger S, Dabrowski PW, Couacy-Hymann E, Schuenadel L, Kurth A, Matz-Rensing K, Boesch C, Leendertz FH, Nitsche A. 2014. Fatal monkeypox in wild-living sooty mangabey, Cote d'Ivoire, 2012. Emerg Infect Dis 20:1009-11.

16. Hutson CL, Damon IK. 2010. Monkeypox virus infections in small animal models for evaluation of anti-poxvirus agents. Viruses 2:2763-76.

17. Hutson CL, Lee KN, Abel J, Carroll DS, Montgomery JM, Olson VA, Li Y, Davidson W, Hughes C, Dillon M, Spurlock P, Kazmierczak JJ, Austin C, Miser L, Sorhage FE, Howell J, Davis JP, Reynolds MG, Braden Z, Karem KL, Damon IK, Regnery RL. 2007. Monkeypox zoonotic associations: insights from laboratory evaluation of animals associated with the multi-state US outbreak. Am J Trop Med Hyg 76:757-68.

18. Magnus PV, Andersen, E.K., Petersen, K.B., Birch-Andersen, A. 1959. A Pox-like Disease in Cynomolgus Monkeys. Acta Pathologica Microbiologica Scandinavica:156-176.

19. Parker S, Buller RM. 2013. A review of experimental and natural infections of animals with monkeypox virus between 1958 and 2012. Future Virol 8:129-157.

20. Americo JL, Moss B, Earl PL. 2010. Identification of wild-derived inbred mouse strains highly susceptible to monkeypox virus infection for use as small animal models. J Virol 84:8172-80.

21. Shchelkunov SN, Marennikova SS, Moyer RW. 2006. Orthopoxviruses Pathogenic for Humans. Springer US.

22. Tesh RB, Watts DM, Sbrana E, Siirin M, Popov VL, Xiao SY. 2004. Experimental infection of ground squirrels (Spermophilus tridecemlineatus) with monkeypox virus. Emerg Infect Dis 10:1563-7.

23. Schultz DA, Sagartz JE, Huso DL, Buller RM. 2009. Experimental infection of an African dormouse (Graphiurus kelleni) with monkeypox virus. Virology 383:86-92.

24. Chapman JL, Nichols DK, Martinez MJ, Raymond JW. 2010. Animal models of orthopoxvirus infection. Vet Pathol 47:852-70.

25. Earl PL, Americo JL, Cotter CA, Moss B. 2015. Comparative live bioluminescence imaging of monkeypox virus dissemination in a wild-derived inbred mouse (Mus musculus castaneus) and outbred African dormouse (Graphiurus kelleni). Virology 475:150-8.

26. Earl PL, Americo JL, Moss B. 2012. Lethal monkeypox virus infection of CAST/EiJ mice is associated with a deficient gamma interferon response. J Virol 86:9105-12.

27. Hooper JW, Thompson E, Wilhelmsen C, Zimmerman M, Ichou MA, Steffen SE, Schmaljohn CS, Schmaljohn AL, Jahrling PB. 2004. Smallpox DNA vaccine protects nonhuman primates against lethal monkeypox. J Virol 78:4433-43.

28. Huggins J, Goff A, Hensley L, Mucker E, Shamblin J, Wlazlowski C, Johnson W, Chapman J, Larsen T, Twenhafel N, Karem K, Damon IK, Byrd CM, Bolken TC, Jordan R, Hruby D. 2009. Nonhuman primates are protected from smallpox virus or monkeypox virus challenges by the antiviral drug ST-246. Antimicrob Agents Chemother 53:2620-5.

29. Zaucha GM, Jahrling PB, Geisbert TW, Swearengen JR, Hensley L. 2001. The pathology of experimental aerosolized monkeypox virus infection in cynomolgus monkeys (Macaca fascicularis). Lab Invest 81:1581-600.

30. Hutson CL, Carroll DS, Gallardo-Romero N, Drew C, Zaki SR, Nagy T, Hughes C, Olson VA, Sanders J, Patel N, Smith SK, Keckler MS, Karem K, Damon IK. 2015. Comparison of Monkeypox Virus Clade Kinetics and Pathology within the Prairie Dog Animal Model Using a Serial Sacrifice Study Design. Biomed Res Int 2015:965710.

31. Hutson CL, Kondas, A.V., Mauldin, M., Doty, J., Sickle, K.V., Morgan, C., Dietz-Ostergaard, S., Nakazawa, Y., Kling, C., Martin, B., Ellison, J.A., Carroll, C., Gallardo-Romero, N., Olson, V. 2019. Pharmacokinetics and Activity of Brincidofovir in a Lethal Prairie Dog Animal Model of Monkeypox. Manuscript in draft.

32. Smith SK, Self J, Weiss S, Carroll D, Braden Z, Regnery RL, Davidson W, Jordan R, Hruby DE, Damon IK. 2011. Effective antiviral treatment of systemic orthopoxvirus disease: ST-246 treatment of prairie dogs infected with monkeypox virus. J Virol 85:9176-87.

33. Keckler MS, Carroll DS, Gallardo-Romero NF, Lash RR, Salzer JS, Weiss SL, Patel N, Clemmons CJ, Smith SK, Hutson CL, Karem KL, Damon IK. 2011. Establishment of the black-tailed prairie dog (Cynomys ludovicianus) as a novel animal model for comparing smallpox vaccines administered preexposure in both high- and low-dose monkeypox virus challenges. J Virol 85:7683-98.

34. Lederman ER, Davidson W, Groff HL, Smith SK, Warkentien T, Li Y, Wilkins KA, Karem KL, Akondy RS, Ahmed R, Frace M, Shieh WJ, Zaki S, Hruby DE, Painter WP, Bergman KL, Cohen JI, Damon IK. 2012. Progressive vaccinia: case description and laboratory-guided therapy with vaccinia immune globulin, ST-246, and CMX001. J Infect Dis 206:1372-85.

35. Payne LG. 1980. Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J Gen Virol 50:89-100.

36. Smith GL, Vanderplasschen A, Law M. 2002. The formation and function of extracellular enveloped vaccinia virus. J Gen Virol 83:2915-31.

37. Moss B. 2011. Smallpox vaccines: targets of protective immunity. Immunol Rev 239:8-26.

38. Gilchuk I, Gilchuk P, Sapparapu G, Lampley R, Singh V, Kose N, Blum DL, Hughes LJ, Satheshkumar PS, Townsend MB, Kondas AV, Reed Z, Weiner Z, Olson VA, Hammarlund E, Raue HP, Slifka MK, Slaughter JC, Graham BS, Edwards KM, Eisenberg RJ, Cohen GH, Joyce S, Crowe JE, Jr. 2016. Cross-Neutralizing and Protective Human Antibody Specificities to Poxvirus Infections. Cell 167:684-694 e9.

39. Hughes L, Wilkins K, Goldsmith CS, Smith S, Hudson P, Patel N, Karem K, Damon I, Li Y, Olson VA, Satheshkumar PS. 2017. A rapid Orthopoxvirus purification protocol suitable for high-containment laboratories. J Virol Methods 243:68-73.

40. Hutson CL, Gallardo-Romero N, Carroll DS, Salzer JS, Ayers JD, Doty JB, Hughes CM, Nakazawa Y, Hudson P, Patel N, Keckler MS, Olson VA, Nagy T. 2019. Analgesia during Monkeypox Virus Experimental Challenge Studies in Prairie Dogs (Cynomys ludovicianus). J Am Assoc Lab Anim Sci 58:485-500.

41. Li Y, Olson VA, Laue T, Laker MT, Damon IK. 2006. Detection of monkeypox virus with real-time PCR assays. J Clin Virol 36:194-203.

42. Benhnia MR, McCausland MM, Moyron J, Laudenslager J, Granger S, Rickert S, Koriazova L, Kubo R, Kato S, Crotty S. 2009. Vaccinia virus extracellular enveloped virion neutralization in vitro and protection in vivo depend on complement. J Virol 83:1201-15.

43. Weiner ZP, Salzer JS, LeMasters E, Ellison JA, Kondas AV, Morgan CN, Doty JB, Martin BE, Satheshkumar PS, Olson VA, Hutson CL. 2019. Characterization of Monkeypox virus dissemination in the black-tailed prairie dog (Cynomys ludovicianus) through in vivo bioluminescent imaging. PLoS One 14:e0222612.

44. Mack TM, Noble J, Jr., Thomas DB. 1972. A prospective study of serum antibody and protection against smallpox. Am J Trop Med Hyg 21:214-8.

45. Sarkar JK, Mitra AC, Mukherjee MK. 1975. The minimum protective level of antibodies in smallpox. Bull World Health Organ 52:307-11.

46. Edghill-Smith Y, Golding H, Manischewitz J, King LR, Scott D, Bray M, Nalca A, Hooper JW, Whitehouse CA, Schmitz JE, Reimann KA, Franchini G. 2005. Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. Nat Med 11:740-7.

47. Galmiche MC, Goenaga J, Wittek R, Rindisbacher L. 1999. Neutralizing and protective antibodies directed against vaccinia virus envelope antigens. Virology 254:71-80.

48. Chen Z, Earl P, Americo J, Damon I, Smith SK, Yu F, Sebrell A, Emerson S, Cohen G, Eisenberg RJ, Gorshkova I, Schuck P, Satterfield W, Moss B, Purcell R. 2007. Characterization of chimpanzee/human monoclonal antibodies to vaccinia virus A33 glycoprotein and its variola virus homolog in vitro and in a vaccinia virus mouse protection model. J Virol 81:8989-95.

49. Lustig S, Fogg C, Whitbeck JC, Eisenberg RJ, Cohen GH, Moss B. 2005. Combinations of polyclonal or monoclonal antibodies to proteins of the outer membranes of the two infectious forms of vaccinia virus protect mice against a lethal respiratory challenge. J Virol 79:13454-62.

50. Lustig S, Fogg C, Whitbeck JC, Moss B. 2004. Synergistic neutralizing activities of antibodies to outer membrane proteins of the two infectious forms of vaccinia virus in the presence of complement. Virology 328:30-5.

51. McCausland MM, Benhnia MR, Crickard L, Laudenslager J, Granger SW, Tahara T, Kubo R, Koriazova L, Kato S, Crotty S. 2010. Combination therapy of vaccinia virus infection with human anti-H3 and anti-B5 monoclonal antibodies in a small animal model. Antivir Ther 15:661-75.

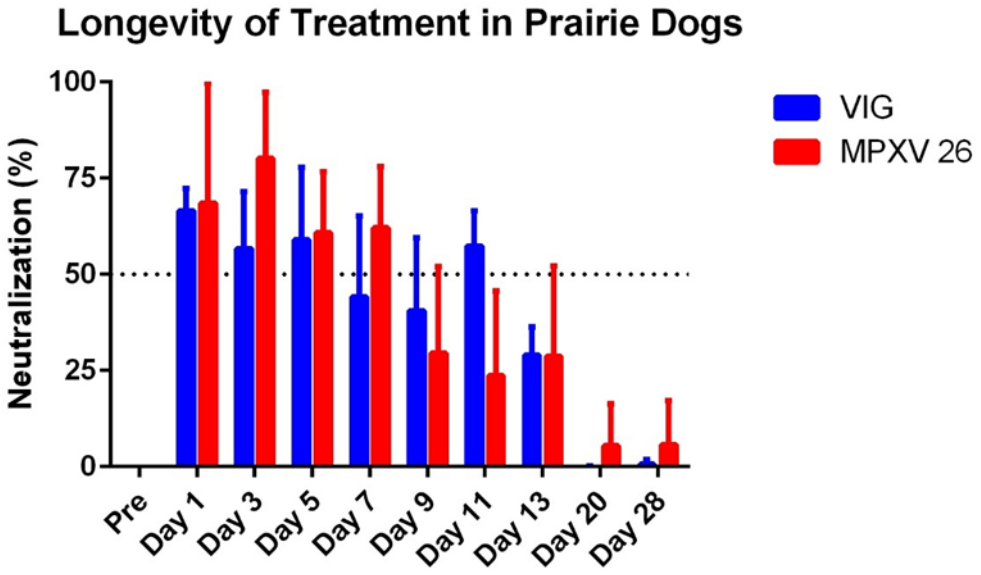


Figure 2.1. Human Antibodies Neutralize MPXV until at ≥7 days Post Treatment. Serum taken from prairie dogs treated with VIG at 12,000 IU/kg or 8 mg/kg of MPXV-26 was screened in a PRNT assay against MPXV IMV to determine neutralization capability of MPXV. As expected, variation was seen between animals in the same group in this outbred animal model. Neutralization at ≥50% was seen in both treatment groups in a majority of the animals until ≥7 days post treatment. The group average suggests ≥50% ends at 5 days post treatment for VIG. Samples were not available from Animal 13068 day 13 and 9996 Day 1 and Day 3.

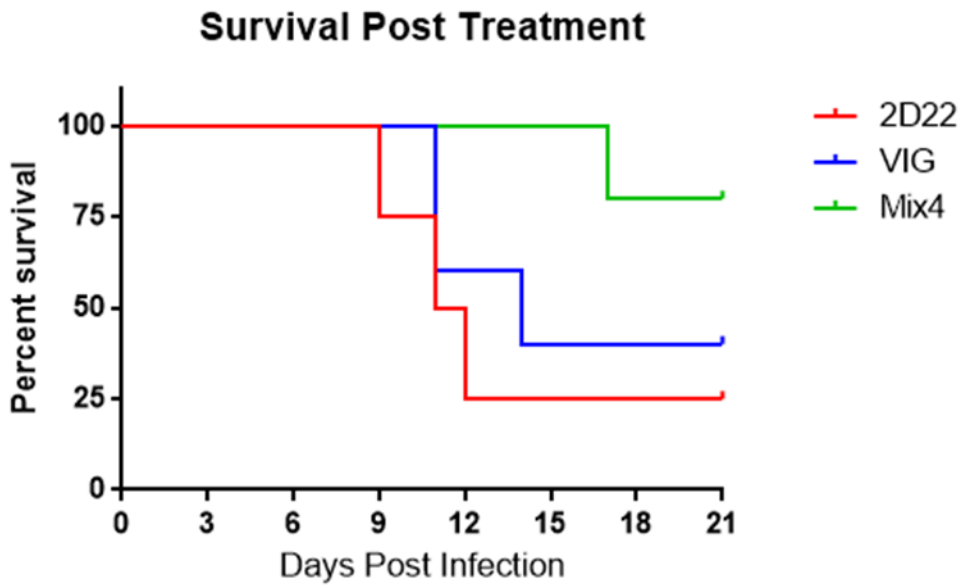
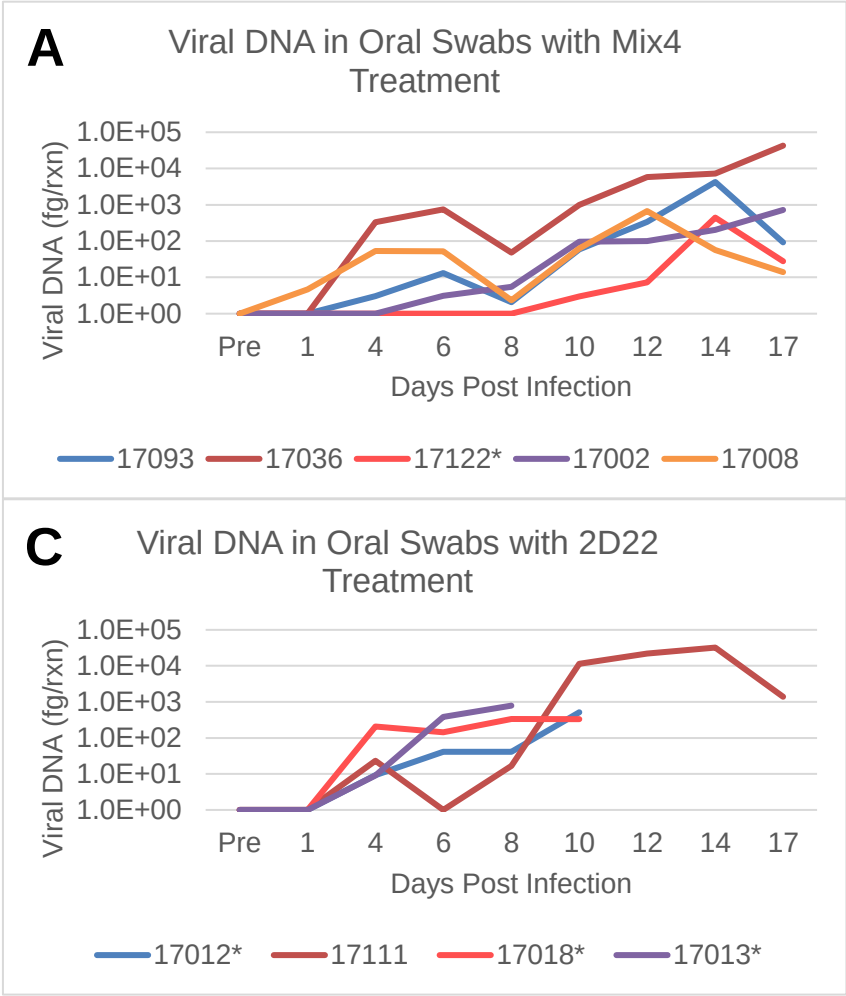
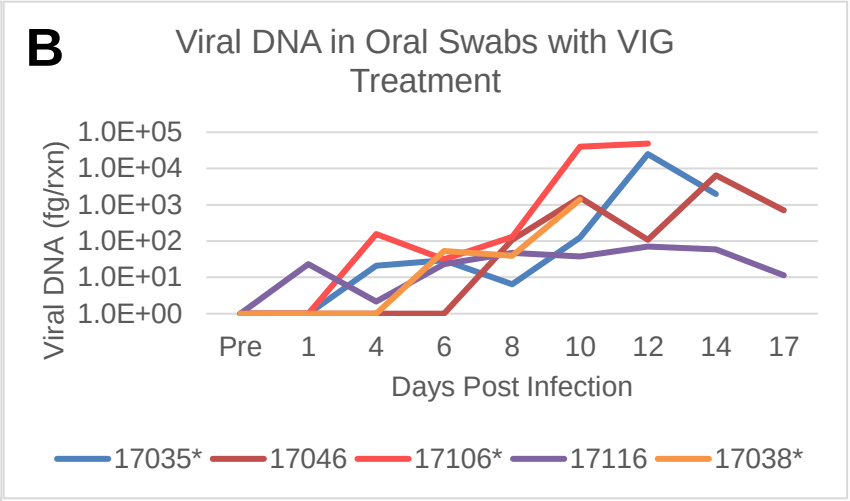


Figure 2.2. Survival Curve Post Treatment. Animals were treated at 1 day pre-infection and 6 days post infection with 48 mg/kg Mix4, 48 mg/kg 2D22, or 12,000 IU/kg VIG intraperitoneal. Mix4 provided greater protection than VIG and 2D22 with survival rates of 80%, 40% and 25% respectively.

Treatment Group	DPI Lesion Onset	Maximum Lesion Amount	Weight Loss	Observed Clinical Signs	Clinical Score Range	Mortality Date	Viral DNA Tissue (fg/rxn) Maximum and Mean	Viral DNA Oral Swab (fg/rxn) Maximum and Mean	Viremia (fg/rxn) Maximum and Mean
Mix4	14-17	4	Minimal <5%	Mild RD, Mild GI bloat, nasal discharge	3-10	17 (n=1)	3.43E+06	4.27E+04	1.54E+02
							1.81E+05	1.63E+03	2.78E+01
VIG	10-16	3	Moderate <15%	Nasal discharge, Mod RD, GI bloat	5-11	11 (n=1) 13 (n=1) 14 (n=1)	6.89E+06	4.88E+04	1.38E+03
							2.83E+05	3.75E+03	1.26E+02
2D22	3-10	50+	Moderate ≤15%	Nasal discharge, Severe RD, mild GI bloat, decreased appetite	7-16	9 (n=1) 12 (n=2)	1.45E+06	3.22E+04	5.81E+03
							1.04E+05	3.18E+03	5.76E+02

Table 2.1: A summary of the clinical outcome of treatment with Mix4, 2D22 or VIG. Clinical signs were included in “observed clinical signs” column if they were present in greater than 50% of the animals in any one group. Respiratory distress is abbreviated as “RD”. Gastrointestinal tract is abbreviated as “GI”.


A
Viral DNA in Oral Swabs with Mix4 Treatment
Viral DNA (fg/rxn)
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
Pre 1 4 6 8 10 12 14 17
Days Post Infection
17093 17036 17122* 17002 17008


B
Viral DNA in Oral Swabs with VIG Treatment
Viral DNA (fg/rxn)
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
Pre 1 4 6 8 10 12 14 17
Days Post Infection
17035* 17046 17106* 17116 17038*

C
Viral DNA in Oral Swabs with 2D22 Treatment
Viral DNA (fg/rxn)
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
Pre 1 4 6 8 10 12 14 17
Days Post Infection
17012* 17111 17018* 17013*

Figure 2.3. Viral Loads in Oral Swabs Post Treatment. Oral swabs from each treatment group, Mix4 (A), VIG (B) and 2D22 (C) were collected throughout the study. The viral load in oral swabs was determined using real-time PCR targeting the OPXV E9L gene. Oral swabs were not collected on the last sample day (21 dpi) as tongue was collected at necropsy. Animal IDs with an asterisk indicate the animal reached a pain score of ≥10 and was euthanized prior to study end (21 dpi). Samples from animals that died on non-sample days were included in the dataset for the next sample day. In order for samples to be considered positive, samples must be positive in duplicate reactions and contain ≥2fg/rxn.

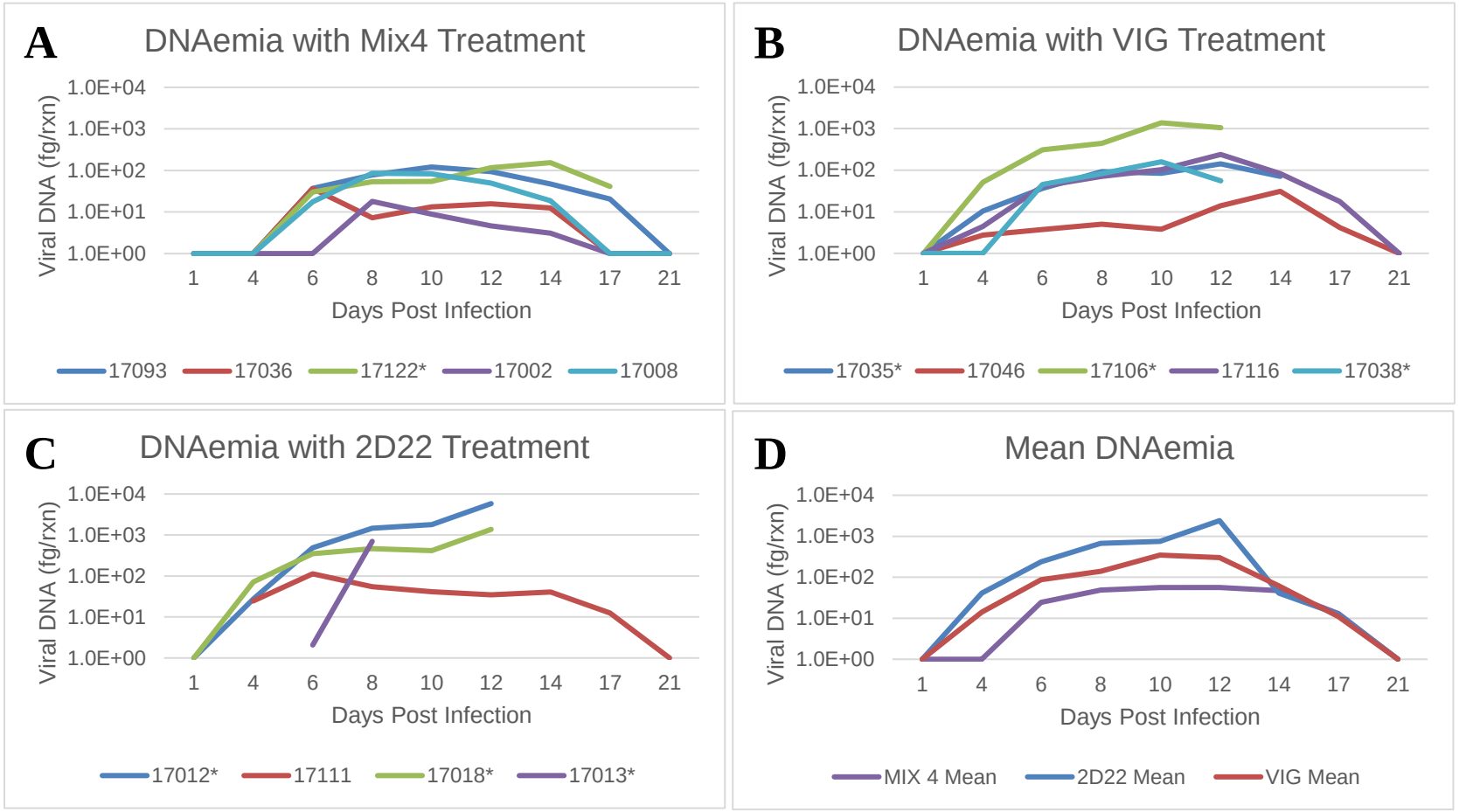


Figure 2.4. Delayed DNAemia in Mix4 Treated Animals. Whole blood was collected throughout the study to compare DNAemia between Mix4 (A), VIG (B) and irrelevant 2D22 (C) treatment groups. The DNAemia levels were statistically significant at 4 dpi when comparing 2D22 to Mix4 (p-value 0.018) and when comparing Mix4 to VIG (p-value 0.008). The treatment group mean (D) is presented for comparison. Viral loads were determined using real-time PCR targeting the OPXV E9L gene. Samples were not able to

be collected from Animal 17111 on 1 dpi and Animal 17013 4 dpi. Animal IDs with an asterisk indicate the animal reached a pain score of ≥10 and was euthanized prior to study end (21 dpi). Samples from animals that died on non-sample days were included in the dataset for the next sample day. In order to be considered positive, samples must be positive in duplicate reactions and contain ≥2fg/rxn.

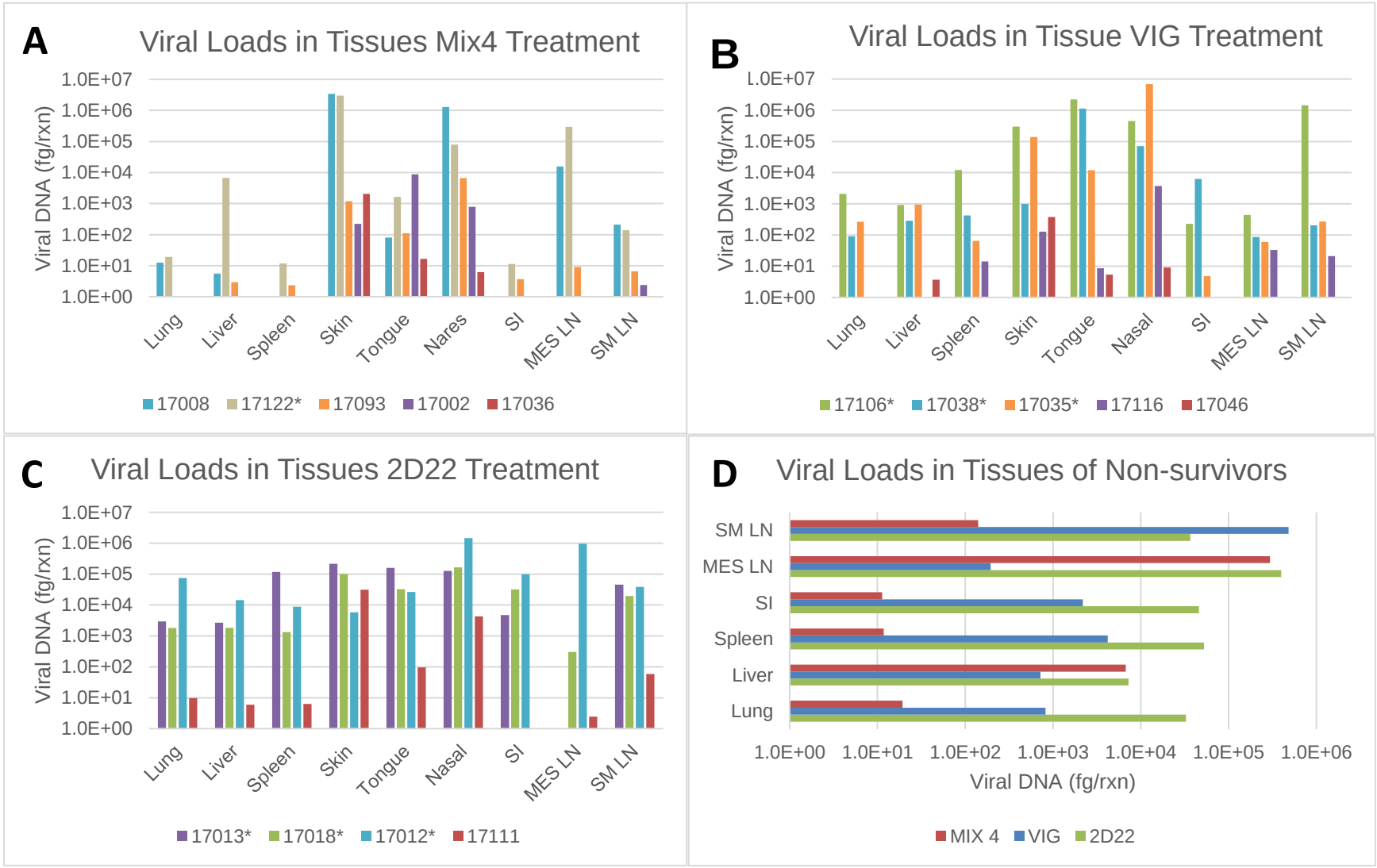


Figure 2.5. Mix4 Treated Animals had Lowest Number of Tissues Positive for Viral DNA Compared to Other Treatment Groups. Tissues were collected at euthanasia to compare viral loads in Mix4 (A), VIG (B) and irrelevant mAb 2D22 (C) treatment

groups. Samples were processed and underwent extraction to isolate viral DNA. Viral loads were determined using real-time PCR targeting the OPXV E9L gene. Animal IDs with an asterisk indicate the animal reached a pain score of ≥10 and was euthanized prior to study end (21 dpi). In order to be considered positive, samples must be positive in duplicate reactions and contain ≥2fg/rxn. As each group had at least one animal that did not survive until study end (21 dpi), viral loads in internal organs were averaged and compared (D). In this analysis, the Mix4 group had one animal (17122), the VIG group had three animals (17106, 17038 and 17035) and the 2D22 group had three animals (17013, 17018, and 17012).

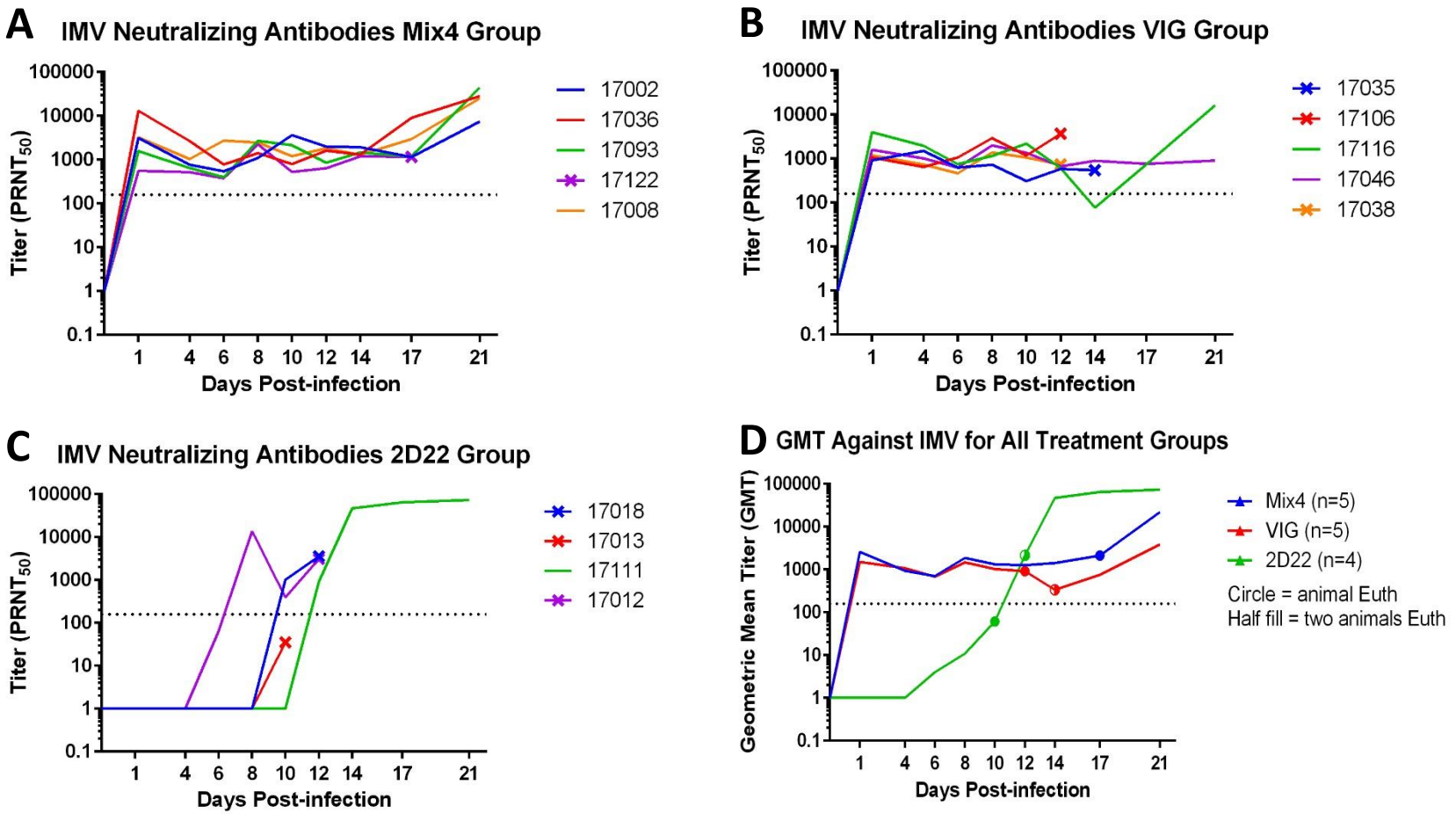


Figure 2.6. IMV Neutralizing Antibodies Detected Earliest in Mix4 and VIG Treatment Groups. The levels of IMV neutralizing antibodies was determined using an IMV PRNT assay starting at a 1:160 dilution and identified 50% virus neutralization. IMV neutralizing antibodies were detected as early as 1 dpi for Mix4 (A) and VIG (B) treatment groups. 2D22 (C) shows the host antibody

response to IMV. The geometric mean titer (GMT) was calculated for all treatment groups (D) and animals that were euthanized prior to 21 dpi are show on the graph. The dotted lines indicate the minimum dilution tested and values below that line were extrapolated. Samples from animals that died on non-sample days were included in the dataset for the next sample day.

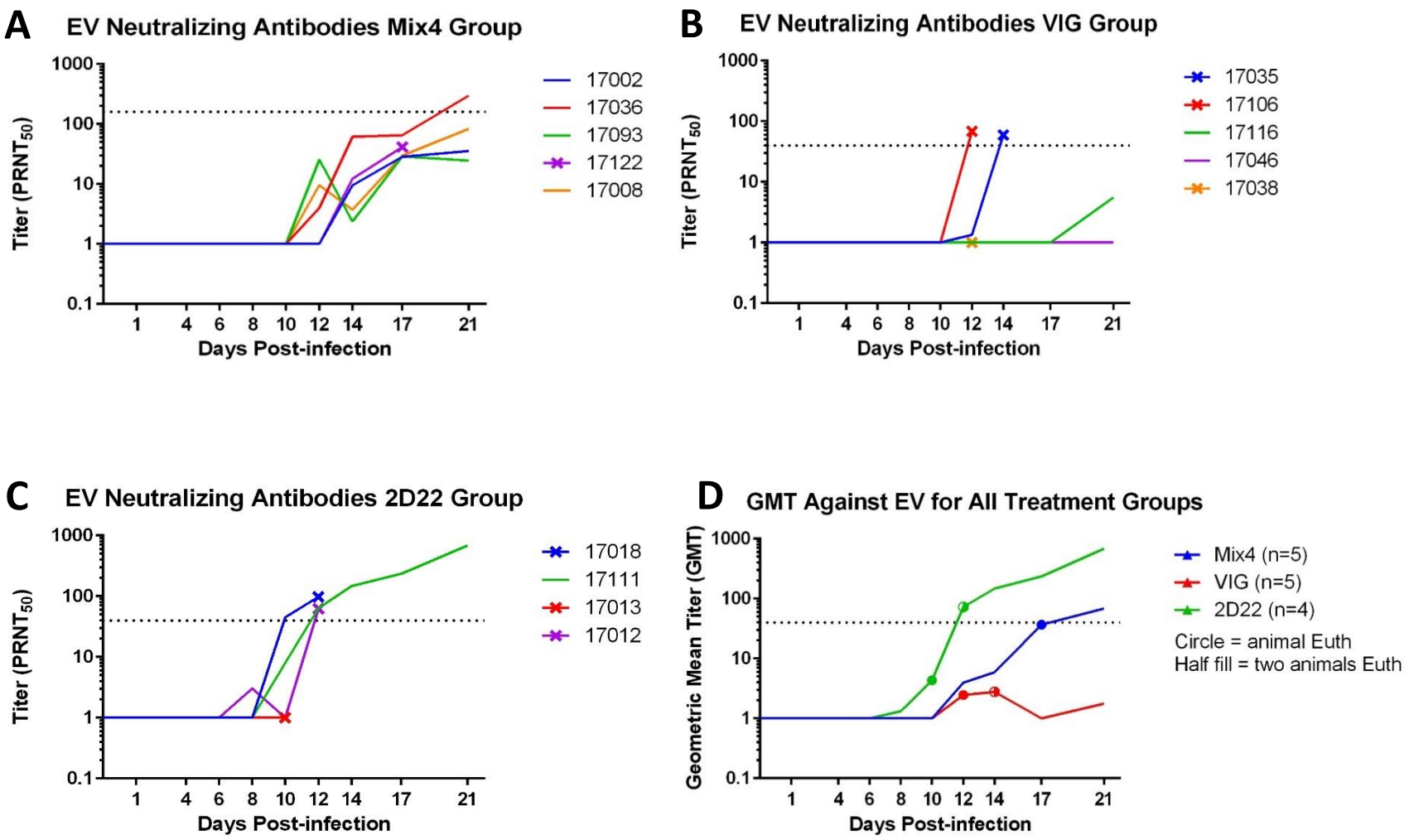


Figure 2.7. Minimal Levels of EV Neutralizing Antibodies Detected in Mix4 and VIG Treatment Groups. The levels of EV neutralizing antibodies was determined using an EV with complement PRNT assay starting at a 1:40 dilution and identified 50% virus neutralization. Low levels of EV neutralizing antibodies were detected 12 dpi for Mix4 (A) and VIG (B) treatment groups. 2D22 (C)

shows the host antibody response to EV beginning ~ 10 dpi and the strongest response was seen by the survivor in this group. The geometric mean titer (GMT) was calculated for all treatment groups (D) and animals that were euthanized prior to 21 dpi are show on the graph. The dotted lines indicate the minimum dilution tested and values below that line were extrapolated. Samples from animals that died on non-sample days were included in the dataset for the next sample day.

CHAPTER 3

DEVELOPMENT OF AN UNIVERSAL POX MONOCLONAL ANTIBODY MIX[3]

[3]Ashley V. Kondas, Iuliia Gilchuk, James E. Crowe Jr., Victoria A. Olson, Christina L. Hutson. To be submitted to the Journal of Infectious Diseases.

Abstract

Variola virus (VARV), the causative agent of smallpox, was declared eradicated by the World Health Organization in 1980 yet bioterrorism concerns persist today due to possible re-emergence and medical countermeasures are limited. While one antiviral is approved for smallpox treatment, orthopoxviruses are capable of becoming resistant to treatment, suggesting a multi-therapeutic regimen would be most effective. Monoclonal antibodies (mAbs) or mAb cocktails could fill the need for a second therapeutic by targeting both main forms of the virus: the intracellular mature virions and extracellular enveloped virions (EV). Here we present evidence suggesting individual mAbs and mAbs cocktails are capable of neutralizing VARV *in vitro.* Our data highlights the importance of testing therapeutics against the intended agent VARV and not relying solely on surrogate *Orthopoxvirus* (OPXV) models. While Mix4 and Mix6, looked promising against nonvariola orthopoxviruses, our data revealed that the mAbs in these mixes do not neutralize the extracellular enveloped virions of VARV. However, several mAbs, such as MPXV-13 and MPXV-56, neutralize both VARV and *Monkeypox virus* EV suggesting a different combination of mAbs, called the Universal Pox Mix, could be a potential candidate for not only a smallpox therapeutic, but an OPXV therapeutic.

Introduction

Smallpox, caused by *Variola virus* (VARV), has been called the most feared disease humankind was ever faced, causing an estimated 300 million deaths in the 20th century alone. Prior to its eradication in 1980, smallpox was found worldwide with a 1%-30% case fatality rate (CFR) depending on the strain and outbreak (1). VARV is transmitted through large respiratory droplets or contact with infected fomites (i.e. bodily fluids and bedding) and is highly transmissible with an estimated R_0 of 6.87 (2). During the course of infectivity, the virus is very stable and capable of surviving in scabs for years (3, 4). These traits of VARV have allowed the virus to be used as a biological weapon throughout history (1, 5, 6). The last naturally occurring case of smallpox occurred in Merca, Somalia in 1977. The disease was officially declared

eradicated in 1980 after an extensive vaccination campaign led by the World Health Organization (WHO) which is estimated to save $2 billion globally each year (7). While the disease is eradicated, the threat of an intentional release or the recreation of the virus through synthetic biology remains (8, 9) and would result in devastating consequences due to lack of widespread vaccination and resulting waning immunity within todays population (10). Based on historical data, a smallpox outbreak would be far more devastating than the 2014 West African Ebola outbreak and 2019 COVID-19 pandemic. These global incidents stress the importance of preparedness for highly transmissible infectious diseases. Due to these concerns, the virus is classified as a Tier 1 Select Agent by the Centers and Disease Control and Prevention (CDC) and it is part of the military's "dirty dozen" pathogens of concern (11). Although VARV is a grave pathogen of concern, medical countermeasures (MCMs) for prevention and treatment are limited.

In recent years, monoclonal antibodies (mAbs) have received Food and Drug Administration (FDA) licensure for treatment of multiple conditions and infectious diseases. Individual mAbs or mAbs cocktails could be an option for a smallpox MCM. The *Orthopoxvirus* (OPXV) lifecycle is comprised of two main forms of virions, intracellular mature virion (IMV) and external enveloped virion (EV), which express different surface proteins (12). Therefore, a mAb cocktail comprised of at least one EV neutralizing mAb and at least one IMV neutralizing mAb is hypothesized to have the greatest chance of success as a smallpox therapeutic. Several mAbs and mAb cocktails have been shown to neutralize nonvariola OPXVs which cause human disease, such as *Vaccinia virus* (VACV)*, Cowpox virus* (CPXV) *and Monkeypox virus* (MPXV), *in vitro* as well as *in vivo* (13-18). MPXV is also a pathogen of concern with increasing case reports in Africa (19-21) and importations of the virus to non-endemic areas including the United States (22-26). MPXV is the most similar OPXV to VARV in terms of disease progression and transmission and has up to a 11% case fatality rate (27, 28). It is commonly used as a surrogate model for VARV under the FDA's Animal Rule (21 CFR 314.600-650 for drugs; 21 CFR 601.90-

95 for biologics) since thus far there has been no representative smallpox animal model using VARV as the challenge virus. OPXVs have very similar genomes sharing ≥95% identify (29), however VARV is a solely human pathogen and contains genes not present in other OPXVs (30-32). Evaluation of these potential therapeutics directly against VARV is also a critical step in preparedness particularly for mAbs which bind to surface proteins. Since many of these mAb products have not been evaluated against VARV, their ability to be employed as a therapeutic agent should smallpox reemerge has not been confirmed.

The Vanderbilt Vaccine Center identified n=48 mAbs that were capable of neutralizing the IMV or EV form of representative nonvariola OPXVs (33). Based on the nonvariola neutralizing data, two different cocktails were created, Mix4 and Mix6, with the goal to be OPXV generic and provide protection against not only VARV but the three most common OPXVs that cause human disease: VACV, MPXV, CPXV. It was previously shown that these cocktails had high capacity to neutralize nonvariola OPXVs *in vitro* and *in vivo* in several VACV mouse models (33). Mix4 and Mix6 were also tested against VARV *in vitro* in a plaque reduction neutralization assay (PRNT) against the IMV form of the virus without complement and had promising results (33). However, to ensure adequate protection by the mAb cocktail against smallpox, it is important to ascertain neutralization capacity of both forms of infectious viral progeny. We hypothesized that Mix4 and Mix6 would also neutralize the EV form of the virus based on the promising results of the mixes (33) and due to genome similarity (29). Here we present evidence highlighting the importance on testing therapeutics against VARV and not relying exclusively on surrogate OPXV models. While Mix4 and Mix6 looked promising against nonvariola OPXVs, we identified that none of the EV neutralizing mAbs present in the mixes strongly neutralize VARV *in vitro*. Our complete data set including VARV suggests that a cocktail comprised of a different combination of mAbs, the Universal Pox Mix, than those currently within Mix4 and Mix6 would likely have greater efficacy as a smallpox therapeutic.

Materials and Methods

Cells, Viral Stocks and Titer Techniques

For all experiments, Vero E6 cells (ATCC CRL-1586) were used at passage 12-40. Cells were grown at 37°C with 6% CO_2 until confluence and then plated in six (EV viral stock growth) or twelve well plates (titration).

For IMV, VARV BSH74_sol (34) was selected as the representative virus for the IMV PRNT and working stocks of the virus were stored at -80°C. This strain was selected due to the high CFR and that it was used in non-human primate studies (35-37). For EV, VARV SLN68_258 (34) and MPXV-ROC-2003-358 (22) were selected as the representative virus for the VARV EV PRNT and MPXV EV PRNT respectively. VARV SLN68_258 was selected as it produces a large number of EV compared to other VARV strains (38). MPXV-ROC-2003-358 was selected due to its CFR. EV was produced for each PRNT by infecting cells at a multiplicity of infection (MOI) of 1 with an IMV viral stock that had been stored at -80°C. After incubating for 1 hour at 35.5°C with 6% CO_2, the inoculation was removed and 1.2 ml of RPMI-1640 (Thermo Fisher) containing 2% FBS (Atlanta biologicals; heat inactivated at 56°C for 1 hour) and 10 µg/ml of 7D11 (USAMRIID) was added to wells to neutralize IMV. The cells were incubated for ~48 hours at 35.5°C with 6% CO_2 before the supernatant was collected, centrifuged at 1000 x g for 20 minutes and transferred to a new tube to remove any remaining cellular debris (39). The EV stocks were stored at 4°C and used within two weeks.

Viruses were titered in the presence or absence of 10% baby rabbit complement following the appropriate PRNT procedure. For both IMV and EV titers, we selected tissue culture grade baby rabbit complement (Cedar Lane Labs); the complement was aliquoted into single use vials and those vials were used within 6 hours of thawing. Briefly, virus was diluted in Hank's Balanced Salt Solution (HBSS; Corning) and 1% bovine serum albumin (BSA; Sigma Aldrich) in the absence or presence of 10% baby rabbit complement and incubated for 1 hour at 35.5°C with 6% CO_2. After the incubation, 0.5 ml of the viral dilution was applied to cells and

incubated for 2 hours at 35.5°C with 6% CO_2. Next, 0.5 ml of Opti-MEM I (Gibco) supplemented with 2% Low IgG FBS (Thermo Fisher; heat inactivated at 56°C for 1 hour) was added to each well and plates were incubated for 96 hours for MPXV or 120 hours for VARV at 35.5°C with 6% CO_2. Viable cells were stained with crystal violet by adding a 1:1 ratio of stain to media, incubated for 15 minutes at room temperature and subsequent washing with water. After drying, plates were scanned by the CTL analyzer (ImmunoSpot) and plaques were counted using the Biospot software (ImmunoSpot). The obtained titer was used to calculate the amount of virus within the inoculum used in the PRNT.

mAbs

The mAbs were generated as previously described (33). Mix6 is comprised of VACV-301, VACV-249, MPXV-72, MPXV-26, VACV-22 and VACV-283 which target IMV proteins A27, D8, H3, L1, and EV proteins A33 and B5 respectively; Mix4 lacks VACV-249 and MPXV-72 (33).

PRNTs

Individual mAbs and cocktails were screened against VARV IMV and EV and MPXV EV depending on the testing results against nonvariola OPXVs (33). mAbs were screened at 100 µg/ml and 50 µg/ml in duplicate in two independent experiments. If neutralization was >50%, the effective concentration 50 (EC_{50}) and, when percent neutralization reached 90%, effective concentration 90 (EC_{90}) was identified by doing two-fold serial dilutions in HBSS + 1% BSA from 100 µg/ml to 0.01221 µg/ml for all mAbs excluding VACV-301 which was diluted to 0.00076 µg/ml. GraphPad Prism 6 software was used to calculate the EC values. EC experiments were done in duplicate in two independent experiments. If the average value between the two runs was negative, that concentration and concentrations below it were excluded from analysis. The viral stock was diluted in HBSS + 1% BSA and 20% baby rabbit complement (Cedar Lane) if applicable. Complement was treated the same as above. The viral inoculum was added to the monoclonal antibody or cocktail dilution and incubated for 1 hour at 35.5°C with 6% CO_2. After

the incubation, 0.5 ml of the samples were applied to cells and incubated for 2 hours at 35.5°C with 6% CO_2. Next, 0.5 ml of Opti-MEM I supplemented with 2% Low IgG FBS (heat inactivated at 56°C for 1 hour) was added to each well and plates were incubated for 96 hours for MPXV or 120 hours for VARV at 35.5°C with 6% CO_2. Viable cells were stained with crystal violet by adding a 1:1 ratio of stain to media, incubating for 15 minutes at room temperature and subsequent washing with water. After drying, plates were scanned by the CTL analyzer (ImmunoSpot) and plaques were counted using the Biospot software (ImmunoSpot). The virus only count was used to calculate the amount of virus within the inoculum neutralized by the different concentrations of monoclonal antibodies. For the PRNT to pass quality control and be considered reliable, the following criteria were developed:

1. The cell only control plate must have less than 5 imperfections in the monolayer.
2. The virus only control plate must provide an average for viral plaques within acceptable ranges (IMV PRNT 74.8 – 194.4; EV PRNT 59.8 - 179.4). Exceptions were made depending on plaque clarity.
3. An internal control, Vaccinia Immune Globulin (VIG), was also used within each PRNT. If appropriate neutralization of VARV was not seen with VIG, the experiment was repeated.
4. The non-specific poxvirus mAb (2D22) must show, on average, below 30% neutralization for each concentration.

Results

VARV IMV No Complement PRNT Results

Based on the previous results, n=48 mAbs were identified to neutralize a nonvariola OPXV (VACV, MPXV, CPXV) by at least 50% when present at 100 mg/ml (33). The protein target was identified for 44 of those mAbs to be one of six major poxvirus proteins: IMV proteins D8, L1, B5, A27 or H3 and EV proteins A33 and B5. These n=44 mAbs were screened against VARV in the IMV no complement PRNT. Due to quality control issues during production, 6/44

mAbs were excluded from analysis. Of the n=38 mAbs that were included in analysis, we identified n=8 mAbs that had sufficient neutralization to undergo attempts to determine the EC_{50} and EC_{90} (data not shown). The EC_{50} was determined for 3/8 mAbs and EC_{90} was determined for 2/8 mAbs (Table 3.1). All n=3 mAbs, MPXV-83, MPXV-26 and VACV-34, target the L1 protein. MPXV-26 had the most promising EC values for VARV with an EC_{50} value of 0.2875 and EC_{90} value of 2.752 µg/ml. MPXV-26 is a component of Mix4 and Mix6, confirming that both mixes have one mAb that effectively neutralizes the IMV form of VARV *in vitro*. The results of the cocktails (Mix4 and Mix6) in the VARV no complement PRNT have been published elsewhere (33).

VARV IMV with Complement PRNT Results

Since the IMV form of VARV was effectively neutralized even in the absence of complement (Table 3.1), the large panel (n=48) of mAbs was not screened in the IMV with complement PRNT. One mAb was screened with this assay (VACV-301), which targets the A27 protein, because it is a component of Mix4 and Mix6 and was previously shown to be complement dependent in neutralizing other OPXV (33). Preliminary data (one run done in duplicate) suggests VACV-301 neutralizing VARV with a preliminary EC_{50} of 0.006593 µg/ml and EC_{90} of 0.0212 µg/ml. The EC values will not be finalized until the mAbs are produced under standard cell platforms instead of hybridoma derived antibody preparations due to high lot to lot variability (40). Nonetheless, based on the promising VARV IMV no complement PRNT results we have confirmed that both mixes have at least one mAb (MPXV-26) that effectively neutralizes the IMV form of VARV *in vitro*.

VARV EV with Complement PRNT Results

The activity of the mAbs was not tested in the EV without complement PRNT as neutralization was not expected in this assay based on nonvariola OPXV results (33). EV mAbs are primarily complement dependent (33, 41). A smaller subset of n=30 mAbs was selected for the initial screens in the VARV EV complement PRNT due to limited volume and previous data

suggesting that only 16/48 mAbs neutralized EV from at least one nonvariola OPXV (CPXV, MPXV or VACV). Two mAbs had quality control issues during production and were excluded from analysis. In total, 7/28 mAbs passed quality control and demonstrated sufficient neutralization of VARV EV to undergo further analysis to determine the EC_{50} and EC_{90} (data not shown). The EC_{50} values were determined for all 7 mAbs as well as Mix4 and Mix6 (Table 3.2). Six out of seven individual mAbs target the B5 protein and MPXV-56 targets the A33 protein (33). MPXV-2 had the most promising EC_{50} value of 0.03528 µg/ml. None of the individual mAbs that neutralized VARV EV are present in Mix4 or Mix6 (Table 3.2). The two mAbs that are within these mAb cocktails, VACV-22 and VACV-283, target the A33 and B5 proteins respectively in nonvariola OPXVs (33). In the initial PRNT screens, neither VACV-22 or VACV-283 reached ≥50% neutralization even at 100 µg/ml. Additional PRNTs were conducted with VACV-22, VACV-283 and the combination of the two mAbs. The combination was the most successful with an EC_{50} of 0.6042 µg/ml which is higher than the other 7 identified mAbs (Table 3.2). Mix4 and Mix6 did generate EC_{50} values in the EV PRNT of 1.263 and 1.95 µg/ml respectively. These results suggested the Mix4 and Mix6 may not be the ideal combination of mAbs for treatment of smallpox and the EV neutralizing mAbs within the mixes should be replaced with another mAb, such as MPXV-2, based on the VARV EV PRNT data.

MPXV EV with Complement PRNT Results

The VARV PRNT dataset suggested that Mix4 and Mix6 may not be the ideal combination of mAbs for treatment of smallpox. While the evaluation of the mAbs in which we saw strong neutralization against VARV had previously been tested against MPXV in a different laboratory, we retested several of the mAbs in our EV with complement PRNT against MPXV. This retesting was due to discrepancies between the ELISA binding and PRNT results (33) and this data was crucial prior to determining if a more effective universal mAb cocktail could be created. Based on the VARV neutralization results, n=9 individual mAbs and one mAb combination were selected for further testing, including the most effective at neutralizing VARV

EV, as well as VACV-22, VACV-283 and VACV-22 + VACV283 combination to compare to VARV PRNT results (Table 3.3). EC_{50} values were only determined for two individual mAbs, MPXV-13 and MPXV-56, and one of the combination mixes comprising of MPXV-56 and MPXV-66 with values of 0.01731, 0.2331 and 0.2919 µg/ml respectively. Since an EC_{50} could not be determined for MPXV-66 against MPXV even at 100 µg/ml, this suggests that all neutralization activity seen in the MPXV-56 and MPXV-66 mix is generated by MPXV-56 and that MPXV-66 is likely not a good candidate for the Universal Pox Mix based on the MPXV neutralization. This was surprising as MPXV-66 performed well against VARV (Table 3.2). Similar to the VARV EV with complement PRNT, EC_{50} values for VACV-22 and VACV-283 were not able to be determined even at 100 µg/ml and the combination of the two mAbs also did not generate strong neutralization activity against the EV form of MPXV. However, both MPXV-13 and MPXV-56 neutralized both MPXV and VARV EV in the presence of complement suggesting possible alternatives in a new Mix (Table 3.4).

Discussion

The United States, in efforts coordinated with those of the international community, has supported a pipeline process for the development of smallpox MCMs. Two vaccines (VACV; ACAM 2000® [Sanofi Pasteur Biologics Co.] and MVA JYNNEOS® [Bavarian Nordic]), have received approval from the FDA. Despite the existence of an effective vaccine, the need for therapeutic MCMs to treat smallpox disease remains as historical data suggests that vaccination post exposure to smallpox was effective when administered within 3 days (42). This could be difficult to achieve in a large-scale outbreak. In 2019, a small molecule compound, TPOXX® (tecovirimat; SIGA), which targets the F13L protein involved in late morphogenesis stage of the viral life cycle, gained FDA licensure for treatment of smallpox. However, VARV is capable of becoming resistant to TPOXX® treatment (43) suggesting a multi-therapeutic approach would provide the best protection in an outbreak. Current considerations by the WHO Advisory Committee of Variola Virus Research (ACVVR) have recommended the need for at

least two antiviral compounds, with discrete mechanisms of action, to be licensed and available for smallpox preparedness. Studies in surrogate OPXV animal models have provided evidence administration of mAb cocktails provide better protection than a single mAb or VIG (13-18).

The World Health Assembly has mandated that all studies utilizing live VARV must occur within biosafety level 4 laboratories which undergo routine international biosafety inspections to minimize risk of release of the virus. There are only two WHO Collaborating Centers currently approved to work with live VARV, and all studies are presented to the WHO ACVVR annually to attain approval prior to initiation of investigation. Within the US, VARV is a Tier 1 Select Agent and requires annual inspections from the Department of Select Agents and Toxins. These challenges in combination with the fact that smallpox is a solely human pathogen, have dictated that almost all evaluation of MCMs for smallpox occur with surrogate OPXVs, such as MPXV and VACV, under The Animal Rule. While surrogate OPXV models are useful for understanding the potential of small compounds to halt the OPXV life cycle, the main limitation is efficacy testing against the authentic agent, an unique OPXV only pathogenic to humans. Here we present data highlighting the importance of testing MCMs against the authentic agent opposed to relying solely on surrogate OPXVs. Previous nonvariola *in vitro* data suggested that Mix4 and Mix6 provided protection against the main zoonotic OPXVs that cause disease in humans (33). These mAb cocktails also provide protection against VACV *in vivo* (33). Our neutralization data evaluating the individual mAbs ability to neutralize VARV without complement identified n=3 mAbs one of which, MPXV-26, is present in Mix4 and Mix6 (Table 3.1). Our evaluation of the individual mAbs capability to neutralize the EV form of VARV with complement identified n=7 mAbs (Table 3.2). Unfortunately, none of these mAbs are included in Mix4 or Mix6. While neutralization was seen with Mix4 and Mix6 in the VARV EV with complement PRNT, it required 10-fold higher concentration compared to other individual mAbs. The VARV *in vitro* results suggests that another combination of mAbs would be more effective as a smallpox therapeutic than Mix4 and Mix6.

Since the VARV *in vitro* data prompted the exploration of developing a new mAb cocktail, re-evaluation of several mAbs in the MPXV EV with complement PRNT were conducted. While a smallpox MCM is the ultimate goal, a MCM with cross protection for both MPXV and VARV is desirable. This decision was made as MPXV is also a pathogen of concern due to increased prevalence, recent importation to other countries including an importation with human-to-human transmission (24-26). Our PRNTs further supported that the two EV mAbs in Mix4 and Mix6 are not optimal and revealed that MPXV-13 and MPXV-56 neutralize both MPXV and VARV EV *in vitro* (Table 3.4). These would be ideal candidates for a new mAb cocktail as both of these mAbs neutralize all 4 OPXVs of concern (VARV, MPXV, VACV and CPXV) (33). No IMV PRNTs were repeated with MPXV and the higher EC_{50} values reported are likely due to differences in the assays from previously published results (33). While the VARV dataset supports the potential use of several α-L1 mAbs (MPXV-26, MPXV-83 or VACV-34), only MPXV-26 performed strongly against both VARV and MPXV (Table 3.4). Previous data against MPXV (33) and preliminary data for VARV suggests that VACV-301 could also be a potential candidate for the Pox Universal Mix.

The goal of smallpox MCMs is to save lives if smallpox were to ever re-emerge. However, MCMs that provide protection against multiple OPXVs are desirable. Due to the OPXV life cycle, a mAb cocktail has the best chance of success if it targets both main forms of the progeny virus, as a large portion of the virions produced are IMV and EV is important in dissemination (44, 45). While we hypothesized that Mix4 and Mix6 would contain a strong EV neutralizer based on the nonvariola OPXV data (33), that was not the case when we examined the individuals mAbs in the cocktails. However, our VARV and MPXV data presented here in combination with the previously published nonvariola data (33) suggested that several mAbs developed by the Vanderbilt Vaccine Center could be used not only as a potential smallpox therapeutic but as a Universal Pox MCM. It is important to note that the nonvariola neutralization data sets did not always agree with the VARV dataset highlighting the importance of testing

MCMs against the target agent. Several IMV mAbs such as MPXV-83 and VACV-34, neutralized VARV well but lacked the same efficacy against MPXV (33). Furthermore, drastic differences were seen in the EV PRNTs when comparing MPXV and VARV. Seven mAbs had promising EC_{50} values for VARV but only 2 of those 7 performed well against MPXV. Future work should be done to better characterize the surface proteins on VARV and testing against VARV should continue for potential smallpox MCMs to provide the best chance of protection if smallpox ever did re-emerge. Although the mechanism of action has not been confirmed for the mAbs, as these mAbs were not effective without complement (33) it suggests they neutralize the virus through the complement pathway compared to phagocytosis or antibody-dependent cellular cytotoxicity at least *in vitro*.

We propose that the most promising mAbs undergo production using cell culture platforms such as CHO cells as opposed to human hybridomas as this production technology has been used for other Investigational Drug Protocols (IND) (40). Postproduction, the following mAbs should be re-evaluated in the appropriate PRNT assays for all 4 OPXVs (VARV, MPXV, CPXV, VACV): IMV targeting mAbs MPXV-26, and VACV-301 and EV targeting mAbs, MPXV-56 and MPXV-13 (Table 3.4). Based on these results, potential Universal Pox cocktails should be tested *in vitro* comprising of n=2 or n=3 mAbs total due to the cost of manufacturing products for use under an IND protocol. Additionally, the best potential Universal Pox cocktails should be tested utilizing relevant OPXV animal models prior to making the final mAb selection for the Universal Pox cocktail. The Universal Pox cocktail as a MCM for not only smallpox but other human pathogenic OPXVs as well is an exciting step forward in our preparedness for OPXV outbreaks.

Acknowledgements

The authors would like to thank Todd Smith, Laurie Seigler, and Matthew R. Mauldin for their technical support in the PRNT assays, Christine M. Hughes for GraphPad Prism6

assistance and the Unites States Army Medical Research Institute of Infectious Diseases for providing the 7D11 antibody.

References

1. Fenner F, Henderson, D.A., Arita, I., Jezek, Z., Ladnyi, I.D. 1988. Smallpox and its eradication. World Health Organization.

2. Eichner M, Dietz K. 2003. Transmission potential of smallpox: estimates based on detailed data from an outbreak. Am J Epidemiol 158:110-7.

3. Wolff HL, Croon JJ. 1968. The survival of smallpox virus (variola minor) in natural circumstances. Bull World Health Organ 38:492-3.

4. Maccallum FO, McDonald JR. 1957. Survival of variola virus in raw cotton. Bull World Health Organ 16:247-54.

5. Robertson RG. 2001. Rotting Face: Smallpox and the American Indian. Caxton Press.

6. Hopkins DR, Lythcott GI. 1985. Princes and Peasants: Smallpox in History. University of Chicago Press.

7. Ehreth J. 2003. The global value of vaccination. Vaccine 21:596-600.

8. Reardon S. 2014. NIH finds forgotten smallpox store, *on* Nature. https://www.nature.com/news/nih-finds-forgotten-smallpox-store-1.15526. Accessed

9. Noyce RS, Lederman S, Evans DH. 2018. Construction of an infectious horsepox virus vaccine from chemically synthesized DNA fragments. PLoS One 13:e0188453.

10. Henderson DA, Inglesby TV, Bartlett JG, Ascher MS, Eitzen E, Jahrling PB, Hauer J, Layton M, McDade J, Osterholm MT, O'Toole T, Parker G, Perl T, Russell PK, Tonat K. 1999. Smallpox as a biological weapon: medical and public health management. Working Group on Civilian Biodefense. JAMA 281:2127-37.

11. Cieslak TJ, Kortepeter MG, Wojtyk RJ, Jansen HJ, Reyes RA, Smith JO, Panel NBMA. 2018. Beyond the Dirty Dozen: A Proposed Methodology for Assessing Future Bioweapon Threats. Mil Med 183:e59-e65.

12. Smith GL, Vanderplasschen A, Law M. 2002. The formation and function of extracellular enveloped vaccinia virus. J Gen Virol 83:2915-31.

13. Chen Z, Earl P, Americo J, Damon I, Smith SK, Yu F, Sebrell A, Emerson S, Cohen G, Eisenberg RJ, Gorshkova I, Schuck P, Satterfield W, Moss B, Purcell R. 2007. Characterization of chimpanzee/human monoclonal antibodies to vaccinia virus A33 glycoprotein and its variola virus homolog in vitro and in a vaccinia virus mouse protection model. J Virol 81:8989-95.

14. McCausland MM, Benhnia MR, Crickard L, Laudenslager J, Granger SW, Tahara T, Kubo R, Koriazova L, Kato S, Crotty S. 2010. Combination therapy of vaccinia virus infection with human anti-H3 and anti-B5 monoclonal antibodies in a small animal model. Antivir Ther 15:661-75.

15. Lustig S, Fogg C, Whitbeck JC, Eisenberg RJ, Cohen GH, Moss B. 2005. Combinations of polyclonal or monoclonal antibodies to proteins of the outer membranes of the two infectious forms of vaccinia virus protect mice against a lethal respiratory challenge. J Virol 79:13454-62.

16. Moss B. 2011. Smallpox vaccines: targets of protective immunity. Immunol Rev 239:8-26.

17. Mucker EM, Wollen-Roberts SE, Kimmel A, Shamblin J, Sampey D, Hooper JW. 2018. Intranasal monkeypox marmoset model: Prophylactic antibody treatment provides benefit against severe monkeypox virus disease. PLoS Negl Trop Dis 12:e0006581.

18. Crickard L, Babas T, Seth S, Silvera P, Koriazova L, Crotty S. 2012. Protection of rabbits and immunodeficient mice against lethal poxvirus infections by human monoclonal antibodies. PLoS One 7:e48706.

19. Sklenovska N, Van Ranst M. 2018. Emergence of Monkeypox as the Most Important Orthopoxvirus Infection in Humans. Front Public Health 6:241.

20. Mwamba DK KB, Shongo RL, Pukuta E, Kayembe NJM. 2014. Profil épidemiologique du monkeypox en RDC, 2010-2014. Ann African Med doi:Available online at: https://anafrimed.net/profil-epidemiologique-du-monkeypox-en-rdc-2010-2014-monkeypox-in-drc-epidemiological-profile-2010-2014/:1855-60.

21. Durski KN, McCollum AM, Nakazawa Y, Petersen BW, Reynolds MG, Briand S, Djingarey MH, Olson V, Damon IK, Khalakdina A. 2018. Emergence of Monkeypox - West and Central Africa, 1970-2017. MMWR Morb Mortal Wkly Rep 67:306-310.

22. Likos AM, Sammons SA, Olson VA, Frace AM, Li Y, Olsen-Rasmussen M, Davidson W, Galloway R, Khristova ML, Reynolds MG, Zhao H, Carroll DS, Curns A, Formenty P, Esposito JJ, Regnery RL, Damon IK. 2005. A tale of two clades: monkeypox viruses. J Gen Virol 86:2661-72.

23. Reed KD, Melski JW, Graham MB, Regnery RL, Sotir MJ, Wegner MV, Kazmierczak JJ, Stratman EJ, Li Y, Fairley JA, Swain GR, Olson VA, Sargent EK, Kehl SC, Frace MA, Kline R, Foldy SL, Davis JP, Damon IK. 2004. The detection of monkeypox in humans in the Western Hemisphere. N Engl J Med 350:342-50.

24. Anonymous. December 5 2018. Monkeypox case in England. https://www.gov.uk/government/news/monkeypox-case-in-england. Accessed September 8 2018.

25. Erez N, Achdout H, Milrot E, Schwartz Y, Wiener-Well Y, Paran N, Politi B, Tamir H, Israely T, Weiss S, Beth-Din A, Shifman O, Israeli O, Yitzhaki S, Shapira SC, Melamed S, Schwartz E. 2019. Diagnosis of Imported Monkeypox, Israel, 2018. Emerg Infect Dis 25:980-983.

26. Anonymous. 2019. Monkeypox - Singapore, *on* World Health Organization. https://www.who.int/csr/don/16-may-2019-monkeypox-singapore/en/. Accessed

27. Nolen LD, Osadebe L, Katomba J, Likofata J, Mukadi D, Monroe B, Doty J, Hughes CM, Kabamba J, Malekani J, Bomponda PL, Lokota JI, Balilo MP, Likafi T, Lushima RS, Ilunga BK,

Nkawa F, Pukuta E, Karhemere S, Tamfum JJ, Nguete B, Wemakoy EO, McCollum AM, Reynolds MG. 2016. Extended Human-to-Human Transmission during a Monkeypox Outbreak in the Democratic Republic of the Congo. Emerg Infect Dis 22:1014-21.

28. Jezek Z, Szczeniowski M, Paluku KM, Mutombo M. 1987. Human monkeypox: clinical features of 282 patients. J Infect Dis 156:293-8.

29. Mauldin MR, Antwerpen M, Emerson GL, Li Y, Zoeller G, Carroll DS, Meyer H. 2017. Cowpox virus: What's in a Name? Viruses 9.

30. Yadav VN, Pyaram K, Mullick J, Sahu A. 2008. Identification of hot spots in the variola virus complement inhibitor (SPICE) for human complement regulation. J Virol 82:3283-94.

31. Rosengard AM, Liu Y, Nie Z, Jimenez R. 2002. Variola virus immune evasion design: expression of a highly efficient inhibitor of human complement. Proc Natl Acad Sci U S A 99:8808-13.

32. Van Vliet K, Mohamed MR, Zhang L, Villa NY, Werden SJ, Liu J, McFadden G. 2009. Poxvirus proteomics and virus-host protein interactions. Microbiol Mol Biol Rev 73:730-49.

33. Gilchuk I, Gilchuk P, Sapparapu G, Lampley R, Singh V, Kose N, Blum DL, Hughes LJ, Satheshkumar PS, Townsend MB, Kondas AV, Reed Z, Weiner Z, Olson VA, Hammarlund E, Raue HP, Slifka MK, Slaughter JC, Graham BS, Edwards KM, Eisenberg RJ, Cohen GH, Joyce S, Crowe JE, Jr. 2016. Cross-Neutralizing and Protective Human Antibody Specificities to Poxvirus Infections. Cell 167:684-694 e9.

34. Esposito JJ, Sammons SA, Frace AM, Osborne JD, Olsen-Rasmussen M, Zhang M, Govil D, Damon IK, Kline R, Laker M, Li Y, Smith GL, Meyer H, Leduc JW, Wohlhueter RM. 2006. Genome sequence diversity and clues to the evolution of variola (smallpox) virus. Science 313:807-12.

35. Jahrling PB, Hensley LE, Martinez MJ, Leduc JW, Rubins KH, Relman DA, Huggins JW. 2004. Exploring the potential of variola virus infection of cynomolgus macaques as a model for human smallpox. Proc Natl Acad Sci U S A 101:15196-200.

36. Wahl-Jensen V, Cann JA, Rubins KH, Huggins JW, Fisher RW, Johnson AJ, de Kok-Mercado F, Larsen T, Raymond JL, Hensley LE, Jahrling PB. 2011. Progression of pathogenic events in cynomolgus macaques infected with variola virus. PLoS One 6:e24832.

37. Huggins J, Goff A, Hensley L, Mucker E, Shamblin J, Wlazlowski C, Johnson W, Chapman J, Larsen T, Twenhafel N, Karem K, Damon IK, Byrd CM, Bolken TC, Jordan R, Hruby D. 2009. Nonhuman primates are protected from smallpox virus or monkeypox virus challenges by the antiviral drug ST-246. Antimicrob Agents Chemother 53:2620-5.

38. Olson VA, Karem KL, Smith SK, Hughes CM, Damon IK. 2009. Smallpox virus plaque phenotypes: genetic, geographical and case fatality relationships. J Gen Virol 90:792-8.

39. Benhnia MR, McCausland MM, Moyron J, Laudenslager J, Granger S, Rickert S, Koriazova L, Kubo R, Kato S, Crotty S. 2009. Vaccinia virus extracellular enveloped virion neutralization in vitro and protection in vivo depend on complement. J Virol 83:1201-15.

40. Li F, Vijayasankaran N, Shen AY, Kiss R, Amanullah A. 2010. Cell culture processes for monoclonal antibody production. MAbs 2:466-79.

41. Benhnia MR, Maybeno M, Blum D, Aguilar-Sino R, Matho M, Meng X, Head S, Felgner PL, Zajonc DM, Koriazova L, Kato S, Burton DR, Xiang Y, Crowe JE, Jr., Peters B, Crotty S. 2013. Unusual features of vaccinia virus extracellular virion form neutralization resistance revealed in human antibody responses to the smallpox vaccine. J Virol 87:1569-85.

42. Massoudi MS, Barker L, Schwartz B. 2003. Effectiveness of postexposure vaccination for the prevention of smallpox: results of a delphi analysis. J Infect Dis 188:973-6.

43. Lederman ER, Davidson W, Groff HL, Smith SK, Warkentien T, Li Y, Wilkins KA, Karem KL, Akondy RS, Ahmed R, Frace M, Shieh WJ, Zaki S, Hruby DE, Painter WP, Bergman KL, Cohen JI, Damon IK. 2012. Progressive vaccinia: case description and laboratory-guided therapy with vaccinia immune globulin, ST-246, and CMX001. J Infect Dis 206:1372-85.

44. Chan WM, Ward BM. 2012. The A33-dependent incorporation of B5 into extracellular enveloped vaccinia virions is mediated through an interaction between their lumenal domains. J Virol 86:8210-20.

45. Payne LG. 1980. Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J Gen Virol 50:89-100.

Table 3.1. The Effective Concentrations of 3 mAbs in the VARV IMV no complement PRNT

µg/ml	**MPXV-26**	**VACV-34**	**MPXV-83**
EC_{50}	0.2875	0.8352	0.6938
EC_{90}	2.752	9.701	ND

Samples will run in duplicate per PRNT and in two independent experiments to determine effective concentration values. The effective concentration 50 and effective concentration 90 was identified whenever possible using GraphPad Prism 6. The protein target of all three mAbs is L1.

Table 3.2. The EC_{50} values of 7 mAbs and 3 Mixes in the VARV EV with complement PRNT

mAb	µg/ml
VACV-1	0.1677
VACV-151	0.1441
MPXV-2	0.03528
MPXV-13	0.1029
MPXV-25	0.2383
MPXV-56	0.2114
MPXV-66	0.1086
Mix4	1.263
Mix6	1.95
VACV-22 + VACV-283	0.6042

Samples were run in duplicate per PRNT and in two independent experiments to determine effective concentration values. The effective concentration 50 were identified whenever possible using GraphPad Prism 6. The concentrations tested did not allow for the effective concentration 90 to be determined. The protein target of the mAbs is B5 excluding MPXV-56 which targets A33. In addition to VACV-22 and VACV-283, Mix4 also has MPXV-26 and VACV-301 targeting L1 and A27 protein respectively. To generate Mix6, MPXV-72 and VACV-249 were also included targeting H3 and D8 proteins respectively.

Table 3.3. The Effective Concentrations of mAbs in the MPXV EV with complement PRNT

Target	mAb	MPXV EC_{50} (µg/ml)
B5	MPXV-2	ND
B5	MPXV-13	0.01731
B5	MPXV-25	ND
A33	MPXV-56	0.2331
B5	MPXV-66	ND
B5	VACV-1	ND
A33	VACV-22	ND
B5	VACV-151	ND
B5	VACV-283	ND
A33, B5	VACV-22 + VACV-283	ND
A33, B5	MPXV-56 + MPXV-66	0.2919

mAbs chosen for neutralization against MPXV were based on promising results seen against VARV or because they are part of the Mix4 or Mix6 cocktails. Samples were run in duplicate per PRNT and in two independent experiments to determine effective concentration values. The effective concentration 50 were identified whenever possible using GraphPad Prism 6. The concentrations tested did not allow for the effective concentration 90 to be determined. Values were not always to be determined as noted by “ND” indicating not determined even when tested at 100 µg/ml.

Table 3.4. Individual mAbs Options for the Universal Pox Mix that Neutralized both VARV and MPXV

PRNT	Target	mAb	MPXV EC_{50} (µg/ml)	VARV EC_{50} (µg/ml)
IMV NC	L1	MPXV-26	3^{+}	0.2875
IMV C	A27	VACV-301	0.8^{+}	0.006593
EV C	B5	MPXV-13	0.01731	0.1029
EV C	A33	MPXV-56	0.2331	0.2114

A summary of the PRNTs determining the neutralization of MPXV and VARV highlighting mAbs that could be utilized in a Universal Pox Mix. $^{+}$Some of the data was taken from a previous publication (33). “NC” indicates no complement was used compared to “C” should indicates complement was used in the PRNT.

CHAPTER 4

CAN YOU TEACH A NEW MOUSE OLD TRICKS? HUMANIZED MICE AS A MODEL FOR VARIOLA VIRUS[3]

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Abstract

Smallpox, caused by the solely human pathogen *Variola virus* (VARV), was declared eradicated in 1980. While known VARV stocks are secure, smallpox remains a bioterrorist threat agent. Recent Food and Drug Administration (FDA) approval of the first smallpox anti-viral (TPOXX®; tecovirimat) therapeutic was a successful step forward in smallpox preparedness; however, orthopoxviruses can become resistant to treatment, suggesting a multi-therapeutic approach is necessary. Animal models are required for testing medical countermeasures (MCMs) and ideally MCMs are tested directly against the pathogen of interest. Since VARV only infects humans, a representative animal model for testing therapeutics directly against VARV remains a challenge. Here we show that three different humanized mice strains are highly susceptible to VARV infection, establishing the first small animal model using VARV. In comparison, the non-humanized, immunosuppressed background mouse was not susceptible to systemic VARV infection. Following an intranasal VARV challenge that mimics the natural route for human smallpox transmission, the virus spread systemically within the humanized mouse before mortality (~ 13 days post infection), similar to the time from exposure to symptom onset for “ordinary” human smallpox. Our identification of a permissive/representative VARV animal model can facilitate testing of MCMs in a manner consistent with their intended use, aid in identifying why VARV is a solely human pathogen and provide valuable samples for understanding the human immune response to VARV infection.

Introduction

Variola virus (VARV), the causative agent of smallpox, is a solely human pathogen. The World Health Organization (WHO) certified the global eradication of smallpox in 1980 with the last reported naturally occurring case in Somalia in 1977. While the known viral stocks are secure in two laboratories (Centers for Disease Control and Prevention [CDC] and VECTOR Institute), the threat of unknown sources outside the repositories remains and could be used with malicious intent. VARV is a select agent and it is subject to the requirements of the select

agent regulations 42 CFR 73. Forgotten lyophilized VARV vials from the 1950's were discovered in 2014 at a non-WHO approved variola virus laboratory (1); these vials contained high-titer viable virus (unpublished data). Additionally, a recently published article provided detailed instructions on how to reconstruct *Horsepox virus*, a virus closely related to VARV (2). A recent tabletop exercise evaluating what would happen if smallpox was used as a bioweapon indicated potentially devastating consequences (3). While two vaccines (*Vaccinia virus*; ACAM2000® and JYNNEOS®) and one anti-viral (TPOXX® [ST-246®]) have received licensure by the Food and Drug Administration (FDA) as medical countermeasures (MCMs) for use against smallpox infection, these MCMs were developed after the eradication of human smallpox and have never been tested against the authentic agent in humans. Because VARV causes smallpox only in humans, and thus far no satisfactory VARV animal model has been developed, the FDA has approved The Animal Model Rule as a pathway for regulatory approval. Under this rule, potential anti-virals must be tested in at least two surrogate *Orthopoxvirus* animal models, such as *Monkeypox virus* in non-human primates (NHP), *Rabbitpox virus* in rabbits and/or *Ectromelia virus* in mice (21 CFR 601.90). While this route to FDA licensure is critical for preparedness, the development of a small animal model susceptible to VARV infection would be ideal for testing MCMs directly against the authentic agent.

Humanized mice have become a valuable tool for studying infectious diseases (4-6). An animal model with a human-like immune system, would be advantageous to the study of VARV, potentially identifying why VARV is a solely human pathogen and providing valuable samples for understanding aspects of the human immune response to VARV infection. Given this, we sought to determine if humanized mice can support a productive VARV infection. Three different strains of humanized mice (hu-PBMC, hu-CD34^{+}, and hu-BLT) were evaluated and compared to the susceptibility of the immunodeficient NSG background mouse. This sequence of studies allowed us to better understand if susceptibility to VARV infection is attributable to a lack of murine immune response, the humanization process or some combination of both. We found

that all three strains of humanized mice were susceptible to systemic VARV infection following intranasal (IN) inoculation, while NSG background mice were not. Herein, we present novel humanized mouse models of smallpox that utilize the exact etiologic agent of human disease and provide further evidence that VARV dissemination requires some component of the human immune system.

Materials and Methods

Mice

Female Hu-PBMC, hu-CD34^{+} and hu-BLT (NSG background) and NSG mice, were purchased from The Jackson Laboratory. Hu-CD34^{+} and hu-BLT were received at 15 weeks old (12 weeks post engraftment) while hu-PBMC mice were 8 weeks old (3 days post engraftment) during part one of the study. For part two, NSG mice were received at 7 weeks old and hu-PBMC mice were 7 weeks old (~1 week post engraftment). Animal care and use procedures were approved by the CDC IACUC under protocol number 2671GALMOUC. Precautions were taken including the use of sterile drinking water and sterilization by autoclaving of bedding, enrichment and food used during the study. Mice were grouped housed (n=3-5) upon being received and acclimated for at least 3 days prior to beginning the studies. After acclimation but prior to inoculation, serum was collected via the submandibular vein (CDC IACUC policy 026).

Challenge virus

Work with live VARV is conducted in the Biosafety Level 4 laboratory (BSL-4), approved by the World Health Organization Advisory Committee on Variola Virus Research (WHO ACVVR), is done in accordance with all applicable select agent regulations (42 CFR part 73) and all inactivation procedures used had been reviewed and approved by the CDC Laboratory Safety Review Board. A semi-purified preparation of VARV_JAP51_hrpr (primary clade I) was selected as the challenge virus, as it has been used in historic NHP studies (7-9) as well as other mouse studies (10). This virus was isolated from a United States solider in Japan in 1951 and was part of the U.S. Army repository before being transferred to the U.S. CDC VARV

collection (7). The isolate underwent 5 passages on chorioallantoic membrane before being passaged twice in African green monkey kidney cells (BSC-40) and undergoing purification as previously described (11-16). Inoculum was diluted in phosphate-buffered saline (PBS). For ɣ-irradiation inactivation, the virus was treated with 5 x 10^6 rads. All challenge doses were confirmed via standard cell culture techniques (back-titration) immediately following inoculation.

Animal inoculation

All mice were inoculated via the IN route to mimic the natural route of human smallpox infection. Inoculum was diluted in phosphate-buffered saline (PBS) and 0.05% bovine serum albumin. For part one of the study, Hu-PBMC, hu-$CD34^+$ and hu-BLT mice were inoculated with 7 x 10^3 or 7 x 10^5 plaque forming unit (pfu) (n=4 per group). To serve as controls, two hu-PBMC mice were inoculated with diluent and two of each mouse strain were inoculated with ɣ-irradiated VARV_JAP51_hrpr equivalent to 7 x 10^5 pfu. For part two of the study, NSG mice were inoculated with 4 x 10^6 or 5 x 10^4 pfu (n=5 per group). For negative controls, NSG mice were uninfected or mock-infected with diluent (n=3 per group). Three hu-PBMC mice were also included in the part two of the study and challenged with 4 x 10^6 pfu to serve as positive controls.

Animal sampling, observations and euthanasia

Clinical signs were recorded daily, and oral swabs, physicals and weights were taken three times weekly under 3-5% inhalation anesthesia. Euthanasia/pain scores were determined by weight loss, behavior and appearance, with clinical scoring performed twice daily once animals reached a score of ≥5. We only considered a score of ≥5 as clinical signs attributed to viral infection because a score of 4 was often seen in non-infected controls due to weight loss alone; weight loss was not utilized as a pain score for control mice unless observed for two consecutive weight recording days. At 21 days post infection (dpi) or a pain score of 10, euthanasia was performed via exsanguination and cervical dislocation under 5% inhalation anesthesia. Oral swab (polyester frozen dry), tissues, whole blood and serum (Starstedt) were

collected upon euthanasia. The following tissues were collected: nose, lung, liver, spleen, ovaries, heart, kidney and any other tissue with abnormal appearance. Between samples, necropsy tools were decontaminated in 5% micro-chem, scrubbed with a brush and rinsed with water. All samples were frozen at -80°C for future analysis.

Sample processing and DNA extraction

Swabs were processed as previously described (17). Tissues and whole blood were thawed on ice. Aliquots of 1 mm zirconia/silica beads (Biospec) and PBS for tissue homogenizing were made by mixing 0.5 ml and 0.7 ml, respectively. Each beads/PBS tube was weighed prior to pouring contents into the tissue tube; the tissue tube was weighed and change in readings was used as an approximate tissue weight. Samples were homogenized using the Mini-BeadBeater-16 (Biospec) by grinding twice for one minute, and icing samples for one minute between runs. Sample inactivation (per internally validated/approved inactivation method) and DNA extraction were performed as previously described except samples were heated at ≥56°C (17).The remaining sample was re-frozen at -80°C.

Pathology and immunohistochemistry

During necropsy, a portion of each collected tissue and the remaining mouse carcass were placed into 10% neutral-buffered formalin for 7 days for virus inactivation (per internally validated/approved inactivation method) and tissue fixation and were transferred to a BSL-2 laboratory. Tissues were processed for routine paraffin histology, and sections were cut at 4 microns and stained with hematoxylin-eosin (H&E). Immunohistochemical (IHC) detection of VARV was performed as previously described (18), using a rabbit polyclonal anti-VARV antibody (CDC). Formalin-fixed, ɣ-irradiated, paraffin-embedded monkey kidney cells infected with VARV were used as a positive control. Negative control utilized normal rabbit serum in place of the primary antibody. Tissues for electron microscopic examination were either formalin-fixed and placed in phosphate buffer and then buffered with 2.5% glutaraldehyde or

were processed by the on-slide embedding technique (19). Samples were then embedded in Epon/Araldite by standard methods (20).

Molecular assays

Real-time PCR was performed previously described (21). A standard dilution series of semi-purified VARV DNA was assayed on each PCR plate in order to quantify DNA levels within samples. Two fg viral DNA/rxn was the positive cut-off value. Samples that crossed the threshold (Ct value) before cycle 38 in duplicate underwent a modified version of with a 96 hour incubation (17). Samples were thawed on ice prior to sonication at 40% output twice at one-minute intervals, with 10 seconds of icing between sonication. Samples were titrated on BSC-40 cells with a 96-hour incubation prior to staining with crystal violet stain. Detection of anti-Orthopoxvirus IgG and IgM was performed as previously described (22).

Statistics

Comparisons of the tissue viral load (pfu/ml) between mouse strains and dose were made using the Wilcoxon rank-sum test, as the data are not normally distributed. Differences in mortality rates for three humanized mouse strains were compared using Fisher's exact test, due to small sample size. Kaplan-Meier survivorship curves were calculated and compared using the log-rank test. A p-value < 0.05 was considered statistically significant. Data analysis was performed using SAS version 9.4 (SAS Institute).

Results

VARV disease in humanized mice

For part one of the study, the first clinical signs observed were suspect pox lesions on the hocks of a subset of mice beginning at 7 dpi. Swabs of the lesions were collected. The hock lesions were sporadic, with only five infected animals affected, but did include animals of all three strains and no uninfected control animals (Table 4.1). For hu-CD34$^+$, and hu-BLT infected groups, onset of other clinical signs was variable but generally began between 7-17 dpi (hu-CD34$^+$ high dose), 17-19 dpi (hu-CD34$^+$ low dose), and 12-14 dpi (hu-BLT both doses). The

majority of hu-PBMC infected mice did not show clinical signs attributed to viral infection, with only three animals displaying clinical signs close to study end (17-21) dpi for both high (1/4) and low-dose groups (2/4). Clinical disease progressed rapidly for hu-CD34$^+$ and hu-BLT and animals were not always able to be euthanized before succumbing to disease. Hu-CD34$^+$ 8 (low dose) was believed to have an unsuccessful inoculation. While the animal had very low levels of viral DNA in the spleen, kidney and ovaries (data not shown), this animal displayed no clinical signs and survived to the study end. Unsuccessful inoculation was further supported by lack of pathologic lesions, viral immunostaining, and absence of detectable viable virus. Upon review of the clinical record, this mouse experienced a “bubble” during the inoculation supporting the hypothesis that the animal was not successfully inoculated (i.e., entire inoculum not delivered into nares). This animal was excluded in comparisons between infected mouse strains.

Dose-dependent mortality was seen in the hu-CD34$^+$ and hu-BLT mice beginning at day 13 (Figure 4.1A). High dose hu-CD34$^+$ mice were significantly more likely to succumb earlier than those in the low dose group (p=0.02). While differences in survivorship between dose groups for hu-BLT mice were not significant (p=0.2), there was a trend suggesting the high dose group succumbed earlier than those challenged with the low dose. All hu-BLT and hu-CD34$^+$ mice in the high dose group and all the hu-BLT mice low dose group succumbed to disease. The hu-PBMC mice did not have high morbidity, and only one from the high dose group was euthanized before study end (19 dpi). Hu-PBMC mice were significantly more likely to survive VARV infection compared to hu-BLT and hu-CD34$^+$, controlling for dose (high dose p=0.004, low dose p = 0.008; Figure 4.1B). There was no significant difference in survivorship between hu-BLT and hu-CD34$^+$ mice when controlling for dose (high dose p=0.20; low dose = 0.63). One ɣ-irradiated control hu-PBMC mouse was found deceased on day 13, which may have been related to anesthesia complications; this was the only control animal to die during the study. All necropsy samples from this animal were negative for VARV DNA.

Pathology and immunohistochemistry

Cutaneous lesions (vesicles or ulcers) were observed on the hocks of n=5 mice. At necropsy, the most common gross pathologic finding was multifocal and coalescing regions of hepatic necrosis involving up to 50% of liver in hu-CD34$^+$ (n=3) and hu-BLT (n=5) mice. Sporadic splenomegaly and gallbladder hemorrhage were also seen in these strains. Hu-PBMC mice had no remarkable gross lesions (Table 4.2). The following tissues were examined by histopathology, when available: brain, lung, liver, kidney, spleen, adrenal gland, lymph nodes, bone marrow, female reproductive tract, gastrointestinal tract, oronasal tissues, and skin from hock lesions when present. These findings are summarized in Table 4.2. Hock skin lesions were identified microscopically for 1 hu-PBMC and 1 hu-CD34$^+$ mouse, with histopathological findings including epidermal hyperplasia and hyperkeratosis with epithelial staining by VARV immunohistochemistry at the chronic ulcer margins. Periarticular tissues (synovium, tendon, periosteum) also had mild inflammation and VARV immunostaining (Figures 4.2, A-B).

Histopathologic findings in extracutaneous tissues were generally similar between low and high dose groups for each mouse strain (Figures 4.2 – 4.3 and Table 4.2). Hu-CD34$^+$ and hu-BLT mice had tissue necrosis with minimal inflammation in the liver, adrenal gland, lymphoid tissues, and reproductive tract. Abundant VARV antigen was detected within necrotic foci, but also sometimes within morphologically unaffected tissue, by immunohistochemistry. Livers showed confluent and lobular hepatocellular necrosis, with immunostaining localized prominently within necrotic and intact hepatocytes, as well as scattered Kupffer and endothelial cells. Rarely, eosinophilic globular cytoplasmic material reminiscent of viral inclusions was present within hepatocytes (Figure 4.3, inset). Adrenal glands had discrete foci of necrosis most commonly in the cortex, and occasionally in the medulla. Poxviral antigen localized to foci of necrosis, as well as other foci of intact cells. Examined spleens uniformly showed diffuse necrosis with severe lymphoid depletion and red pulp expansion by fibrin and hemorrhage. Poxviral antigen localized to macrophages and mesenchymal cells around central arteries in the

regions of periarteriolar lymphoid sheath depletion. Lymph nodes from various sites similarly showed lymphoid necrosis and depletion, with poxviral immunostaining in reticuloendothelial cells. Bone marrow showed extensive necrosis and hemorrhage with immunostaining in the hematopoietic compartment, and patchy staining of endosteum and periosteum. Lungs had a mild increase in interstitial cellularity, with scattered interstitial immunostaining. One hu-BLT mouse (hu-BLT6) also had multifocal bronchiolar epithelial necrosis and immunostaining. Nasal tissues had scattered small foci of submucosal, and rarely respiratory epithelial or serous glandular, immunostaining, without notable inflammation or necrosis. Teeth had multifocal to extensive immunostaining that was concentrated in the dental pulp, sometimes in association with necrosis, and the periodontal ligament. For available female reproductive tract tissues, the ovarian stroma consistently had scattered viral immunostaining, without overt morphological alterations; follicles also stained to a lesser extent. 2/7 hu-CD34^{+} uteri, and 3/7 hu-BLT uteri, had moderate to extensive immunostaining of smooth muscle and stromal (including perivascular) cells, variably accompanied by necrosis. Oviduct and vagina had scattered or patchy immunostaining in a pattern similar to that observed in the uterus, without overt necrosis. Gastrointestinal tissues also showed inconsistent and rare, scattered staining in the smooth muscle, serosa, and rarely submucosa. Kidneys showed very rare, scattered immunostaining within glomeruli and interstitial cells. For hu-BLT mice with remnant subcapsular grafts, grafted human tissues showed extensive immunostaining. Brain had no histopathologic findings and no immunostaining, and heart was not available, from any animal of these strains. Disseminated bacteremia was seen histologically in all virally challenged animals of these two strains, with bacterial emboli consistently found in brain, lung, liver, spleen, and kidney. Immunohistochemical testing of a subset of tissues from four animals (2 hu-CD34^{+} and 2 hu-BLT) detected involvement of multiple, mixed bacteria, including *Staphylococcus spp*., *Enterococcus spp.*, and *Streptococcus spp.* Gram-negative bacteria were not identified.

Hu-PBMC mice had overall similar findings, but with less liver, bone marrow, and tooth involvement, increased nasal and lung involvement, slightly more prominent inflammation overall, and absence of bacteremia (Figures 4.2 – 4.3 and Table 4.2). Lungs from hu-PBMC mice had more consistent and numerous foci of bronchiolar epithelial necrosis accompanied by perivascular and peribronchiolar edema and mild inflammation, and mild interstitial pneumonitis. Immunostaining localized to bronchiolar epithelial cells, peribronchiolar and perivascular stromal and inflammatory cells, and scattered interstitial cells. Nasal tissues had patchy edema, necrosis, and mild inflammation, with necrosis and atrophy of serous glands, with corresponding multifocal to widespread immunostaining of epithelium and submucosal stroma and glands. These changes were more prominent in the high dose hu-PBMC group. One animal in the low dose group (hu-PBMC 9) had a fibrinocellular and hemorrhagic exudate, which showed granular intra- and extracellular VARV immunostaining, in the middle nasal meatus. All uninfected, and n=2/8 infected hu-PBMC animals had systemic, atypical lymphoid proliferation suggestive of the development of graft vs host (GVH) disease.

Select tissues were examined by transmission electron microscopy (Figure 4.4). Ultrastructural evaluation revealed almost exclusively immature VARV particles in the hepatocytes of the native mouse liver tissue of infected hu-CD34^{+} (n=2), hu-BLT (n=2) and hu-PBMC (n=1) mice (Figure 4.4A); however, both mature and immature particles were located in the sinusoidal endothelial cells and free in the sinusoids of the livers (Fig 4.4B). In hu-PBMC murine ovary (n=1), uterus (n=1), and adventitial cells surrounding the bile duct (n=1), and in human fetal thymic allograft (n=1) from hu-BLT mouse (Fig 4.4C), both mature and immature particles were present.

Molecular findings

Of the n=222 oral swabs that were collected throughout the study, only n=4 were positive for viral DNA. Two oral swabs contained viable virus and were from the lower dose groups: hu-BLT 8 on 12 dpi (46.2 pfu/ml) and hu-PBMC 11 on 19 dpi (594 pfu/ml). All hock

lesion swabs were negative for viral DNA, but all five hock lesion tissues contained viral DNA and viable virus (Figure 4.5). Remarkably high loads of viable virus were present in multiple tissues, including heart, kidney, liver, lung, ovaries and spleen, and in some instances reached as high as 1.66 x 10^{11} pfu/gram of tissue (Figure 4.5). The hu-BLT mice had the highest viral loads (both dose groups) followed by slightly lower levels in hu-CD34^{+} mice. Tissue viral load comparisons between hu-BLT and hu-CD34^{+} were not significantly different when controlling for dose. Despite little mortality in the hu-PBMC mice, high viral loads were detected in most of the tissues tested. Minimal whole blood euthanasia samples were available for evaluation of viremia. Viral DNA was detected in 9/10 infected animals (3/4 high dose hu-PBMC, 4/4 low dose hu-PBMC, 2/2 high dose hu-BLT) ranging from 1.67x10^{2} to 2.05x10^{5} fg/µl with hu-BLT mice having the highest quantities. Post DNA evaluation, three samples had sufficient volume remaining for viral titration, all from the hu-PBMC low dose group, and 2/3 contained viable virus (5.4x10^{2} and 4.4x10^{3} pfu/ml). To look for evidence of antibodies post infection, serum (when available) was tested for the presence of human IgM and IgG by ELISAs. None of the tested serum samples had detectable levels of human IgM or IgG in ELISAs designed for human serum. Serum available for testing included all hu-PBMC mice excluding one ɣ-irradiated infected control animal , all hu-CD34^{+} control mice, two from the high and two from the low dose group, and for hu-BLT mice only serum from the ɣ-irradiated control group, one uninfected animal and four from the high dose group.

Non-humanized NSG mice are not susceptible to systemic VARV disease

Part two of the study determined whether the immunosuppressed NSG background mouse was susceptible to systemic VARV infection. Clinical signs and weight loss were minimal/absent in the NSG mice (both doses) and all NSG animals survived until study end (21 dpi). Gross findings were absent during necropsy. On pathologic evaluation, 1/5 in the low dose group, and 4/5 in the high dose group had very mild inflammatory changes associated with VARV immunostaining in the nasal submucosa (Figure 4.6). No significant histopathologic

changes, and no VARV immunostaining, were seen in extranasal tissues (lung, liver, spleen, kidney, lymphoid tissues, and female reproductive tract) (Figure 4.6; data not show). The oral and skin swabs were negative for viral DNA. Several necropsy tissues, mainly the lung, liver and nasal cavity, from several NSG mice were positive for viral DNA (Figure 4.7). However, the only tissues that contained viable virus were two nasal cavities, one from the low and one from the high dose group, which was the site of inoculation (Figure 4.7C). All NSG negative control mice were negative for viral DNA and had no significant pathologic findings and no VARV immunostaining.

We utilized hu-PBMC mice as positive controls during this part of the study; similar to part one of the study, hu-PBMC mice began displaying clinical signs late in the study (~day 19) and one mouse had to be euthanized on day 19. Molecular and pathology results were overall similar to part one of the study, with the virus spreading systemically and high loads of viable virus found throughout most tissues tested (Figure 4.7D).

Discussion

Due to the discontinuation of smallpox vaccination after disease eradication, the human population is extremely susceptible to smallpox. Its malicious release could have devastating consequences, making imperative the continued development of preventive and therapeutic countermeasures. Animal models are an invaluable tools for the development of MCMs; an ideal animal model would mimic natural human smallpox disease using the authentic agent (VARV), realistic infectious dose, aerosol droplet route of infection, 8-14 day incubation period, identifiable prodrome, detectable immune response, high mortality and systemic rash 1-4 days post prodrome (23, 24). Various models have successfully fulfilled parts, but not all, of this ideal. Historically, most adult animals have been insusceptible to VARV challenge, even when susceptible to other orthopoxviruses. While CAST/EiJ mice and prairie dogs (*Cynomys ludovicianus*) are susceptible to an IN *Monkeypox virus* challenge(25-27), neither developed a systemic infection following IN VARV challenge (10, 17, 28, 29). Similar findings were observed

in ICR and SCID mice following IN VARV challenge (30). NHPs are susceptible to VARV infection, with development of systemic rash illness and mortality; however, this model has several disadvantages including: use of infectious doses much higher ($1x10^8$ to $1x10^9$ pfu) than the suspected dose required for human infection, and requires intravenous inoculation, an unnatural route of infection which bypasses the initial local replication and viremia, eliminating the incubation and prodromal periods (7). In this model, only infectious doses at $1x10^9$ pfu resulted in high mortality which manifested as “hemorrhagic” smallpox with animals succumbing to the disease as early as 4 dpi (7, 8) limiting the window for testing post-exposure MCMs. Due to these difficulties, animal models using surrogate orthopoxviruses have been utilized to test MCMs. While each surrogate orthopoxvirus animal model has its strengths and weaknesses, none have the potential for investigating the role of the human immunologic response specifically, in the progression and resolution of smallpox infection

Here we present the first small animal models of human smallpox, utilizing humanized mice that are highly susceptible to VARV infection. We have shown that these novel humanized mouse models will be useful for studying VARV infection, and potentially, features of the human immune system’s response to infection. We have shown that these novel humanized mouse models will be useful for studying VARV infection, and potentially, features of the human immune system’s response to infection. While all three humanized mouse strains were susceptible to VARV and supported a productive infection, the hu-$CD34^+$ and hu-BLT mice are the best candidates for further model characterization. For both strains, high and low doses of VARV administered IN produced systemic disease and high mortality, with pathologic features resembling aspects of the severe, highly lethal ‘hemorrhagic’ form of human smallpox (24) and also corroborated what has been shown in high dose intravenous inoculation of VARV in macaques (7, 8). Pertinent features include hepatosplenic, lymphoid, and hematopoietic necrosis, with widespread VARV antigen and nucleic acid detection in these and other tissues, and the uniform presence of bacteremia with a variety of gram-positive cocci at the time of

death. Systemic bacterial infections due to gram-positive cocci are a common feature of fatal human smallpox (24, 31, 32), and were also uniformly seen in intravenously inoculated macaques that developed hemorrhagic disease (8). The role of bacterial infections as potentiators of VARV infection and/or secondary infections being the immediate cause of death in human smallpox has been debated (24, 31, 33-36), and these mice models may be valuable in investigating this important aspect of severe human smallpox. Although the model developed pathologic features resembling aspects of the 'hemorrhagic' form of human smallpox, the approximate 13-day incubation period in these mice better approximates that of "ordinary" human smallpox, and allows a broader window for testing potential new MCMs than some other animal models which have a more rapid onset mortality (23, 25).

Cutaneous lesions (vesicles and ulcers) were occasionally, but not consistently, identified in these mice, suggesting that their disease progression may most closely resemble that of 'early hemorrhagic' smallpox, in which severe disease develops before cutaneous vesicular lesions. It could be that the animals did not live long enough for a systemic rash to form, or subtle skin lesions were obstructed by the animal's fur. The presence of infectious virions in the skin was not evaluated in this study and should be examined in future studies as the skin was seeded with virions before the eruption of the rash and infected individuals were not infectious until rash eruption (33). Moreover, hemorrhage per se was not a feature of VARV infection in these mice and therefore, disease in this model may not fit neatly into one of the well-defined clinical types of human smallpox (7, 8). As with other animal models, the time from onset of clinical signs to death was short (often <4 hours) in these mice. A defined prodrome was not apparent, and antemortem noninvasive samples (oral swabs, skin swabs) were not useful for confirmation of infection. The absence of mucocutaneous exudates and lack of deroofing prior to swab collection may account for the futility of these samples. Further studies with lower doses of VARV are warranted to attempt to both recreate 'ordinary smallpox and to increase the prodromal period and potentially identify distinct biomarkers of infection that may

be useful when evaluating new antivirals in a post exposure setting. Additional antemortem blood sampling should be considered in future studies as a method for confirmation of infection prior to development of clinical signs and to evaluate hematologic alterations in disease as well as determine if any biomarkers predict outcome. While human IgG and IgM were not detected in the limited blood samples available in this study, future studies should include other detection methods for evaluation of human immune response which would strengthen the model. Subsequent studies to evaluate the detection of human immune response (in particular the hu-BLT model since IgG has been reported (37-39), as well as studies to understand the viral trafficking of the virus in the host will provide greater understanding of the utility of the model to evaluate MCMs as well as potentially provide valuable samples to understand the human immune response in these animals.

Of the assessed strains of humanized mice, the hu-PBMC mice were deemed the least promising for studying systemic VARV infection, based on their apparent delayed disease course evidenced by little mortality despite high viral loads in tissue, and the development of systemic lymphoproliferative lesions, attributed to GVH (40). GVH develops in all three of these humanized mice strains, but onset is earlier (within 4 weeks) in hu-PBMC mice, compared to 20 weeks and 12 months in hu-BLT and hu-CD34^{+}, respectively. However, the hu-PBMC model may be useful for investigating specific aspects of VARV disease, such as respiratory or reproductive tract effects.

By contrast, we showed that the non-humanized, immunosuppressed NSG background mouse supported virus replication only at the site of inoculation (nasal tissue) but was insusceptible to disseminated VARV infection. These findings indicate that VARV requires some component of the human immune system in order to spread systemically. Numerous studies have been performed looking at the VARV genome in order to help elucidate why this virus is solely a human pathogen by comparing hypothesized VARV virulence genes with the corresponding genes of other orthopoxviruses (41-44). When analyzing the VARV genome and

other closely related poxviruses, authors detected a “hotspot” of genome variation within the VARV ortholog of the vaccinia virus O1L gene, at the level of single nucleotide polymorphisms (45). The O1L gene has been shown to be necessary for efficient replication of Vaccinia virus in human cells (46). This gene is non-functional in the two most closely related viruses to VARV (*Camelpox virus* and *Taterapox virus*), which rarely infect humans, and typically cause self-limiting infections when they do (45, 47, 48). The genetic and epidemiological difference between closely related pathogens suggests selection at the OL1 gene may have played a role in the adaptation of VARV to human hosts. Although these types of genetic studies are important in investigating why VARV only infects humans, using this newly characterized humanized mouse model to understand what human component is necessary for VARV spread will be informative. Further studies should explore which human characteristic(s) contribute to a productive infection in these humanized mice.

In conclusion, our results indicate that hu-CD34^{+} and hu-BLT mice will be valuable as models of human smallpox for continued development of pre- and post-exposure treatments. There is currently only one antiviral compound licensed by the FDA (TPOXX®, SIGA) for treatment of smallpox infection. Previous *in vivo* studies have found that multi-drug treatment (TPOXX® and CMX-001®) has higher levels of protection from mortality than single drug therapy (49, 50) and resistance has been reported with single-drug treatment (51). The WHO ACVVR has recommended the licensure of at least two therapeutics for treatment of smallpox prior to destruction of all viral stocks. While surrogate models of smallpox infection have utility, they lack *in vivo* testing against the authentic agent of smallpox, VARV. Additionally, surrogate animal models do not allow investigation of the role of the human immunologic response in VARV infection. Our results showed that non-humanized NSG mice were not susceptible to systemic disease, indicating that a human component is required in these mice for severe smallpox disease development. The further characterization and development of these models may both strengthen the correlation of animal model and human disease, and also provide the

novel opportunity to investigate the role of human-specific immunologic responses in VARV infection and smallpox disease.

Acknowledgements

Michael Townsend, Jillybeth Burgado, Brock Martin, Zachary Weiner, Scott Smith, Theodora Khan, and Felix Jackson for various supportive roles throughout the study; and Heather Hayes and Brigid Bollweg for histology and immunohistochemistry support.

References

1. Reardon S. 2014. NIH finds forgotten smallpox store, *on* Nature. https://www.nature.com/news/nih-finds-forgotten-smallpox-store-1.15526. Accessed

2. Noyce RS, Lederman S, Evans DH. 2018. Construction of an infectious horsepox virus vaccine from chemically synthesized DNA fragments. PLoS One 13:e0188453.

3. MacIntyre CR, Heslop, D.J., Nand, D., Schramm, C., Butel, M., Rawlinson, W., Baker, M., Kiedrzynski, T., Nelson, C., Rosewell, A., Fotualii, L., Yeo, K., Elsgaard, J., Fonua, L. and Lane, J.M. 2019. White Paper on response to a smallpox bioterrorism release in the Pacific. Global Biosecurity 1:91-105.

4. Kim KC, Choi BS, Kim KC, Park KH, Lee HJ, Cho YK, Kim SI, Kim SS, Oh YK, Kim YB. 2016. A Simple Mouse Model for the Study of Human Immunodeficiency Virus. AIDS Res Hum Retroviruses 32:194-202.

5. Bird BH, Spengler JR, Chakrabarti AK, Khristova ML, Sealy TK, Coleman-McCray JD, Martin BE, Dodd KA, Goldsmith CS, Sanders J, Zaki SR, Nichol ST, Spiropoulou CF. 2016. Humanized Mouse Model of Ebola Virus Disease Mimics the Immune Responses in Human Disease. J Infect Dis 213:703-11.

6. Jaiswal S, Smith K, Ramirez A, Woda M, Pazoles P, Shultz LD, Greiner DL, Brehm MA, Mathew A. 2015. Dengue virus infection induces broadly cross-reactive human IgM antibodies that recognize intact virions in humanized BLT-NSG mice. Exp Biol Med (Maywood) 240:67-78.

7. Jahrling PB, Hensley LE, Martinez MJ, Leduc JW, Rubins KH, Relman DA, Huggins JW. 2004. Exploring the potential of variola virus infection of cynomolgus macaques as a model for human smallpox. Proc Natl Acad Sci U S A 101:15196-200.

8. Wahl-Jensen V, Cann JA, Rubins KH, Huggins JW, Fisher RW, Johnson AJ, de Kok-Mercado F, Larsen T, Raymond JL, Hensley LE, Jahrling PB. 2011. Progression of pathogenic events in cynomolgus macaques infected with variola virus. PLoS One 6:e24832.

9. Huggins J, Goff A, Hensley L, Mucker E, Shamblin J, Wlazlowski C, Johnson W, Chapman J, Larsen T, Twenhafel N, Karem K, Damon IK, Byrd CM, Bolken TC, Jordan R, Hruby D. 2009. Nonhuman primates are protected from smallpox virus or monkeypox virus challenges by the antiviral drug ST-246. Antimicrob Agents Chemother 53:2620-5.

10. Gallardo-Romero NF, Hutson CL, Carroll D, Kondas AV, Salzer JS, Dietz-Ostergaard S, Smith S, Hudson P, Olson V, Damon I. 2020. Use of live Variola virus to determine whether CAST/EiJ mice are a suitable surrogate animal model for human smallpox. Virus Res 275:197772.

11. Esposito J, Condit R, Obijeski J. 1981. The preparation of orthopoxvirus DNA. J Virol Methods 2:175-9.

12. Esposito JJ, Knight JC. 1985. Orthopoxvirus DNA: a comparison of restriction profiles and maps. Virology 143:230-51.

13. Esposito JJ, Obijeski JF, Nakano JH. 1978. Orthopoxvirus DNA: strain differentiation by electrophoresis of restriction endonuclease fragmented virion DNA. Virology 89:53-66.

14. Olson VA, Laue T, Laker MT, Babkin IV, Drosten C, Shchelkunov SN, Niedrig M, Damon IK, Meyer H. 2004. Real-time PCR system for detection of orthopoxviruses and simultaneous identification of smallpox virus. J Clin Microbiol 42:1940-6.

15. Ropp SL, Jin Q, Knight JC, Massung RF, Esposito JJ. 1995. PCR strategy for identification and differentiation of small pox and other orthopoxviruses. J Clin Microbiol 33:2069-76.

16. Sarmiento M, Haffey M, Spear PG. 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B2) in virion infectivity. J Virol 29:1149-58.

17. Hutson CL, Carroll DS, Gallardo-Romero N, Drew C, Zaki SR, Nagy T, Hughes C, Olson VA, Sanders J, Patel N, Smith SK, Keckler MS, Karem K, Damon IK. 2015. Comparison of Monkeypox Virus Clade Kinetics and Pathology within the Prairie Dog Animal Model Using a Serial Sacrifice Study Design. Biomed Res Int 2015:965710.

18. Guarner J, Johnson BJ, Paddock CD, Shieh WJ, Goldsmith CS, Reynolds MG, Damon IK, Regnery RL, Zaki SR, Veterinary Monkeypox Virus Working G. 2004. Monkeypox transmission and pathogenesis in prairie dogs. Emerg Infect Dis 10:426-31.

19. Hayat MA. 2000. Principles and techniques of electron microscopy : biological applications, 4th ed. Cambridge University Press, Cambridge, UK ; New York.

20. Goldsmith CS, Whistler T, Rollin PE, Ksiazek TG, Rota PA, Bellini WJ, Daszak P, Wong KT, Shieh WJ, Zaki SR. 2003. Elucidation of Nipah virus morphogenesis and replication using ultrastructural and molecular approaches. Virus Res 92:89-98.

21. Kondas AV, Olson VA, Li Y, Abel J, Laker M, Rose L, Wilkins K, Turner J, Kline R, Damon IK. 2015. Variola virus-specific diagnostic assays: characterization, sensitivity, and specificity. J Clin Microbiol 53:1406-10.

22. Karem KL, Reynolds M, Braden Z, Lou G, Bernard N, Patton J, Damon IK. 2005. characterization of acute-phase humoral immunity to monkeypox: use of immunoglobulin M enzyme-linked immunosorbent assay for detection of monkeypox infection during the 2003 North American outbreak. Clin Diagn Lab Immunol 12:867-72.

23. Chapman JL, Nichols DK, Martinez MJ, Raymond JW. 2010. Animal models of orthopoxvirus infection. Vet Pathol 47:852-70.

24. Fenner F, Henderson, D.A., Arita, I., Jezek, Z., Ladnyi, I.D. 1988. Smallpox and its eradication. World Health Organization.

25. Americo JL, Moss B, Earl PL. 2010. Identification of wild-derived inbred mouse strains highly susceptible to monkeypox virus infection for use as small animal models. J Virol 84:8172-80.

26. Earl PL, Americo JL, Cotter CA, Moss B. 2015. Comparative live bioluminescence imaging of monkeypox virus dissemination in a wild-derived inbred mouse (Mus musculus castaneus) and outbred African dormouse (Graphiurus kelleni). Virology 475:150-8.

27. Earl PL, Americo JL, Moss B. 2012. Lethal monkeypox virus infection of CAST/EiJ mice is associated with a deficient gamma interferon response. J Virol 86:9105-12.

28. Carroll DS, Olson VA, Smith SK, Braden ZH, Patel N, Abel J, Li Y, Damon IK, Karem KL. 2013. Orthopoxvirus variola infection of Cynomys ludovicianus (North American black tailed prairie dog). Virology 443:358-62.

29. Hutson CL, Olson VA, Carroll DS, Abel JA, Hughes CM, Braden ZH, Weiss S, Self J, Osorio JE, Hudson PN, Dillon M, Karem KL, Damon IK, Regnery RL. 2009. A prairie dog animal model of systemic orthopoxvirus disease using West African and Congo Basin strains of monkeypox virus. J Gen Virol 90:323-33.

30. Titova KA, Sergeev AA, Zamedyanskaya AS, Galahova DO, Kabanov AS, Morozova AA, Bulychev LE, Sergeev AA, Glotova TI, Shishkina LN, Taranov OS, Omigov VV, Zavjalov EL, Agafonov AP, Sergeev AN. 2015. Using ICR and SCID mice as animal models for smallpox to assess antiviral drug efficacy. J Gen Virol 96:2832-43.

31. Councilman WT, Magrath GB, Brinckerhoff WR. 1904. The Pathological Anatomy and Histology of Variola. J Med Res 11:12-135.

32. Lillie RD. 1930. Smallpox and Vaccinia The Pathologic Histology. Archives of Pathology 10.

33. Downie AW, Macdonald A. 1953. Smallpox and related virus infections in man. Br Med Bull 9:191-5.

34. Martin DB. 2002. The cause of death in smallpox: an examination of the pathology record. Mil Med 167:546-51.

35. Bras G. 1952. The morbid anatomy of smallpox. Doc Med Geogr Trop 4:303-51.

36. Moore ZS, Seward JF, Lane JM. 2006. Smallpox. Lancet 367:425-35.

37. Biswas S, Chang H, Sarkis PT, Fikrig E, Zhu Q, Marasco WA. 2011. Humoral immune responses in humanized BLT mice immunized with West Nile virus and HIV-1 envelope proteins are largely mediated via human CD5+ B cells. Immunology 134:419-33.

38. Lan P, Tonomura N, Shimizu A, Wang S, Yang YG. 2006. Reconstitution of a functional human immune system in immunodeficient mice through combined human fetal thymus/liver and CD34+ cell transplantation. Blood 108:487-92.

39. Melkus MW, Estes JD, Padgett-Thomas A, Gatlin J, Denton PW, Othieno FA, Wege AK, Haase AT, Garcia JV. 2006. Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. Nat Med 12:1316-22.

40. Shultz LD, Ishikawa F, Greiner DL. 2007. Humanized mice in translational biomedical research. Nat Rev Immunol 7:118-30.

41. Yadav VN, Pyaram K, Mullick J, Sahu A. 2008. Identification of hot spots in the variola virus complement inhibitor (SPICE) for human complement regulation. J Virol 82:3283-94.

42. Lee SH, Jung JU, Means RE. 2003. 'Complementing' viral infection: mechanisms for evading innate immunity. Trends Microbiol 11:449-52.

43. Rosengard AM, Liu Y, Nie Z, Jimenez R. 2002. Variola virus immune evasion design: expression of a highly efficient inhibitor of human complement. Proc Natl Acad Sci U S A 99:8808-13.

44. Van Vliet K, Mohamed MR, Zhang L, Villa NY, Werden SJ, Liu J, McFadden G. 2009. Poxvirus proteomics and virus-host protein interactions. Microbiol Mol Biol Rev 73:730-49.

45. Smithson C, Purdy A, Verster AJ, Upton C. 2014. Prediction of steps in the evolution of variola virus host range. PLoS One 9:e91520.

46. Schweneker M, Lukassen S, Spath M, Wolferstatter M, Babel E, Brinkmann K, Wielert U, Chaplin P, Suter M, Hausmann J. 2012. The vaccinia virus O1 protein is required for sustained activation of extracellular signal-regulated kinase 1/2 and promotes viral virulence. J Virol 86:2323-36.

47. Bera BC, Shanmugasundaram K, Barua S, Venkatesan G, Virmani N, Riyesh T, Gulati BR, Bhanuprakash V, Vaid RK, Kakker NK, Malik P, Bansal M, Gadvi S, Singh RV, Yadav V, Sardarilal, Nagarajan G, Balamurugan V, Hosamani M, Pathak KM, Singh RK. 2011. Zoonotic cases of camelpox infection in India. Vet Microbiol 152:29-38.

48. Khalafalla AI, Abdelazim F. 2017. Human and Dromedary Camel Infection with Camelpox Virus in Eastern Sudan. Vector Borne Zoonotic Dis 17:281-284.

49. Quenelle DC, Prichard MN, Keith KA, Hruby DE, Jordan R, Painter GR, Robertson A, Kern ER. 2007. Synergistic efficacy of the combination of ST-246 with CMX001 against orthopoxviruses. Antimicrob Agents Chemother 51:4118-24.

50. Chen N, Bellone CJ, Schriewer J, Owens G, Fredrickson T, Parker S, Buller RM. 2011. Poxvirus interleukin-4 expression overcomes inherent resistance and vaccine-induced immunity: pathogenesis, prophylaxis, and antiviral therapy. Virology 409:328-37.

51. Lederman ER, Davidson W, Groff HL, Smith SK, Warkentien T, Li Y, Wilkins KA, Karem KL, Akondy RS, Ahmed R, Frace M, Shieh WJ, Zaki S, Hruby DE, Painter WP, Bergman KL, Cohen JI, Damon IK. 2012. Progressive vaccinia: case description and laboratory-guided therapy with vaccinia immune globulin, ST-246, and CMX001. J Infect Dis 206:1372-85.

Table 4.1: Summary of clinical signs, mortality and gross necropsy findings from part 1 of the study.

		Clinical Signs					Mortality		Gross necropsy findings			
	Group	>20% Wt Loss	Minimally Subdued Activity	Unresponsive when stimulated	Ruffled/ piloerection	Skin lesions	Found Deceased	Did not survive Day 21	Hepatic necrosis	Gallbladder hemorrhage	Splenomegaly	Lymphadenomegaly
hu-PBMC	Diluent Non-infectous Control	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	1/2
hu-PBMC	γ-irradiated Non-infectious VARV Control	0/2	0/2	0/2	0/2	0/2	1/2	1/2	0/2	0/2	0/2	0/2
hu-PBMC	VARV $7x10^5$	1/4	1/4	0/4	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4
hu-PBMC	VARV $7x10^3$	2/4	0/4	0/4	2/4	1/4	0/4	0/4	0/4	0/4	0/4	0/4
hu-PBMC	Negative Non-infectious Control	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
hu-CD34	γ-irradiated Non-infectious VARV Control	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	1/2	0/2
hu-CD34	VARV $7x10^5$	4/4	0/4	2/4	4/4	0/4	2/4	4/4	3/4	0/4	0/4	0/4
hu-CD34	VARV $7x10^3$	3/3	1/3	0/3	1/3	1/3	1/3	3/3	1/3	2/3	2/3	1/3
hu-CD34	Negative Non-infectious Control	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
hu-BLT	γ-irradiated Non-infectious VARV Control	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
hu-BLT	VARV $7x10^5$	3/4	1/4	2/4	3/4	2/4	1/4	4/4	3/4	0/4	1/4	0/4
hu-BLT	VARV $7x10^3$	3/4	1/4	0/4	3/4	1/4	4/4	4/4	2/4	0/4	0/4	0/4
hu-BLT	Negative Non-infectious Control	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2

Control animals were only considered to have a clinical sign of weight loss if >20% weight loss occurred at two or more time points.

Hu-CD-34+-8 was excluded from analysis.

Table 4.2. Microscopic lesions and VARV immunolocalization in tissues from humanized (PBMC, CD34^{+}, BLT) mice and non-humanized background strain (NSG) mice inoculated intranasally with VARV. Fractions indicate number of animals with finding divided by number of animals with tissue type collected.

+: mild/focal; ++: moderate/multifocal; +++: severe/extensive; 0: tissue not collected; N/A: not applicable

*Infection did not take in one animal from this group; this animal is excluded from summarized results.

**Immunostaining localized to foci of necrosis when present; however, immunostaining was also seen in tissues without morphologic alterations. Reported immunolocalization includes both necrotic and morphologically intact foci.

	Mouse strain				
Histopathologic findings	**PBMC (n=8)**	**CD34+ (n=7)***	**BLT (n=8)**	**NSG (n=10)**	**VARV immunolocalization****
Skin, hock: chronic ulceration	+ (1/4)	+ (1/3)	0	N/A	Hyperplastic epidermis at ulcer margin, periarticular mesenchymal cells
Liver: hepatocellular necrosis, rare intracytoplasmic viral inclusions	+ (2/7)	+++ (7/7)	+++ (8/8)	- (0/10)	Hepatocytes, Kupffer cells, endothelial cells
Adrenal gland: multifocal cortical > medullary necrosis	+ (1/3)	+++ (3/5)	+++ (5/5)	- (0/1)	Necrotic and intact cortical epithelium and medullary chromaffin cells

Spleen: necrosis, hemorrhage, lymphoid depletion	++ (2/3)	+++ (5/5)	+++ (5/5)	- (0/8)	Mesenchymal cells, reticuloendothelial cells
Lymph nodes: necrosis, lymphoid depletion	+++ (2/2)	+++ (4/4)	+++ (2/5)	- (0/10)	Reticuloendothelial cells
Bone marrow: necrosis, hemorrhage	+ (2/5)	+++ (7/7)	+++ (8/8)	- (0/10)	Necrotic hematopoietic cells, endosteum, periosteum
Ovary: multifocal necrosis	++ (2/3)	- (0/3)	- (0/5)	- (0/9)	Stromal and follicular cells
Uterus: multifocal to mural necrosis	+++ (5/7)	++ (2/7)	++ (3/7)	- (0/10)	Stromal and smooth muscle cells
Lung: multifocal bronchial epithelial necrosis; peribronchiolar and perivascular edema; mild interstitial pneumonitis	++ (7/7)	- (0/5)	+ (4/8)	- (0/9)	Bronchial epithelium, interstitial cells, inflammatory cells
Nasal cavity: multifocal submucosal necrosis and edema, serous gland atrophy, minimal inflammation	++ (5/7)	- (0/7)	- (0/8)	- (0/10)	Submucosal stroma, respiratory and serous glandular epithelial cells
Tooth: pulp necrosis	+	++	++	-	Pulp, periodontal

	(2/6)	(3/7)	(2/8)	(0/10)	ligament
Disseminated bacteremia	- (0/7)	+++ (7/7)	+++ (8/8)	- (0/10)	N/A

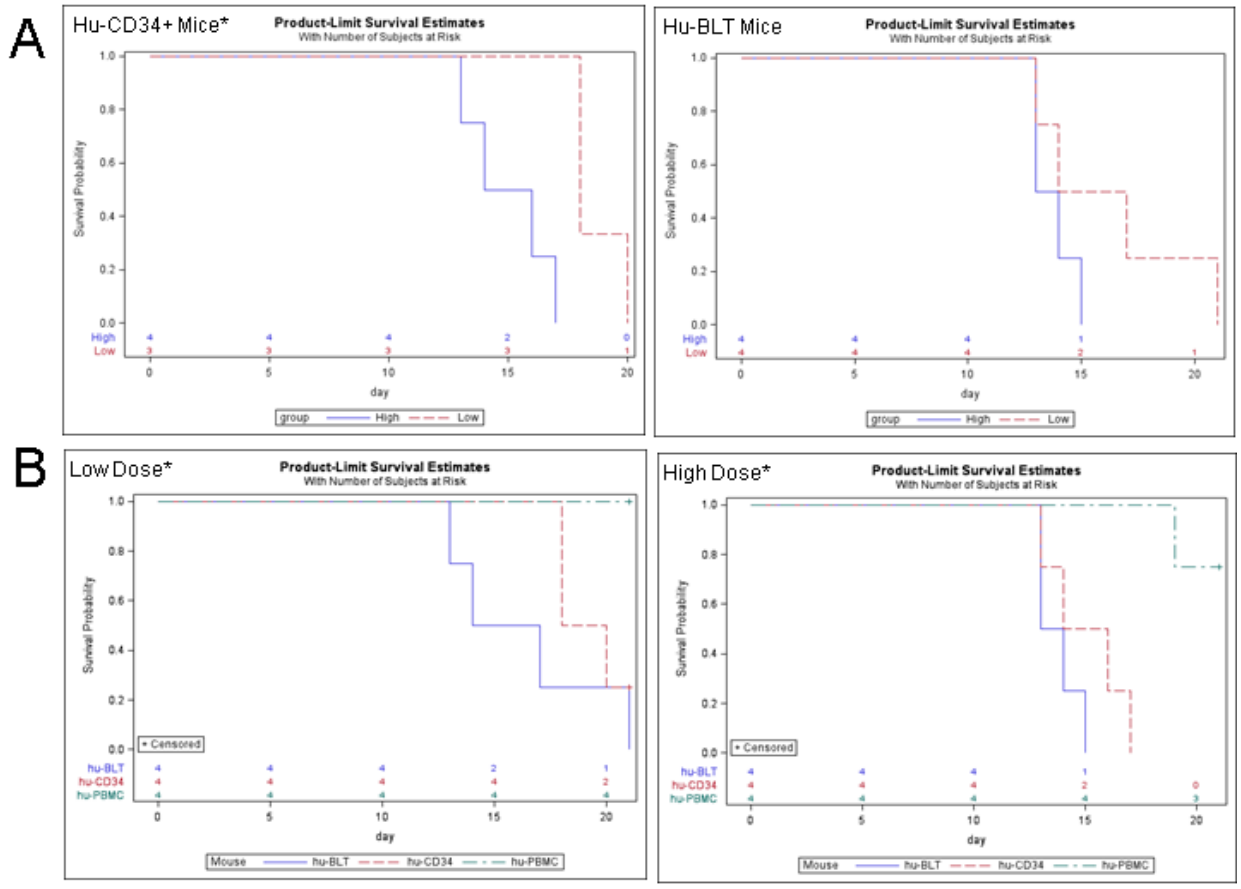


Figure 4.1 Survivorship Curves for mice and dosage groups. A. Dose-dependent mortality was seen in the hu-CD34^{+} and hu-BLT mice starting at day 13. High dose animals succumb to disease earlier than those with a lower dose. *Difference was significant in Hu-CD34_ mice, p = 0.02. B. Survival of three types of humanize mice (BLT, CD34^{+}, and PBMC) were assessed for low dose and high dose independently. *Hu-PBMC mice were significantly more likely to survive

variola infection compared to hu-BLT and hu-CD34^{+} mice in both dose groups (high dose p=0.004, low dose p=0.008). There were no significant differences between hu-BLT and hu-CD34^{+} mice, when controlling for dose.

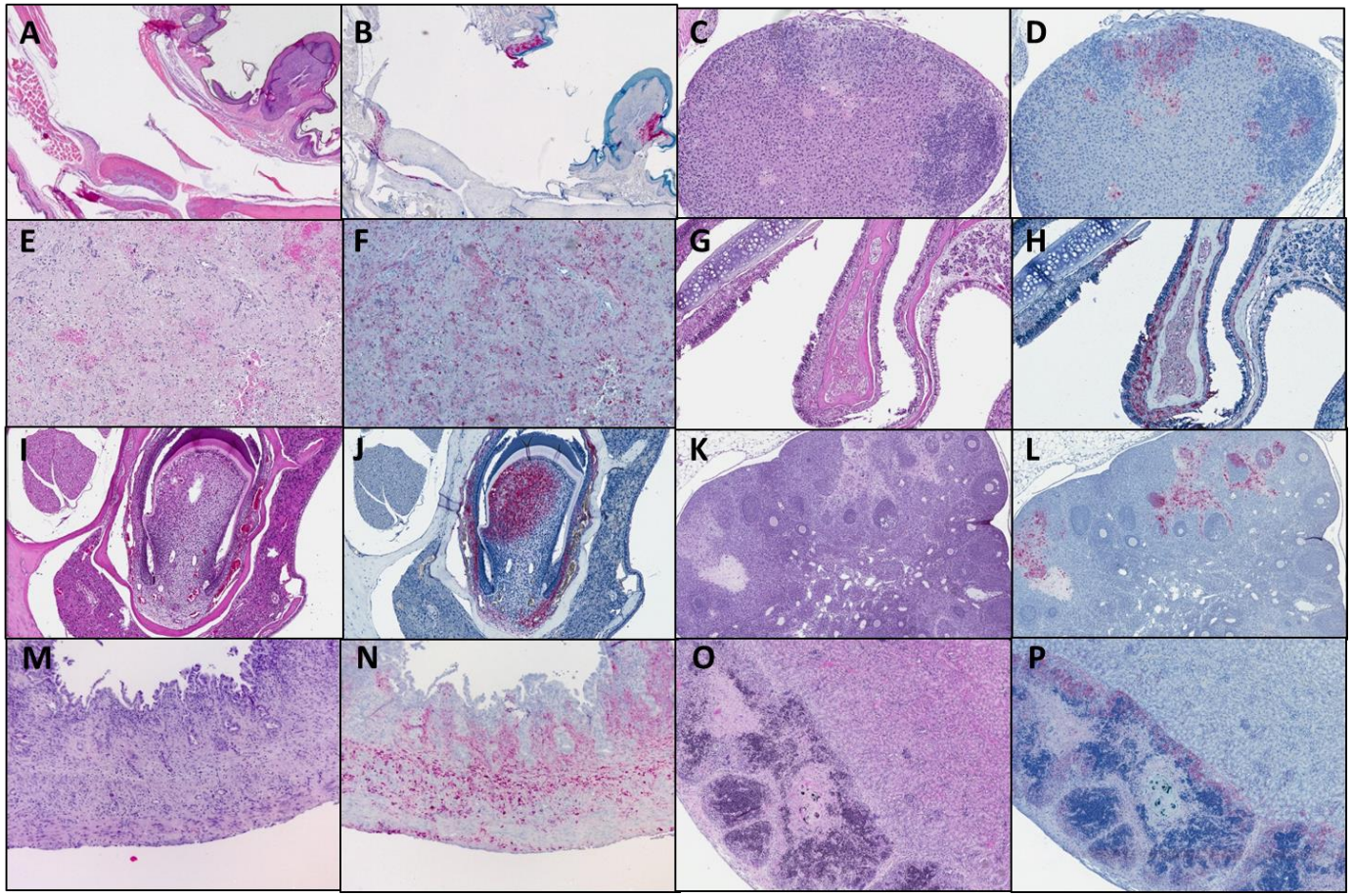


Figure 4.2. Representative histopathology and immunohistochemistry of VARV infection in humanized mice. A, B: Chronic skin ulceration over the hock, with epidermal hyperplasia and VARV immunostaining in skin, tendon, and periosteum (PBMC-1). C, D: Multifocal adrenal gland necrosis with VARV immunostaining (BLT-5). E, F: Diffuse splenic necrosis with lymphoid depletion, fibrin, and hemorrhage; extensive VARV immunostaining in reticuloendothelial and mesenchymal cells ($CD34^{+}$-4). G, H: Nasal

mucosa with submucosal edema and mild inflammation; extensive VARV immunostaining (PBMC-6). I, J: Tooth with dental pulp necrosis; VARV immunostaining of pulp and periodontal ligament ($CD34^{+}$-9). K, L: Ovary with multifocal stromal and follicular necrosis and VARV immunostaining (PBMC-10). M, N: Uterus with transmural necrosis and VARV immunostaining (PBMC-5). O, P: Focal necrosis in renal subcapsular human fetal thymic graft, with extensive VARV immunostaining (BLT-5). A, C, E, G, I, K, M, O (hematoxylin-eosin); B, D, F, H, J, L, N, P (VARV immunohistochemistry). Original magnifications: A, B, O, P (x50); C-J, M, N (x100); K, L (x200).

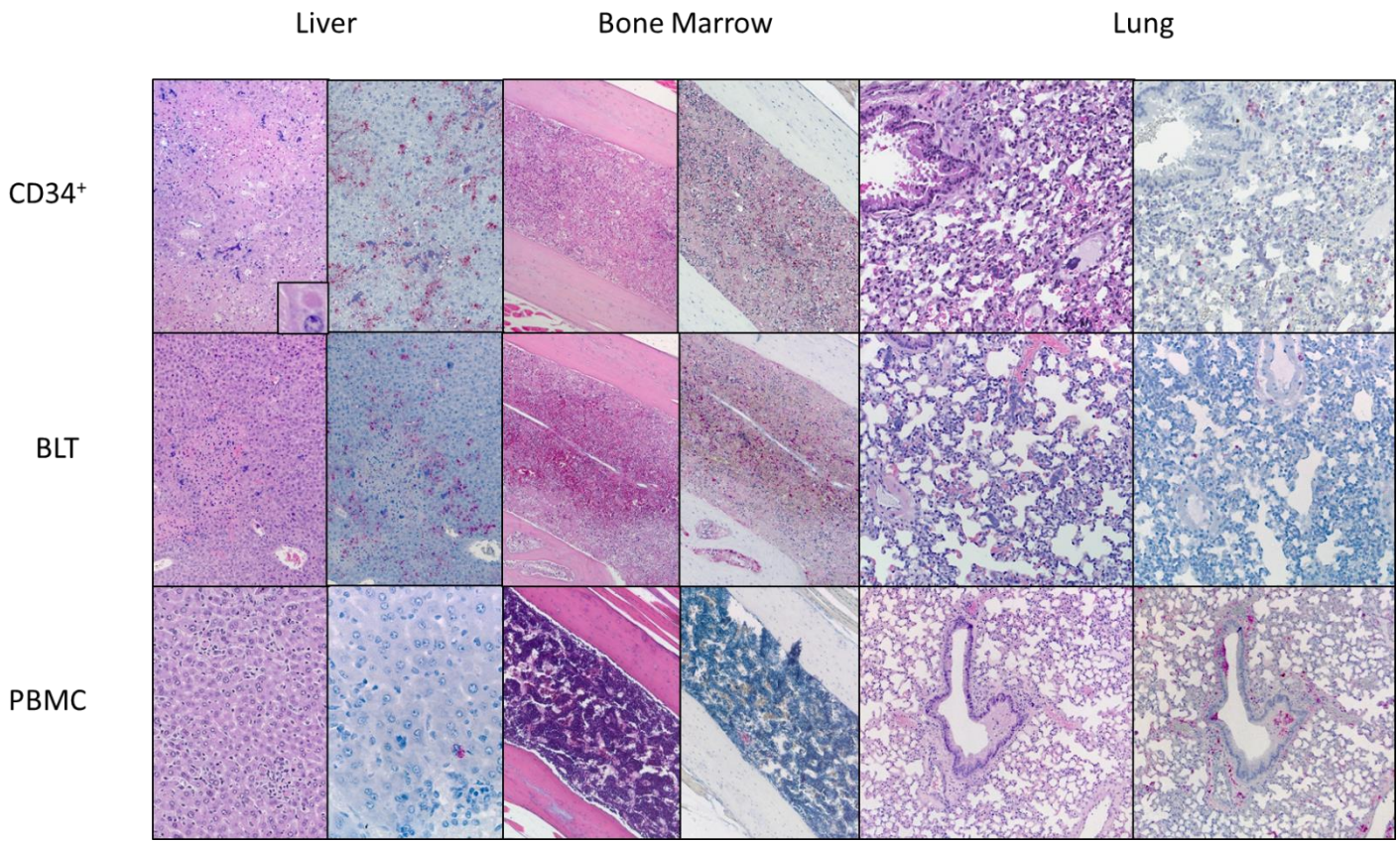


Figure 4.3. Representative differences in pathologic findings among three strains of humanized mice with VARV infection. $CD34^+$ and BLT mice had similar findings in liver, bone marrow, and lung, which contrasted those seen in PBMC mice. $CD34^+$ and BLT livers had confluent and lobular hepatocyte necrosis with very rare eosinophilic globular inclusions (top left, inset), and VARV immunostaining of hepatocytes, Kupffer cells, and occasional endothelial cells. $CD34^+$ and BLT bone marrow specimens similarly showed extensive necrosis and hemorrhage associated with VARV immunostaining. PBMC liver and bone marrow showed minimal

inflammation and no necrosis in liver and bone marrow, and only very rare VARV immunostaining in these tissues. Conversely, $CD34^+$ and BLT lung tissues showed minimal inflammation or VARV immunostaining, while PBMC lungs had more prominent peribronchiolar and perivascular inflammation with VARV immunostaining.$CD34^+$ and BLT mice had disseminated intravascular bacteria (arrows), which were not present in PBMC mice.

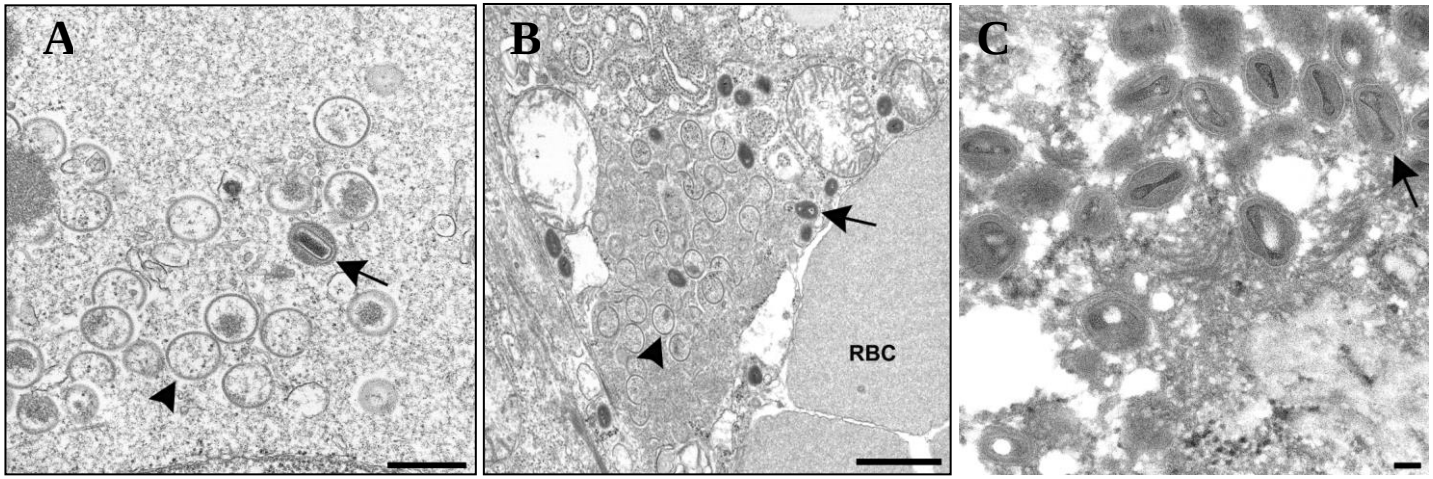
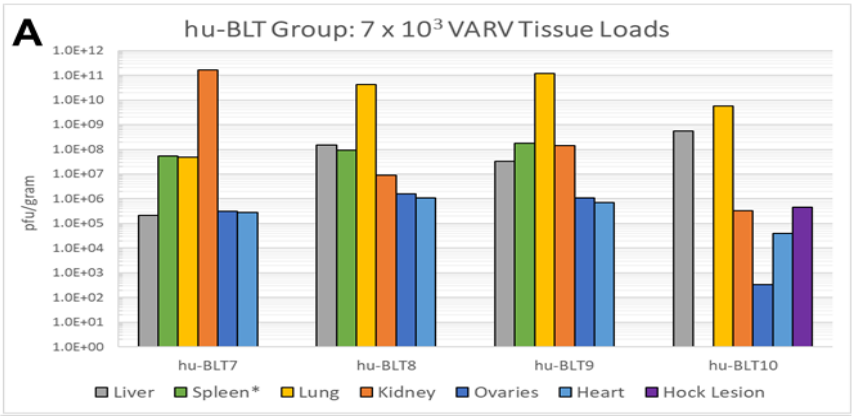
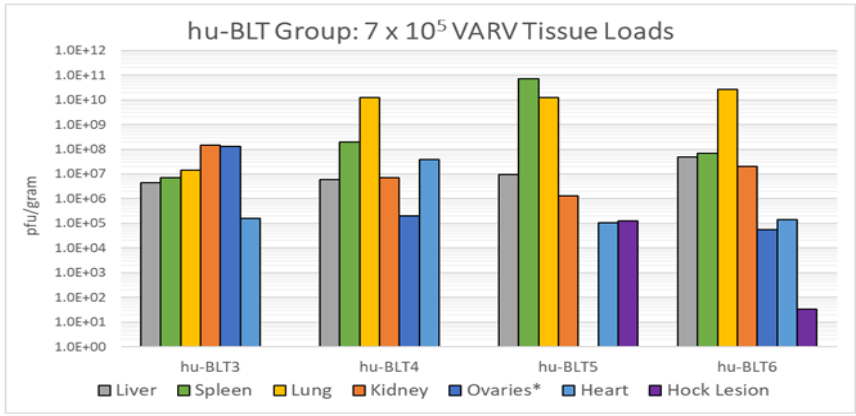
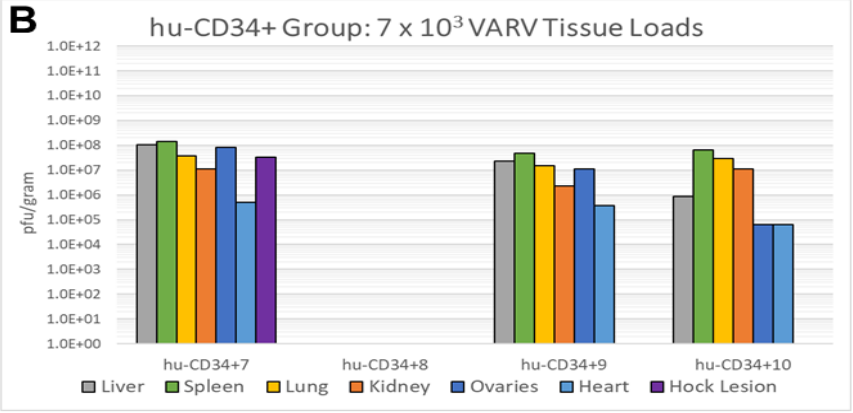
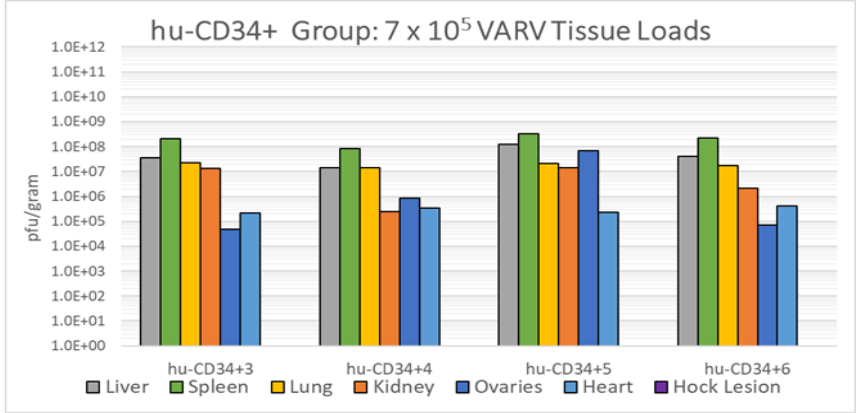


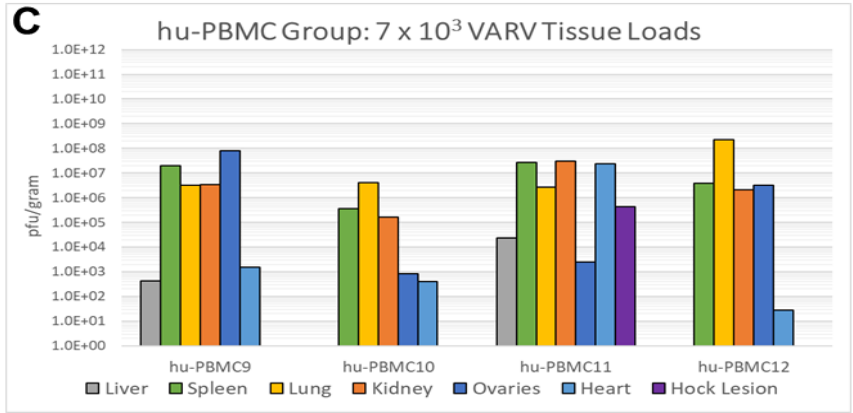
Figure 4.4. Electron microscopic images of humanized mice infected with VARV. A: Hepatocyte with multiple spherical, diffuse immature particles (arrowhead) and a single condensed mature particle (arrow). Bar, 500 nm. B: Sinusoidal endothelial cell in the liver with immature (arrowhead) and mature (arrow) VARV particles. RBC, red blood cell. Bar, 1 um. C: Human fetal thymic allograft containing mature virions with a characteristic dumbbell-shaped nucleoid (arrow). Bar, 100 nm.


A
hu-BLT Group: 7 x 10³ VARV Tissue Loads
pfu/gram
1.0E+12
1.0E+11
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
hu-BLT7
hu-BLT8
hu-BLT9
hu-BLT10
Liver
Spleen*
Lung
Kidney
Ovaries
Heart
Hock Lesion


hu-BLT Group: 7 x 10⁵ VARV Tissue Loads
pfu/gram
1.0E+12
1.0E+11
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
hu-BLT3
hu-BLT4
hu-BLT5
hu-BLT6
Liver
Spleen
Lung
Kidney
Ovaries*
Heart
Hock Lesion


B
hu-CD34+ Group: 7 x 10³ VARV Tissue Loads
pfu/gram
1.0E+12
1.0E+11
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
hu-CD34+7
hu-CD34+8
hu-CD34+9
hu-CD34+10
Liver
Spleen
Lung
Kidney
Ovaries
Heart
Hock Lesion


hu-CD34+ Group: 7 x 10⁵ VARV Tissue Loads
pfu/gram
1.0E+12
1.0E+11
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
hu-CD34+3
hu-CD34+4
hu-CD34+5
hu-CD34+6
Liver
Spleen
Lung
Kidney
Ovaries
Heart
Hock Lesion


C
hu-PBMC Group: 7 x 10³ VARV Tissue Loads
pfu/gram
1.0E+12
1.0E+11
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
hu-PBMC9
hu-PBMC10
hu-PBMC11
hu-PBMC12
Liver
Spleen
Lung
Kidney
Ovaries
Heart
Hock Lesion

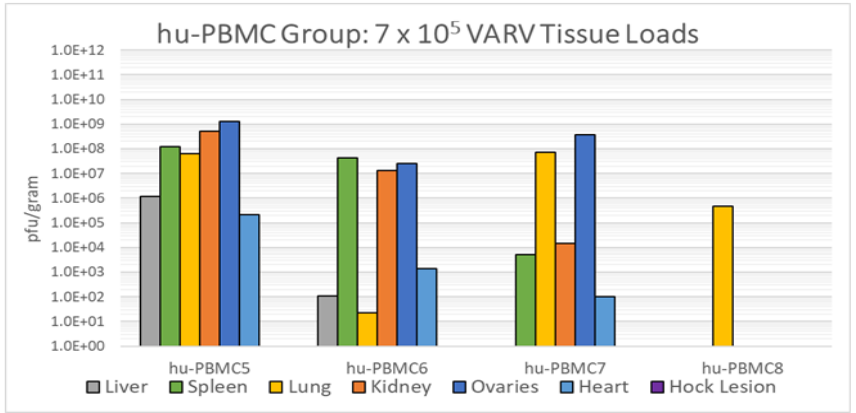

hu-PBMC Group: 7 x 10⁵ VARV Tissue Loads
pfu/gram
1.0E+12
1.0E+11
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
hu-PBMC5
hu-PBMC6
hu-PBMC7
hu-PBMC8
Liver
Spleen
Lung
Kidney
Ovaries
Heart
Hock Lesion

Figure 4.5. High viable virus loads were detected in all three types of humanized mice. Humanized mice (BLT, $CD34^+$ and PBMC) that succumbed to variola virus infection, or were euthanized at 21 dpi, had tissues collected and processed for viral culture. A: The hu-BLT mice had the highest viral loads (both dose groups) with viral loads as high as ~1.66×10^{11} pfu/gram. B: Slightly lower levels were seen in hu-$CD34^+$ mice and one of these mice in the lower dose group (hu-CD34+ 8) had no detectable viable virus (likely not a successful inoculation). C: Viable virus was also seen in most tissues collected from the hu-PBMC mice although little mortality was observed (only 1/8) which suggests there is a delayed disease course in this mouse type. An * indicates one or more of that sample had cell culture monolayer destroyed or plaques were present but below the LOD for this assay.

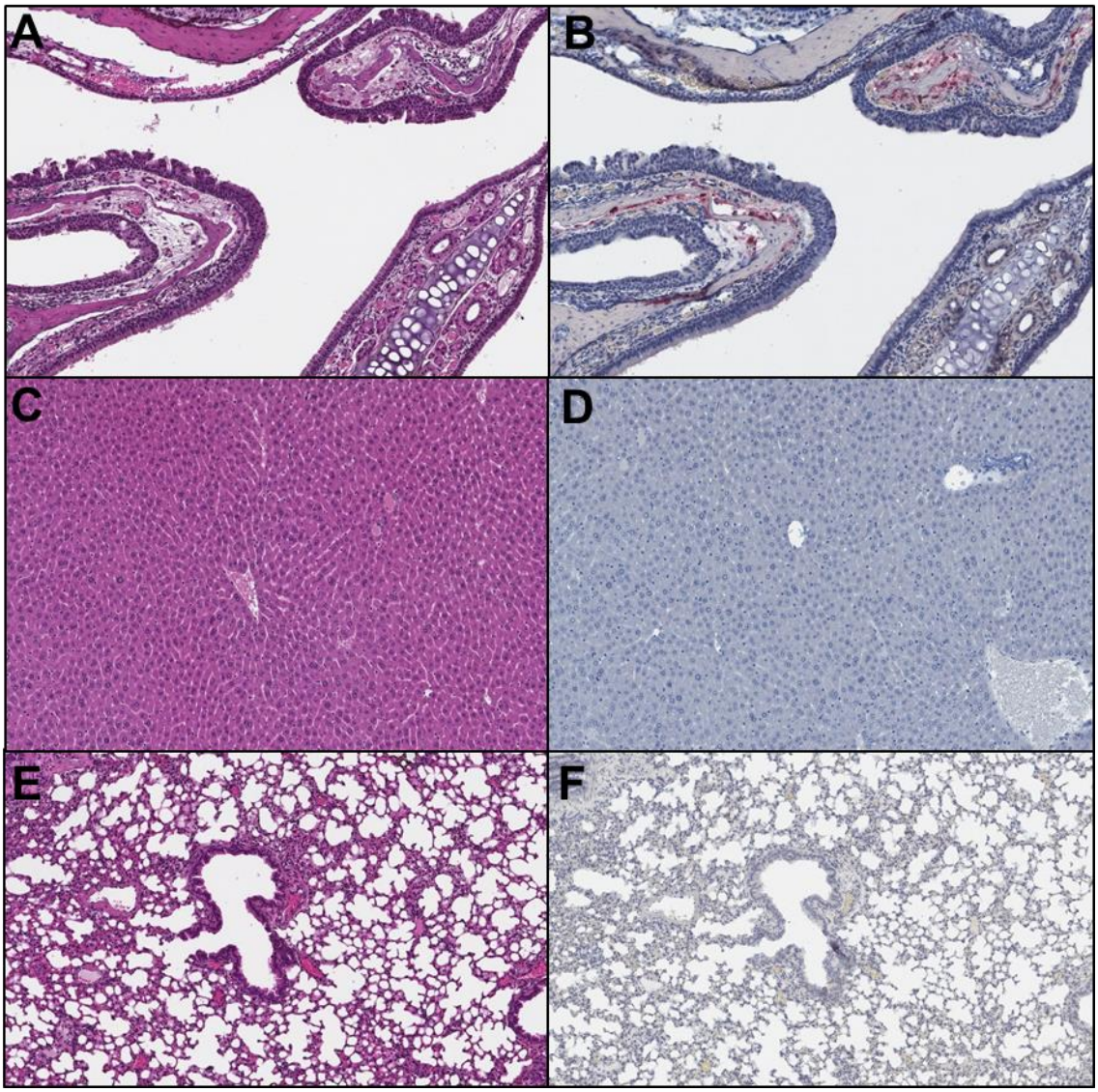


Figure 4.6. Histopathology and VARV immunohistochemistry in non-humanized NSG mice. Nasal tissue (A and B) with mild inflammatory changes and immunostaining of VARV. Liver (C and D) and lung (E and F) tissues without histopathologic changes or immunostaining of VARV.

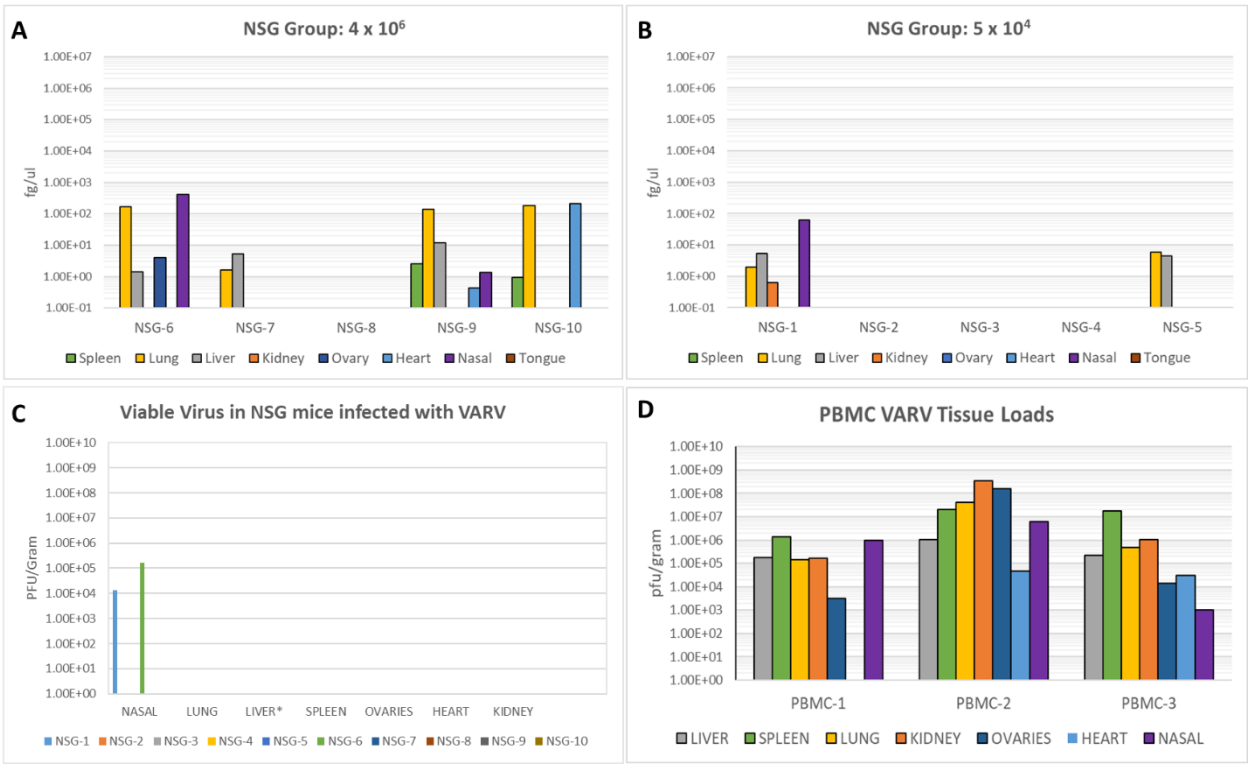


Figure 4.7. High viable virus loads were detected in all three types of humanized mice. Humanized-mice (BLT, CD34^{+} and PBMC) that succumbed to variola virus infection, or were euthanized at 21 dpi, had tissues collected and processed for viral culture. **A:** The hu-BLT mice had the highest viral loads (both dose groups) with viral loads as high as ~1.66 x 10^{11} pfu/gram. **B:** Slightly lower levels were seen in hu-CD34^{+} mice and one of these mice in the lower dose group (hu-CD34+ 8) had no detectable viable virus (likely not a successful inoculation). **C:** Viable virus was also seen in most tissues collected from the hu-PBMC mice although little mortality was observed (only 1/8) which suggests there is a delayed disease course in this mouse type. An * indicates one or more of that sample had cell culture monolayer destroyed or plaques were present but below the LOD for this assay.

CHAPTER 5

CONCLUSIONS

Variola virus (VARV), the causative agent of smallpox, was believed to have killed over 300 million people in the twenty century alone. Through a vaccination campaign led by the World Health Organization, smallpox was declared eradicated in 1980. Although smallpox is eradicated, the threat of re-emergence remains by weaponization of unknown stocks or recreation using synthetic biology. While several medical counter measures (MCMs) are available for the prevention and treatment of smallpox, they are in limited supply and never been tested directly against VARV. Orthopoxviruses (OPXV) have been reported to mutate around antiviral targets suggesting a multiple therapeutic approach would be beneficial. Since VARV is a solely human pathogen, studying the effectiveness of MCMs against the pathogen was been difficult. While many studies have been conducted, no animal model that replicates human smallpox disease progression after the natural route of infection has been identified. Since the eradication of smallpox*, Monkeypox virus* (MPXV) is the most concerning OPXV that causes human disease. Due to disease progression similarities and animal models available, MPXV serves as a surrogate OPXV for testing smallpox medical counter measures (MCMs). Here, we use this model to study the efficacy of a monoclonal antibody (mAb) cocktail, called Mix4 developed by The Vanderbilt Vaccine University (VU), to determine if it has the potential to be a smallpox MCM. Based on our *in vivo* evaluation, we evaluated Mix4, the individual mAbs within Mix4 and other mAbs VU developed to determine if a different combination of mAbs that would strongly neutralize both VARV and MPXV. Lastly, we provide evidence for a small animal model for VARV using three different kinds of humanized mice.

The prairie dog MPXV model has been used to study multiple smallpox MCMs including (ACAM2000®; Sanofi Pasteur Biologics Co.) and small molecular antiviral compounds (TPOXX®; tecovirimat; SIGA). In these studies, we used the prairie dog MPXV model to study the effectiveness of Mix4 which is comprised of two mAbs that target the intracellular mature virion (IMV) and two mAbs that target the extracellular enveloped virion (EV). A longevity study identified that human mAbs can be safely tested in prairie dogs as no negative side effects were seen even at 48 mg/kg. A plaque reduction neutralization assay (PRNT) revealed that mAbs were detected in the blood stream 1 day post injection and remained for at least 7 days for two different products (a mAb and a polyclonal called Vaccinia immune globulin [VIG]). The results from the longevity study suggested a two dose treatment, one day prior to infection and six days post infection, had a good chance of success in an efficacy study. Mix4 treatment also resulted in delayed DNAemia compared to the other treatment groups. While Mix4 provided the highest protection against mortality, one animal had to be euthanized and other animals in this group had mild clinical signs such as respiratory distress. PRNTs revealed that although Mix4 was able to neutralize IMV post injection, Mix4 was not able to neutralize the EV form of MPXV. The PRNT results, along with other study results, suggest that the efficacy of Mix4 could be improved by changing the EV targeting mAbs to other mAbs developed by VU.

Previous work has shown that Mix4 neutralized the IMV form of VARV *in vitro* in a PRNT assay. Based on the results from the prairie dog MPXV efficacy study, it was important to verify that Mix4 neutralized the EV form of VARV prior to deciding if a new mAb cocktail should be created. The IMV PRNT identified three mAbs that neutralized the IMV form of VARV in a PRNT; one of those mAbs, MPXV-26, is present in Mix4. A subset of mAbs developed by VU were also screened against VARV EV in a PRNT with complement. While seven mAbs were identified to neutralize VARV, none of those were present in Mix4. While some neutralization was seen with Mix4 in the EV PRNT with complement, it required 10-fold higher concentration compared to other individual mAbs. These results, along with the prairie dog efficacy results,

suggested that a new mAb cocktail should be strongly considered. Since MPXV is also an OPXV of concern and it is commonly used as a surrogate OPXV for VARV, EV PRNTs with complement were conducted against MPXV. This testing also revealed that three mAbs neutralized MPXV EV well; none of those are present in Mix4. When analyzing the MPXV and VARV PRNT results, four mAbs developed by VU neutralized both pathogens of concern. Two of those mAbs neutralize IMV and two mAbs neutralize EV suggesting that different combination of mAbs developed by VU would be more efficacious than Mix4. Prior to testing the final mixes, we propose that the most promising mAbs undergo production using cell culture platforms such as CHO cells as this production technology has been used for other Investigational Drug Protocols. Post-production, the mAbs and mixes should be rescreened against both MPXV and VARV IMV and EV. This information will result in deciding the best mAbs to include in the Universal Pox Mix which would have the best chance at being efficacious against both MPXV and VARV.

Testing in animal models with surrogate OPXVs are useful when determining the efficacy of potential smallpox MCMs. However, testing against the authentic agent, VARV, would be extremely useful. Humanized mice have become invaluable tools for studying other human pathogens. Here, we show that three different humanized (hu-) mice strains, hu-BLT, hu-CD34^{+} and hu-PBMC, are highly susceptible to VARV infection, establishing the first small animal model using VARV. Post intranasal VARV challenge, which mimics the natural route for human smallpox transmission, VARV spread systemically within the humanized mice with animals beginning to succumb to disease ~ 13 days post infection. This is similar to what was seen in “ordinary” human smallpox which had a 12-14 day incubation period prior to symptom onset. In the humanized mice, high viral loads were detected in multiple tissues as high as 1.66 x 10^{11} pfu/gram of tissue. Pathology detected features of disease similar to the “hemorrhagic” form of human smallpox suggesting that this model may not fit into the disease categories of human smallpox. Pertinent features include hepatosplenic, lymphoid, and hematopoietic

necrosis, with widespread VARV antigen and nucleic acid detection in these and other tissues, and the uniform presence of bacteremia with a variety of gram-positive cocci at the time of death. Interestingly, secondary bacterial infections were also commonly seen with human smallpox due to gram-positive cocci. In comparison, the non-humanized background mouse used to create the humanized mice was not susceptible to systemic VARV infection even though these mice are immunosuppressed. The humanized mice VARV model can be used for MCM testing, aid in identifying why VARV is a solely human pathogen and provide samples for understanding the human immune response to VARV infection.