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THE DEVELOPMENT OF A REVERSE GENETICS SYSTEM FOR MACHUPO VIRUS

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THE DEVELOPMENT OF A REVERSE GENETICS
SYSTEM FOR MACHUPO VIRUS

by

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THE DEVELOPMENT OF A REVERSE GENETICS

SYSTEM FOR MACHUPO VIRUS

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The etiologic agent of Bolivian hemorrhagic fever (BHF), Machupo virus (MACV), is a highly lethal viral pathogen with no approved vaccine or therapeutic to limit infection or outbreaks. Following implementation of rodent population controls after the initial outbreak, no cases of BHF were reported from 1976 to 1993. Reports in the last five years from the endemic region have identified a surge in reported cases and deaths from the disease. Since then, very little characterization or research of MACV has been accomplished. In this research, I describe the development of two major tools for studying MACV: The development of a mini-genome for both the small and large

segments, and the establishment of a reverse genetics system and rescue of a recombinant MACV. Using these tools, I present the first modern *in vitro* characterization of MACV, the development of a novel and lethal murine animal model, and the generation of a rationally attenuated MACV.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AHF	Argentine hemorrhagic fever
ANOVA	Analysis of Variance
BHF	Bolivian hemorrhagic fever
BHK-21	Baby hamster kidney cells
Bp	base pair (nucleotide)
BSL	Biosafety level
CDC	Centers for Disease Control/Prevention
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPI	Days post infection
dsRNA	Double stranded RNA
eIF2α	Eukaryotic initiation factor 2
ELISA	Enzyme linked immunosorbent assays
ER	Endoplasmic reticulum
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FRhL	Fetal rhesus lung
GNL	Galveston National Laboratory
GP-1	Glycoprotein 1
GP-2	Glycoprotein 2
GPC	Glycoprotein Precursor
HPI	Hours post infection
HRP	Horseradish peroxidase
IC	Intracranial
IFN	Interferon
IGR	Intergenic region
IKKε/TBK-1	Serine/threonine kinases IκB kinase ε/TANK-binding kinase-1
IN	Intranasal
IP	Intraperitoneal
IPS-1	IFN-β promoter stimulator-1
IRF3	IFN regulatory factor 3
ISGs	IFN stimulated genes
JAKs	Janus protein kinases

JUNV	Junin virus
L	Large segment of the arenavirus
L protein	RNA dependent RNA polymerase
LASF	Lassa hemorrhagic fever
LASV	Lassa virus
LCMV	Lymphocytic Choriomeningitis virus
LNS	Late neurological syndrome
MACV	Machupo virus
MEM	Modified eagles medium
MOI	Multiplicity of infection
MOPV	Mopeia virus
MTD	Mean time to death
NF-κB	Nuclear factor kappa B
NHP	Non-human primates
NP	Nucleoprotein
NSAID	National Institute of Allergy and Infectious Diseases
NWAs	New World arenaviruses
ORFs	Open Reading Frames
OWAs	Old World arenaviruses
P/S	Penicillin-Streptomycin Antibiotics
PACT	PKR activating protein
PAMPs	Pathogen associated molecular patterns
PCR	Polyermase chain reaction
p-eIF2α	Phosphorylated eukaryotic initiation factor 2
PFU	Plaque forming units
PKR	Protein kinase R
Poly I:C	Polyinosinic:polycytidylic acid
PPE	Personal protection equipment
p-PKR	Phosphorylated Protein kinase R
PRNT	Plaque reducing neutralization test
PRR	Pattern Recognition Receptor
RIG-I	Retinoic acid-inducible gene-I
rMACV	recombinant Machupo virus
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
S	Small segment of the arenavirus
SC	Sub-cutaneous
STAT	Signal transducer and activator of transcription

SSP	Stable Signal Peptide
ssRNA	Single Strand RNA
TCS	Tissue culture supernatant
TRAF-3	Tumor necrosis factor-receptor-associated factor-3
UTMB	University of Texas Medical Branch
UTR	Untranslated region
Z	RING finger protein

CHAPTER 1: INTRODUCTION

1

2   Machupo virus (MACV) is the etiological agent of Bolivian hemorrhagic fever

3 (BHF) (1, 2) and a member of the family *Arenaviridae* (3-9). Bolivian hemorrhagic fever

4 was first described in human patients in the Beni district of northeast Bolivia near the city

5 of San Joaquin during an outbreak that lasted from 1959 to 1963. A team of doctors from

6 the Middle American Research Unit (MARU), led by Dr. Karl Johnson, were the first

7 investigators to identify and characterize BHF in humans (10-12).

8   The prototypical strain of MACV, Carvallo, was isolated from the spleen of a 2

9 year old lethal human case. The spleen homogenate was used to infect newborn hamsters

10 and sick animals were euthanized and brain homogenate used for a second passage. The

11 strain Carvallo has two passages in hamsters (1, 13, 14). Current research with MACV is

12 limited; the virus is classified as a Center for Disease Control and Prevention (CDC)

13 Select Agent and National Institute of Allergy and Infectious Diseases (NIAID) category

14 A pathogen requiring a biosafety level (BSL)-4 laboratory for research within the United

15 States (15). With the reemergence of BHF in the Beni district and the construction of the

16 interoceanic highway along northern Bolivia, the public health threat to the region must

17 be addressed prior to another major outbreak.

18 **VIRUS GENOME**

19   Members of the *Arenaviridae* family are enveloped, bi-segmented, negative-sense

20   RNA viruses (16). The

21   virions are pleomorphic

22   when viewed by electron

23   microscopy and the name

24   A*renaviridae* is derived

25   from the 'sandy'

26   appearance caused by

27   cellular ribosomes found

28   within the virion (17). The

29   large (L) segment (~7.2kb)

30   encodes two viral proteins:

Figure 1: The Genome and Replication Strategy of MACV.
A) Graphical representation of the two segments of MACV. Each segment contains two genes in opposite orientation. The S segment encodes the NP and GPC while the L encodes the Z and L proteins. Each segment has a 5' and 3' untranslated region and each gene is separated by an IGR. B) Replication strategy for the S segment of MACV. The NP gene can be directly transcribed into mRNA by the L protein. NP mRNA is translated into protein. To generate GPC an antigenomic template is replicated by the L protein. This template is transcribed into GPC mRNA, translated into GPC, and post-translationally cleaved into GP1, GP2, and SSP

31   the RNA dependent RNA polymerase (L protein) (18, 19) and a RING finger protein (Z),

32   the arenavirus equivalent to a matrix protein (20-24). The small (S) segment (~3.3kb)

33   encodes two viral proteins: the viral glycoprotein precursor (GPC) and the nucleoprotein

34   (NP) (Fig. 1A). The GPC is post-translationally cleave d in two steps; 1) the cellular

35   signal peptidase cleaves GPC to generate the stable signal peptide (SSP) and 2) the SKI-

36   1/S1P subtilase cleaves the remainder into two glycoproteins, GP-1 and GP-2 (25-30).

37   The SSP is myristoylated following cleavage, and is necessary for the transport of the

38   GP-1/2 polypeptide from the endoplasmic reticulum to the golgi and for the targeted

39   trafficking of the GP-1 and 2 proteins to the cellular membrane prior to virion budding

40   (29, 31). The viral spike comprises a globular head formed by the GP-1 while GP-2 is

41   bound in the lipid bilayer of the cellular membrane anchoring GP-1 to the viral particle

42   (16, 32). NP is the most common viral protein produced during MACV infection and is

43   the primary structural protein in the viral nucleocapsid (16). The L protein of

44   arenaviruses has been shown to have a conserved N-terminal domain which is proposed

45   to have endonuclease activity allowing for 'cap-snatching' and ensuring cellular driven

46   cap-dependent translation of viral mRNAs (33).

47   Both the S and L segments utilize an ambisense encoding strategy with two open

48   reading frames (ORFs), one for each gene, in opposite directions (Fig. 1B). The ORFs of

49   both segments are separated by an intergenic region (IGR). The IGRs are predicted to

50   form secondary RNA structures,

51   which are necessary for terminating

52   transcription (34, 35). At each end of

53   the L and S segments are

54   untranslated regions (UTRs) of

55   which the terminal 17-19 nucleotides

56   are highly conserved within the

57   *Arenaviridae* family (16, 36, 37).

58   These conserved termini regions are

Cases of Bolivian hemorrhagic fever from 1959 to Present

	1959-1962 Outbreak	1963-1964 Outbreak	1965-1970	1970-1975	1975-1980	1980-1985	1985-1990	1990-1995	1995-2000	2000-2005	2005-2010	2010-present
Cases	470	514	34	12	0	0	0	9	0	0	200	131
Deaths	142	114	6	5	0	0	0	7	0	0	12	10
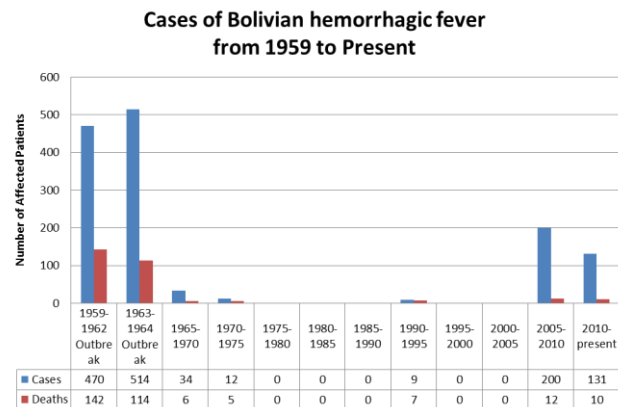
Figure 2: Reported Cases of BHF Since 1959.
A summary of the reported cases and deaths of BHF which have occurred since the original outbreak to present time (July, 2013). The reemergence of the disease is evident in the last few years with a drastic increase in reported cases since 2007. Copyright Current Opinions in Virology

3

59   reported to be vital in segment pan-handle formation for viral template replication and

60   transcription (16, 38, 39).

61   **GEOGRAPHIC DISTRIBUTION AND EPIDEMIOLOGY OF MACHUPO VIRUS**

62   The first outbreak of MACV was reported in Bolivia between 1959 and 1964.

63   Between 1976 and 1993 there were no reported cases of BHF, probably due to both the

implementation of rodent control measures in the populated urban areas and the under-reporting of disease within the region. A limited number of cases and deaths were reported in the mid-1990s including a familial outbreak resulting in 6 infections. Since 2006, there has been an increase in the cases reported compared to the previous decades, with a peak of reported cases in 2008 (1, 40-44) (Fig. 2).

Figure 3: Map of Bolivia.
A map identifying different important locations within Bolivia. 1) The city of Magdalena which was a site of a limited number of cases in 1994. 2) City of San Joaquin and surrounding areas, the site of the original 1959-1964 outbreak. 3) The city of San Roman where 3 cases of BHF were identified in 93 and 08. 4) The capital of Bolivia and highest population city of the country, La Paz. The yellow oval identifies the predicted range of the rodent reservoir of MACV while the green line identifies the location of the pan-oceanic highway. Copyright Current Opinions in Virology

During the 1959 outbreak, researchers identified *Calomys callosus* (2), the large vesper mouse, as the most likely natural

77   vector and reservoir for MACV. *C. callosus* has a wide natural geographical range

78   including portions of Bolivia, Brazil, Paraguay, and Argentina (45). While *C. callosus* are

4

79   found throughout many countries of South America, MACV is endemic within only a

80   small geographic region of Bolivia (Fig. 3). This region of endemic MACV corresponds

81   with the same geographic region in which a specific monophyletic linage of *C. callosus* is

82   found (46). The same phenomenon of a single rodent reservoir is reported with other

83   arenaviruses (47-50).

84   The infection rate of captured and necropsied *C. callosus* animals has ranged from

85   11% to 80% (2, 44, 51). Laboratory testing showed that nearly 100% of neonatal ($\leq$3

86   days) *C. callosus* challenged IP with MACV become persistently infected with

87   detectable viremia and continued to shed from the urine and saliva. The infected animals

88   had no detectable clinical disease and never developed neutralizing antibodies against

89   MACV (52). Contrary to the young animals, older (>2 weeks) animals challenged IP with

90   MACV developed two distinct responses to infection. One group was very similar to the

91   young animals except that they had a higher likelihood of anemia and reduced fertility

92   when compared to the infected neonate animals (14, 52, 53). The second group developed

93   neutralizing antibodies 4 weeks post infection. At the same time, the virus was cleared

94   from the blood it was no longer detectable in the urine and saliva.

95   The route of infection in humans is believed to be similar to other South

96   American hemorrhagic arenaviruses; through breathing in aerosolized excreta or secreta

97   from the rodent reservoir, consumption of contaminated food, or through direct mucus

98   membrane contact with infectious particles (12, 16). Nosocomial transmission has been

99   reported in BHF cases when family members visiting ill patients developed BHF (54,

100   55). Further evidence of human to human transmission occurred in 1971 when four

101   secondary cases of BHF were identified in hospital workers following close contact with

102   a patient suffering from BHF (56). Clinical evidence supports the nosocomial spread of

103   MACV, however, the epidemiologic evidence does not support this form of transmission

104   as a method for maintaining an epidemic (10).

105   In the first two to three years of the 1959 outbreak, most of the cases were in male

106   adults in the rural areas around San Joaquin. The high male case-rate is suspected to be

107   due to the high male-to-female ratio of individuals working in the fields. In 1962, an

108   increase in the number of urban cases was correlated to a decrease in the domestic feline

109   population (10), and to an increase in the rodent populations within the town. The drop in

110   feline population is suspected to have been caused by an over exposure to DDT and not

111   due to infection from MACV. Control of the outbreak was accomplished by 1965

112   following identification of the rodent reservoir and initiation of a systematic trapping of

113   rodents including the importation of a natural predator (12, 14). The cases reported in

114   1994 were also initially identified within a single family unit in which the primary case

115   was a rural worker (41); the most recent cases have been linked to rural/agricultural

116   activities as well (40). All of the recent reported cases of BHF have originated in the Beni

117   district of Bolivia (Fig. 3).

118

CLINICAL MANIFESTATIONS OF BOLIVIAN HEMORRHAGIC FEVER

120   Following aerosol exposure, arenavirus particles are likely engulfed by alveolar

121 macrophages leading to the

122 first cellular infection (57).

123 The incubation period for

124 BHF is 3 to 16 days

125 following exposure (58).

126 Previously, exposure was

127 believed to consistently lead

128 to clinical disease (10).

129 However, recent studies have

130 detected a number of people

131 with detectable IgG

132 antibodies against MACV
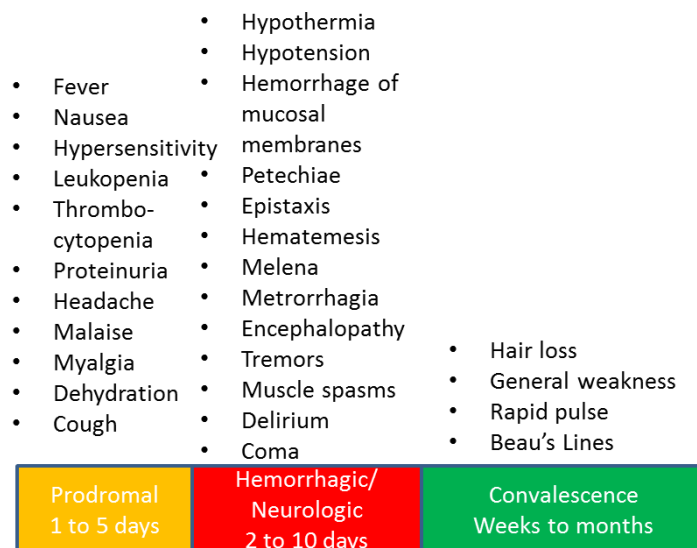
Figure 4: Clinical Disease Progression of Bolivian Hemorrhagic Fever in Humans.
A figure formed from a conglomeration of clinical reports identifying the most commonly reported clinical symptoms in humans infected with Bolivian hemorrhagic fever. Patients commonly present to local health authorities following development of severe symptoms which can develop in the late prodromal to early hemorrhagic/neurologic phases. Copyright Current Opinions in Virology

133 with no history of BHF (16). These unpublished samplings could imply a number of

134 possibilities: MACV might be less lethal than identified in the initial outbreak; MACV

135 might be more widespread than previously thought within the Beni district; or MACV

136 virulence has reduced since the original outbreak in the 1960s (16).

137   The prodromal phase of BHF is similar to that of Argentine hemorrhagic fever

138 (AHF) caused by Junin virus (JUNV), with the onset of fever, malaise, myalgia,

139 headache, and anorexia. This develops into severe symptoms including vomiting,

140 hypersensitivity to physical contact, and early signs of vascular damage. Laboratory

141 findings of clinical samples include leukopenia, thrombocytopenia, and proteinuria

142 during the prodromal phase (12, 16, 55, 58) (Fig. 4).

143   Approximately one third of patients progress into a neurologic or hemorrhagic

144 disease phase within a week of the prodromal phase. Symptoms include flushing of the

145 head and torso, petechiae, hypotension, epistaxis, hematemesis, melena, delirium,

146 convulsions, tremors, coma, and death (59). The cause of these neurological issues is

147 currently unknown. The case fatality rate varies between outbreaks of BHF but was

148 estimated to be around 25% during the initial 1959 to 1964 outbreak (16). While a late

149 neurological syndrome comparable to that reported with JUNV infected patients treated

150 with immune plasma, the neurological disease does appear to be more pronounced in

151 BHF patients (55, 60). If immune plasma treatment is initiated within the region, it is

152 possible cases of LNS will be observed at a comparable rate, 10%, to AHF (61). The

153 convalescent phase can last up to eight weeks and can include fatigue, dizziness and hair

154 loss. The effectiveness of immune serum treatment of non-human primates following

155 exposure to MACV implies clearance is mediated through a humoral immune response

156 (62). This is different from what has been identified in cases of Lassa Fever (LASF), a

157 representative of the Old World arenaviruses (OWAs), where a cellular response is

158 crucial for protection (63, 64).

8

159 **DIAGNOSIS, TREATMENT, AND CARE FOR BOLIVIAN HEMORRHAGIC FEVER**

160   Identification of MACV infection can be accomplished in the late stages of the

161 prodromal phase utilizing an enzyme linked immunosorbent assays (ELISAs) to identify

162 the presence of IgM and IgG antibodies to NP from collected serum or tissue (16).

163 Diagnostic reverse transcription polymerase chain reaction (RT-PCR) tests are also

164 available for quick and accurate identification of the presence of MACV RNA but the kit

165 and equipment is only available at larger hospitals and laboratories in Bolivia. Virus

166 isolated from the blood and tissue samples can be utilized to identify virus infection

167 however, there are several drawback including the length of time and required personal

168 protection equipment (PPE).

169   Currently, there are no Food and Drug Administration (FDA) approved vaccines

170 or therapeutics for BHF. During the 1959 outbreak, supportive care and proper

171 administration of fluids were the best known treatment options for patients in Bolivia

172 (55). Convalescent immune plasma from survivors was utilized in the case of four

173 infected researchers, all of whom recovered (65). Researchers have identified a dose-

174 dependent protection against MACV when rhesus monkeys were treated 4 HPI with

175 human immunoglobulin (62). However, no clinical trials have been completed in human

176 patients. In the same study 75% of infected primates treated with moderate to high (.5 to

177 1.5mL/kg of immunoglobulin) doses of immunoglobulin developed a chronic, late

178 neurological disease (62). All three treated primates that developed signs of neurological

179   impairment died weeks after clinical signs of acute BHF had abated (62). An additional

180   study in non-human primates (NHPs) identified a lethal chronic neurological disease in

181   rhesus monkeys in which six animals receiving convalescent serum succumbed to

182   neurological disease (66).

183   The efficacy of ribavirin, an antiviral therapeutic shown to be effective against

184   Lassa virus (LASV), has been used to treat two patients, both of whom recovered from

185   the disease (67, 68). While both patients recovered, it is impossible to determine if

186   ribavirin played a direct role in their recovery, expansion of clinical trials into the region

187   would be necessary. However, due to the limited number of reported cases in region, the

188   lack of infrastructure, and high costs, no clinical trials have been initiated (41, 67).

189   Preliminary reports also identified vaccination with Candid#1 (a vaccine against AHF) to

190   be protective in NHPs against MACV, but no further testing has been completed to

191   confirm these findings in humans (69). Recent studies in immunocompromised mice have

192   demonstrated a significant efficacy of ribavirin against MACV (70). The lack of clinical

193   infrastructure to support a national convalescent serum stock in Bolivia combined with no

194   proven effective therapeutics or vaccines against MACV will make controlling future

195   outbreaks of MACV difficult.

196

197

ANIMAL MODELS FOR MACHUPO VIRUS

199   Animal models have provided most of the information currently available on

200   MACV pathogenesis (Appendix I). Unlike other arenaviruses, rodent reduction programs

201   successfully controlled MACV from the 1970s to the early 1990s. The number of human

202   cases identified with LASV and JUNV has been important in developing a clearer picture

203   of disease progression and pathogenesis as well as providing key clinical isolates for

204   study within the laboratory. This has not been possible with MACV, making the early

205   NHP and other animal studies important in understanding BHF pathogenesis.

206   **Non-Human Primates:**

207   Four NHP species have been utilized in studying BHF disease pathogenesis.

208   Adult marmosets (*Saquinus geoffroyi)* have been shown to develop a lethal infection

209   following subcutaneous (SC) infection, scarified skin exposure, and corneal instillation,

210   but not through intranasal (IN) or oral administration of MACV (71). The time to death

211   in marmosets ranged from 11 to 21 days following SC infection and was dependent upon

212   infection dose. Virus was successfully isolated from the brains, spleens, kidneys, heart,

213   liver, saliva, and urine (1 sample) of animals that succumbed to disease (53). Clinical

214   signs, such as lethargy, weakness, and hypothermia appeared one to three days prior to

215   death.

11

216   Rhesus macaques *(Macaca mulatta)* have been shown to develop a lethal

217   infection following SC infection with MACV. Disease progression was described as bi-

218   phasic, similar to human disease. Two studies, both utilizing adult and young rhesus

219   macaques, identified clinical illness developing five to six days post infection (DPI).

220   Early symptoms included depression, fever, anorexia, diarrhea, facial rash, and

221   conjunctivitis. Disease progression continued in all macaques with severely ill animals

222   becoming moribund a day or two prior to death. In the first study, animals were infected

223   with either 10^5 or 10^3 plaque forming units (pfu) of MACV, and the mean time to death

224   (MTD) was 14.3 and 19.5 DPI respectively with a 100% case fatality rate (72). A second

225   report utilizing young (2.5-4kg) and adult (5-8kg) rhesus macaques resulted in mortality

226   rates of 85% and 50% following infection with 10^3 pfu (73). The MTD was similar as

227   with the first study. Survivors developed late neurological disease 26 to 41 days after

228   infection in which 66% of the surviving young macaques and all of the adult macaques

229   succumbed to disease (73). Histopathological examination of infected macaques

230   identified moderate to severe encephalitis with vasculitis and internal hemorrhage.

231   Following SC infection, 100% of cynomolgus monkeys (*Macaca fascicularis*) became

232   viremic at five DPI. Minimal clinical signs were identified in diseased animals when

233   compared to rhesus macaques. Infected cynomolgus animals had a reported 70%

234   mortality rate during the acute phase of the disease(73). The MTD was similar to that of

235   the rhesus monkey along with comparable LNS development in survivors of the initial

236   phase of the disease. Animals which developed LNS had a 50% mortality rate (73).

237   MACV disease progression has also been studied in the African green monkey

238   (*Cercopithecus aethiops*). Following SC infection, 100% of animal subjects succumbed

239   to MACV infection, 83% to the acute infection and 17% to late neurological development

240   (74). Histopathological samples taken at the time of death identified necrosis and

241   systemic hemorrhage in the kidneys, liver, and spleen of infected animals. Pneumonia

242   was also identified during necropsies in all of the infected African green monkeys. The

243   clinical development of disease was biphasic, similar to that of the rhesus monkeys, but

244   not cynomologus monkeys or adult marmosets (66, 72-74).

245   **Small Mammals:**

246   Adult small mammals have shown a strong resistance to MACV infection. Inbred

247   adult mice (BALB/C, C3H/HCN, AKR, DBA/2, C57BL/6) challenged by the intracranial

248   (IC) or intraperitoneal (IP) routes had no detectable viremia or illness but developed a

249   strong neutralizing antibody response shown by plaque reducing neutralization test

250   (PRNT) (53). Young and suckling inbred mice, less than two days old, develop a lethal

251   infection following challenge IP or IC but do not develop any hemorrhagic symptoms

252   comparable to BHF described in humans or NHPs (53, 71).

253   A report utilizing signal transducer and activator of transcription (STAT) -1

254   knockout mice described the development of lethal disease following IP (MTD = 7.3

255   days, 100% mortality), SC (MTD=10 days, 66% mortality), and IN (MTD=20, 25%

256   mortality). Virus was detected in the spleen, kidneys, serum, lung, and liver. Clinical

13

257 development of disease including ruffled fur, hunched back, awkward gait, and lethargy

258 were apparent at 5 DPI (70). (53).

259   An additional mouse model utilizing interferon αβ/γ receptor knockout (IFN-αβ/γ

260 R -/-) mice has been reported to develop a lethal disease following challenge with MACV

261 through an IP route of injection (75). Animals were challenged with either wild type

262 MACV or a recombinant MACV virus and were reported to develop two clinical phases

263 of disease. From around 10 to 14 DPI animals were reported to lose a significant percent

264 of body weight when compared to uninfected animals. Peak weight loss observed during

265 the acute phase occurs between 14 to 16 DPI. From this period until severe neurological

266 disease develops, animal bodyweight appears to stabilize, but rarely returns to baseline

267 levels. Starting at 22 DPI, animals developed neurological symptoms including ataxia,

268 rear limb-paralysis, and an awkward gait. One to three days prior to death, infected

269 animals had severe weight and body temperature loss with a MTD around 28 DPI.

270   Adult hamsters, when challenged IN or orally with MACV, did not develop

271 detectable illness. When infected through an IP or IC route at 1,000 pfu with MACV,

272 adult hamsters developed detectable viremia but no observable signs of illness.

273 Neutralizing and complement fixing antibodies are detected 30 days after IC and IP

274 challenge in hamsters (53, 71). Suckling hamsters (less than 6 days old) have been

275 reported to develop a lethal infection following challenge IP, IC, or IN but there are no

276 published reports of disease development or characterization in these animals (53, 71).

14

277   Both outbred (Hartley) and inbred (C-13) species of adult guinea pigs have been

278   reported to develop a lethal infection following challenge with MACV. The

279   characterization of disease development in either species has not been well reported (53,

280   56, 76). There are no reports utilizing young guinea pigs as an animal model. Other adult

281   animals that have been shown to develop a detectable neutralizing antibody response but

282   no disease are horses, cats, rats, and other outbred wild mice species (71).

283   **THE INNATE IMMUNE RESPONSE TO ARENAVIRUS INFECTION**

284   **The Interferon Response to Viral Infection**

285   The recognition of viral targets and activation of the innate immune response is

286   essential to control viral infection. Initiation of an innate immune response in cells is

287   through pattern recognition receptors (PRRs), which can bind to a large number of

288   pathogen associated molecular patterns (PAMPs) (77). Upon recognition of viral

289   infection, production of type-I interferons (IFN) α and β can be up-regulated leading to

290   the secretion of the IFNs (78-81). Three classes of PRRs associated with induction of

291   type I IFN response to viral infection are retinoic acid-inducible gene-I-like receptors

292   (RLRs), toll-like receptors (TLRs), and nucleotide oligomerization domain (NOD)-like

293   receptors (82-84). The RLRs are further subdivided into retinoic acid-inducible gene-I

294   (RIG-I), melanoma differentiation-associated gene 5 (MDA-5), and laboratory of

295   genetics and physiology 2 (LGP2) cytosolic helicases, all of which are capable of

296   recognizing unique facets of RNA associated with viral infection (77, 85-89).

15

297   Recognition by any of these three PRRs leads to activation and translocation into

298 the nucleus of interferon regulatory factors (IRFs) and nuclear factor κB (NF-κB) which

299 stimulate the production of IFN-α/β (84, 90-92). The PRR RIG-I can recognize single

300 stranded RNA (ssRNA) generated

301 during viral replication processes with

302 5'-triphosphates or short double strand

303 RNA (dsRNA) leading to activation of

304 IFN-β promoter stimulator-1 (IPS-1)

305 found on the mitochondrial membrane

306 (88, 93). IPS-1 acts as a binding

307 protein leading to the recruitment of

308 tumor necrosis factor-receptor-

309 associated factor-3 (TRAF-3) which

310 can further activate the

311 serine/threonine kinases IκB kinase

312 ε/TANK-binding kinase (IKKε/TBK)-

313 1 complex. Once activated,

314 IKKε/TBK-1phosphorylates IFN

315 regulatory factor (IRF) -3 and -7

316 which translocates into the nucleus to

317 initiate IFN expression (82, 92, 94-97).
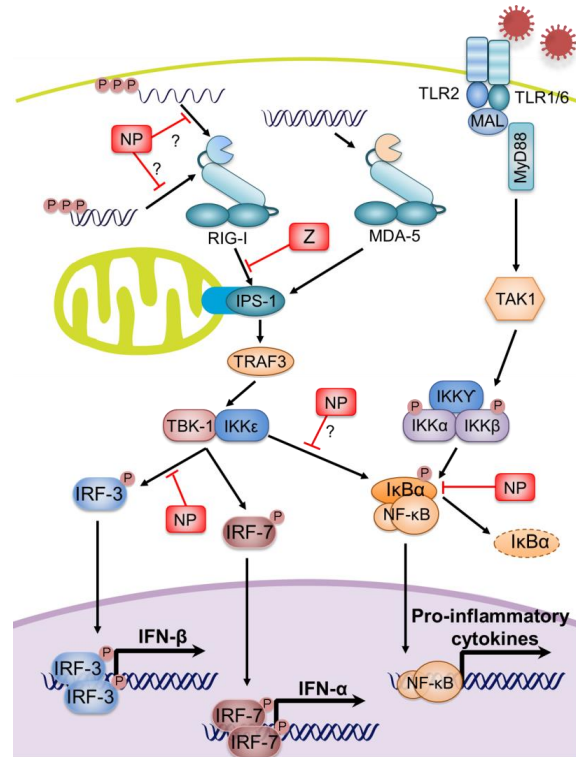
Figure 5: Proposed Mechanisms of Arenavirus Control of the Innate Immune Response.
Graphical representation of the reported mechanisms which arenaviruses utilize to control the innate immune response and induction of IFN. These mechanisms were primarily identified through plasmid expression of genes of utilizing of infectious arenaviruses other than JUNV and MACV. NP is proposed to have 3'to5' exonuclease activity able to degrade viral ds or ssRNA. Z of NW arenaviruses has been shown to interact with RIG-I inhibiting downstream signaling. NP is also shown to interact with IKKε inhibiting the phosphorylation of IRF-3 and -7. NP also inhibits activation of NF-κB. Image Copyright Journal of Molecular Biology

16

318   Upon IFN synthesis, the cytokine is secreted from the cell and binds the cell

319   surface IFN receptors(98). Activation of the IFN receptor leads to downstream activation

320   of the Janus protein kinases (JAKs) Tyk and Jak1 that induce the phosphorylation and

321   activation STAT-1/2, which leads to upregulation of many different IFN stimulated genes

322   (IGSs) and the establishment of an antiviral state in the stimulated cell (87, 99, 100).

323   **Modulation of the Innate Immune Response by Arenaviruses**

324   Reports of LASF describe very low levels of type I IFN, proinflammatory

325   cytokine production, T cell activation, and human dendritic cell activation from a number

326   of *in vitro*, *in vivo*, and clinical data (16, 101-107). In contrast to LASF suppression/lack

327   of activation of the innate immune response, JUNV has been reported to induce strong

328   levels of IFN (2000-64,000 IU/mL) and cytokine production in the serum of patients

329   suffering from AHF (108, 109). High levels of IFN have been linked to disease severity

330   and poor outcome (108, 109). The induction of IFN has also been reported in NHPs

331   infected with MACV or JUNV and from hamsters infected with Pichinde virus (PICV)

332   (110-112). *In vitro* studies have reported that macrophages and monocytes productively

333   infected with JUNV do not induce a cytokine response while infection of A549 cells

334   (human carcinoma epithelial cells) has been shown to induce IFN production through

335   RIG-I recognition resulting in upregulation of ISGs (113, 114).

336   In addition to IFN production and ISG synthesis, cells have the capability to

337   reduce biosynthesis in the cell as one step in developing an antiviral state in the cell with

17

338 the goal resisting viral infection. The protein kinase R (PKR), an ISG, is capable of

339 recognizing double-stranded RNA (dsRNA), which often is formed during viral

340 replication. Once bound to dsRNA, PKR autophosphorylates to become active (p-PKR).

341 Once activated, p-PKR can phosphorylate eukaryotic translation initiation factor 2

342 (eIF2α), an essential element for gene translation. Once eIF2α is phosphorylated, cap

343 dependent translation of genes is severely inhibited as the complex is no longer able

344 recycle bound guanosine diphosphate (100, 115).

345   In recent reports, plasmid driven expression of NP from lymphocytic

346 choriomengitis virus (LCMV), JUNV, PICV, MACV, and LASV inhibited the nuclear

347 translocation of IRF-3 in Vero cells infected with Sendai virus (116). NP has also been

348 shown to prevent the phosphorylation of IRF-3 by binding and blocking IKKε activity

349 (117). As a third proposed mechanism of preventing the establishment of an antiviral

350 state by NP, it has been shown that NP expression can inhibit the activation and

351 therefore, the translocation and transcription activity of nuclear factor kappa B (NF-κB)

352 (118). Both the GPC and NP of JUNV, expressed transiently in IFN incompetent Vero

353 cells, have been shown to inhibit the phosphorylation of eIF2α, inhibiting the

354 establishment of an antiviral state in the infected cell (119). Additionally, the plasmid-

355 expressed Z protein from New World arenaviruses (NWAs) but not OWAs has been

356 shown to bind RIG-I in A549 cells, inhibiting the induction of IFN production (120). This

357 evidence suggests that both OWAs and NWAs have multiple mechanisms of controlling

358 the innate immune response but the clinical evidence from AHF and LASF cases

359 suggests a marked difference on the impact of JUNV on IFN production then what is

360 reported in these *in vitro* studies (Fig. 5).

361 **PROPOSAL AIMS:**

362 This dissertation spans three aims regarding the generation and characterization of

363 a rMACV. Each aim and corresponding hypothesis is stated below:

364 <u>Specific Aim 1: Establish a reverse genetics system for the rescue of rMACV and</u>

365 <u>characterize the virus.</u> My hypothesis is that the rescue of a recombinant MACV can be

366 accomplished utilizing a Pol-I/II plasmid system. The rationale for this aim is based upon

367 previous research in the Paessler laboratory, which successfully rescued a recombinant

368 JUNV utilizing the Pol I/II promoter plasmid driven system.

369 <u>Specific Aim 2:</u> Characterization of the innate immune response to MACV

370 infection <u>*in vitro*.</u> My hypothesis is that infection by MACV will induce an innate

371 immune response comparable to that of JUNV. Previous reports in NHP models

372 identified the strong induction of IFN following challenge with MACV comparable to

373 that reported in JUNV clinical patients (111). There are no reported clinical findings of

374 IFN induction in human cases of BHF.

375 <u>Specific Aim 3: Rationally attenuate rMACV and characterize it *in vivo*.</u> The

376 hypothesis for this aim is that a mutation in the F437 amino acid of the transmembrane

377 region of GP2 will attenuate MACV neurovirulence in a mouse model. Previous work

378 has identified a mutation, F427I, in the transmembrane region of GP2 in Candid#1 as a

379 major determinant for attenuation for neurovirulence in mice (121, 122). This

380 transmembrane region is highly conserved in MACV.

381 **SIGNIFICANCE:**

382   The number of cases of Bolivian hemorrhagic fever has increased in recent years

383 (40, 42-44, 123). The increase in the number of at risk individuals living in endemic

384 regions, expansion of farming land, and mechanization of farming equipment are all

385 potential players in the reemergence of MACV. With the newly completed Transoceanic

386 highway there will be increased trade and travel via the southern portion of Bolivia which

387 increases the risk of disease spread and rodent host expansion to other regions of South

388 America. In addition, the continued threat of MACV as a biological terror threat due to

389 easy aerosol generation and high mortality rates makes MACV a viable threat not just to

390 the endemic Bolivian region but to surrounding countries and the United States.

391   While reverse genetic systems for other arenaviruses has been described, there

392 has been no development of a reverse genetics system for the study of MACV (38, 124-

393 127). Once established, it will provide a mechanism to generate genetically identical and

394 stable stocks of MACV that can be shared with other laboratories and increase the quality

395 and comparability of data coming from different sources. Additionally it will provide us

396 the tools to rationally modify the genome of the virus. This eliminates the need for

397 extensive passaging as was used to generate the attenuated strain of JUNV. Using this

398 tool and the knowledge gained from the attenuation of other arenaviruses we can generate

399 the first attenuated strain of MACV. Knowledge gained from this development can be

400 utilized for generating other attenuated arenaviruses, and may assist in the long term goal

401 of developing a pan-NWA vaccine.

402

403   With the completion of these aims this dissertation will describe the first

404 development of a minigenome system for MACV that can be used for replication studies

405 as well as the reverse genetics system to generate infectious MACV from cDNA. The

406 first reports will be generated on the in vitro immune response of human cells to MACV

407 infection, and a novel murine model to study MACV pathogenesis. Finally, to

408 demonstrate the use of the reverse genetics systems I will rationally modify MACV in an

409 attempt to attenuate the virus. The attenuation of the virus can be analyzed utilizing the

410 murine model characterized in the first aim of my dissertation.

CHAPTER 2: RESCUE OF RECOMBINANT MACHUPO VIRUS

INTRODUCTION:

Background

Reverse genetics systems have been developed for a number of different viruses in the past decades. The development of such systems allows the rational manipulation and modification of the viral genome without the inherent randomness of passaging virus in different cells or tissues to generate mutations. The first reported reverse genetic system was for the dsDNA simian virus 40 (128). Similarly, systems for rescuing positive ssRNA viruses such the bacteriophage Qβ, Sindbis virus, and Semliki forest virus. Reverse genetics systems were developed for these virus by generating viral cDNA, inserting the complete cDNA into a plasmid, and replicating it into infectious mRNA, either through *ex vitro* polymerase or transfection *in vitro* (129-131).

The development of such systems for segmented negative- or ambi- sense ssRNA viruses, such as arenaviruses, is more difficult. The first segmented negative sense ssRNA virus to be rescued completely from cDNA was Bunyamwera virus (132). The systems for negative sense ssRNA segmented viruses can be more complicated than systems for DNA or positive sense RNA viruses. These systems require expression

428 plasmids, transfected in trans, containing the genes for the viral proteins necessary for

429 virus transcription, replication, and sometimes packaging. Additionally, plasmids for

430 each segment must be generated and transfected for each segment of the viral genome.

431   To test the functionality and replication of these reverse genetic systems,

432 minigenome assays have been developed concurrent with many reverse genetics systems.

433 These assays replace the viral genes with genes encoding reporter proteins, such as GFP

434 and firefly luciferase, allowing researchers to study the replication processes of a virus

435 without generating virus. In the case of high containment pathogens, such as MACV,

436 minigenome assays allow researchers to study the life cycle of the virus in a lower

437 containment environment which can expand the number of researchers capable of

438 studying aspects of the virus while reducing the costs associated with high containment

439 pathogens. Additionally, these systems have provided insights and screening strategies

440 for identifying antivirals which can affect the replication of the target virus (16, 127, 133-

441 135).

442   The development of a reverse genetics system is a multistep process. A complete

443 sequence of the L and S segments of MACV is necessary to ensure the accurate

444 translation of viable viral proteins and RNA. As previously reported with JUNV, the

445 sequence of the 19 termini nucleotides at the 5' and 3' UTRs might be inaccurate at the 6

446 and 8 base pair position (38). With these inaccuracies, the rescue of a recombinant JUNV

447 was not possible (38). An essential step in the rescue of a rMACV is to confirm that the

23

448   viral sequence is identical to the published online sequence. The insertion of an L

449   segment into an expression plasmid has been notoriously difficult when establishing

450   reverse genetic systems for other arenaviruses. When inserted, the transfected competent

451   bacterial cells do not grow as efficiently nor is the insertion stable (Personal

452   communication Dr. Paessler, Alexey Seregin). In addition to full segment insertion, the

453   functionality of the RNA dependent RNA polymerase expressed by the expression

454   plasmid has been reported to be problematic (Personal communication Dr. Paessler,

455   Alexey Seregin).

456   The availability of a good animal model for MACV is limited. Adult inbred mice

457   are generally resistant to infection with MACV. Early reports identified suckling

458   hamsters and inbred mice as susceptible following IC challenge (1). Recently,

459   immunoincompetent STAT-1 knockout mice have been reported to develop an acute and

460   lethal infection following intraperitoneal (IP) challenge with MACV, highlighting the

461   importance of an intact IFN pathway in restricting MACV infection but this model did

462   not follow the disease progression which has been reported in BHF cases (70).

463   Guinea pigs have also been reported to succumb to MACV infection following IP

464   challenge but the disease development has not been well characterized (1, 56). Studies

465   utilizing 'chaired' NHPs, a method of restraining the NHPs for extended periods which

466   can induce high levels of stress on the animal, reported lethal disease development

467   following intradermal (ID), intramuscular (IM), and IN routes of infection (66, 72, 74,

468 136). As many of the NHPs utilized for these studies were wild caught, the extended

469 periods of constant restraint could lead to extreme stress in the animals. Interestingly,

470 African green monkeys, rhesus macaques, and cynomolgus monkeys developed a lethal

471 late neurological syndrome (LNS), a disease also reported in guinea pig models infected

472 with JUNV (137). In addition to the neurological involvement identified in serious cases

473 of BHF, both LASV and JUNV have been reported to cause different clinical forms of

474 neurological disease in humans and animal models (65, 138, 139). These clinical signs

475 range from muscle tremors and spasms to delirium, coma, and, in the case of LASF,

476 permanent deafness (55, 140,

477 141).

478 **Gaps in knowledge**

479   A reverse genetics

480 system for MACV has not

481 been established, nor has a

482 minigenome assay for

483 studying replication kinetics.

484 The lack of a small rodent

485 model, which mimics human

486 disease, also makes it very

Figure 6: Graphical representation of Six MACV Plasmids. The MACV S and L antigenomic cDNA inserts are shown (S segment AG and L segment AG, respectively) in the orientation of the full segment insertion into the plasmids. Additionally, four MACV MG reporter plasmids (S-fLuc-GFP, S-GFP-fLuc, L-fLuc-GFP and L-GFP-fLuc) were generated with fLuc and GFP genes replacing viral genes in the S and L segments while leaving the UTR and IGR in place. Copyright Journal of Virology

487 difficult to study MACV *in vivo* without the increased risk and cost of NHPs. These tools

488 are essential for moving forward in studying MACV virulence and the development of

489 antiviral countermeasures.

490 **Hypothesis**

491 Based upon previous research completed within my laboratory developing a

492 JUNV reverse genetics system, I hypothesize that the rescue of a recombinant MACV

493 can be accomplished utilizing a Pol-I/II plasmid system. With the completion of this

494 hypothesis, I expect three novel tools to be established which will further the field of

495 MACV research; a minigenome assay, a reverse genetics system, and a lethal animal

496 model.

497 **Significance**

498 First, the establishment of a reverse genetics system for MACV and subsequent

499 rescue of a rMACV will provide a powerful tool for future studies of the virus.

500 Traditional methods of attenuation would require extensive passaging in different animal

501 and cell types without any control over the mutations, which evolve. The reverse genetics

502 system will provide us the capability to study the genetic determinants of virulence. We

503 will be able to rationally modify entire genes down to specific amino acids. It will also

504 provide us with the ability to generate a genetically stable stock of virus from plasmids,

505 eliminating the need for additional passaging or long term storage of a Select Agent.

506   Second, a minigenome assay will provide us the ability to test the functionality of

507   the MACV NP and L, the proteins essential for viral genome replication, in expressing

508   reporter genes inserted in place of MACV genes in the full segment plasmids (Fig. 6). In

509   addition, the development of a minigenome system would provide a useful tool for

510   studying MACV replication mechanisms outside of the BSL-4 laboratory as has been

511   shown with other arenaviruses (38, 142-148).

512   Third, the modern *in vitro* characterization of MACV and the establishment of a

513   novel murine model would provide the field the knowledge and tools to further study

514   MACV biology. Much of the *in vitro* research characterizing MACV growth kinetics was

515   completed in the 1970s on cell lines, which may no longer be applicable or available.

516   Additionally, the development of a murine model which more closely follows the human

517   disease would be more cost effective, safer, and quicker than utilization of NHPs.

518

519   **METHODS:**

520   **Cells, viruses, and biosafety**

521   Baby hamster kidney (BHK-21) and Vero-CCL81 cells (American Tissue Culture

522   Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with

523   10% fetal calf serum and L-glutamine. The wild-type Carvallo strain of MACV

524   (Genbank accession no. JN794583.1 and JN794584.1) was obtained from Dr. Thomas G.

525   Ksiazek (University of Texas Medical Branch [UTMB]). Viral working stocks of the

526   wild type and recombinant viruses were generated by infecting Vero cells (multiplicity of

527   infection [MOI] =0.01 pfu/cell) and collecting virus containing tissue culture supernatant

528   (TCS) at 96 hours post infection (HPI). Cellular debris was eliminated from the TCS

529   through centrifugation and the viruses were concentrated and purified through Ultra

530   100K Filter Devices (Ultracell 100K centrifugation filter, Amicon) to remove cellular

531   factors, which may affect the immune response. The concentrate containing the virus was

532   diluted with Dulbecco's Modified Eagle Medium (DMEM, Life Technologies 11966-

533   025) containing 2% fetal bovine serum (FBS, Life Technologies 11966-025) to generate

534   a series of working stock aliquots of the virus. All work with infectious MACV and

535   rMACV was performed in the UTMB BSL-4 facility in accordance with institutional and

536   safety guidelines.

537   **Sequencing of Full Length S and L Genomic RNAs from MACV Carvallo**

538   RNA (0.5 to 1.0 mg) was isolated by a RNA purification kit (Zymo Research,

539   DNA-Free RNA kit, R1014) at 96 HPI from MACV-infected Vero cells. Viral cDNA

540   was synthesized by reverse transcription (RT) using either viral specific primers or

541   random primers. Viral specific primers complementary to S and L genome RNAs were

542   used to generate cDNA fragments of each segment. The entire S and L segments were

543   amplified in three and five DNA fragments, respectively, by PCR. PCR products were gel

544   purified (Zymo Research, Zymoclean Gel DNA recovery kit, D4001) and directly

28

545   sequenced to obtain the corresponding master sequences for the MACV S and L genome

546   RNAs. Sequencing data was analyzed using the program Clone Manager V9.

547   **Determination of 5' and 3' Termini of Both S and L Segments**

548   To determine the sequences of the 5' and 3' 19 terminal regions of the S and L

549   segments, the total RNA was isolated from Vero cells infected with MACV. RNA was

550   treated with RNA 5' Tobacco acid pyrophosphatase (Epicentre) and ligated using T4

551   RNA ligase as I previously described (38). The ligated RNA was reverse transcribed

552   utilizing the primers MACV_SsegR312 (5'-AGGGTGACTGACTGGAACTC-3'),

553   MACV_SsegF3129 (5'- GACATGAGCCTATCCACTTC-3') MACV_LsegR349 (5'-

554   TGTGATGGATGTCGGTAGTG-3'), and MACV_LsegF6917 (5'-

555   AGGCGTGTGCTTCACAGGAC-3') for the S and L segments, respectively. The cDNA

556   was used for amplification through PCR utilizing the same primers. Fragments were gel

557   purified and sequenced.

558   **MACV Minigenome Systems**

559   The plasmids expressing MACV L and S segment minigenome were generated

560   similarly as previously described (38). Briefly, viral genes on the pPol-I-MACVSag and

561   pPol-I-MACVLag template plasmids were replaced by the GFP and firefly Luciferase

562   (fLuc) reporter genes (Fig. 6). BHK-21 cells (6×10^4/well in a 12-well plate) were

563   transfected with 0.5µg of pPol-II-NP, 0.5µg of pPol-II-L, and 0.5µg of plasmid

29

564 expressing MACV L or S minigenome segments as indicated. At three days post

565 transfection, cellular lysate was collected and the bioluminescent signal was assayed

566 utilizing a luciferase reporter assay kit (Promega, E1500 or E1910) for luciferase

567 expression.

568 **Rescue of rMACV**

569 The rescue of rMACV was completed in a similar manner as described previously

570 by my laboratory (38). Briefly, equimolar amounts of the two full segment MACV

571 plasmids and the two expression plasmids were transfected into BHK21 cells.

572 Supernatant from these cells was collected at 4 days post transfection. A single passage in

573 Vero cells was performed to generate a higher titer stock of rMACV. The rMACV

574 sequence, including the introduced G1447A gene tag within the NP gene, was confirmed

575 by whole genomic sequence analysis.

576 **Plaque Titrations**

577 All plaque titrations were completed on Vero-CCL81 cell lines, seeded on 6- or

578 12-well plates roughly 16 hours prior to infection. Samples were serially diluted in

579 DMEM with 2% FBS and .5% P/S. Cells were infected with either 200μL or 100μl

580 respectively and incubated for ~1 hour with shaking every 15 minutes. Wells were then

581 overlayed with a 50:50 warmed mixture of 2% agarose in H2O and 2X modified eagles

582 medium (MEM) with 10% FBS and .5% Penicillin-Streptomycin Antibiotics (P/S, Life

583 Technologies, 10378016). The plates were incubated for 8 days post infection (DPI) and

584 fixed with 10% formalin solution. Cells were stained with crystal violet and plaques

585 counted to determine viral load. When calculating the viral load of tissue samples, tissues

586 were weighed prior to homogenization and media was added to the samples consistent to

587 the measured weight, a minimum of 300μL to a maximum of 1000μL. The volume and

588 organ weight were used to calculate viral loads per gram of tissue for each sample.

589 **Animal Experiments**

590 Six to eight week old interferon (IFN)-αβ/γ receptor double knockout (IFN-αβ/γ R

591 -/-) mice on a C57BL/6 background and wild type C57BL/6 mice were utilized for all

592 studies. All animals were housed in a pathogen free environment. All virus infections

593 were performed in the BSL-4 in the Galveston National Laboratory (GNL), UTMB. All

594 animal studies were reviewed and approved by the Institutional Animal Care and Use

595 Committee at UTMB and were conducted according to the National Institutes of Health

596 guidelines. Animals were anesthetized using an isoflurane precision variable-bypass

597 vaporizer prior to virus inoculation by the IP route with 10^4 PFU. Telemetric monitoring

598 of body temperature was accomplished throughout the studies. A BMDS IPTT-300

599 transponder (Bio Medic Data Systems, Inc.) was implanted subcutaneously using a trocar

600 needle assembly. Transponders were read with a DAS-6007 reader (Bio Medic Data

601 Systems, Inc.) and downloaded in accordance with manufacturer's protocol. Body weight

602 measurements were performed throughout the studies by anesthetizing the animals and

31

603   weighing them, weights were compared to baseline collected at 0 DPI (38, 149).

604   Scheduled euthanizations occurred at 14 and 14 DPI during the first study. The

605   experimental endpoints of the two studies were both approximately 40 DPI, where

606   surviving animals were humanely euthanized and necropsied.

607   **Histopathological and Immunohistochemical Analysis**

608   Tissue samples were fixed in 10% buffered formalin for a minimum of 4 days and

609   then transferred to 70% ethanol. Samples were embedded in paraffin and cut into 5μm

610   sections to be mounted on slides. Slides were subjected to standard hematoxylin and

611   eosin staining as described previously (38). Immunohistochemistry targeting MACV

612   antigen was accomplished as described previously by my laboratory (38). In brief, cut

613   sections were deparaffinized and rehydrated through xylene and graded ethanol solutions.

614   Endogenous peroxidase activity was blocked with a solution of Tris-buffered saline

615   containing 0.1% Tween 20, 3% hydrogen peroxide, and 0.03% sodium azide for 15 min,

616   followed by heat antigen retrieval in a water bath at 95°C for 40 min in Dako Target

617   Retrieval Solution, pH 6.1 (Dako Corporation). Endogenous biotin reactivity was blocked

618   through incubation with avidin D and biotin solutions (Vector Laboratories).

619   To detect MACV viral antigen, rabbit anti-peptide primary antibody targeting a

620   14 amino acid residue starting at amino acid residue 220 (KYPRLKKPTIWHKR, ProSci)

621   was utilized at a dilution of 1:500 and incubated on slides for 60 minutes. Tissue samples

622   from uninfected mice were utilized as a negative control. To prevent nonspecific protein

32

623 binding, sections were incubated in blocking solution according to the manufacturer's

624 instructions (Histomouse-SP kit; Zymed).

625 **Statistical Analysis**

626 GraphPad Prism v5 was utilized for all data analysis. To determine significance

627 in weight change, a two-way analysis of variance (ANOVA) test was performed

628 comparing a pooled group of infected wild type mice with both MACV and rMACV

629 infected IFN-$\alpha\beta/\gamma$ R -/-mice. Viral growth curve analysis was completed utilizing a two-

630 way ANOVA test comparing all virus and cell culture types over each day. A Kaplan

631 Meier survival curve was generated and statistical Mantel Cox test was completed to

632 determine significant differences in survival between MACV and rMACV infected

633 animals.

634 **RESULTS:**

635 **5' and 3' Terminal Sequences for Machupo Virus:**

636 I designed primers for the purpose of sequencing via PCR the MACV genome.

637 These primers were based on two full segment S and L sequences submitted to Genbank

638 (accession numbers JN794584, JN794583 and AY619643, AY619642). The terminal 19

639 nucleotides of the 5'- and 3'-ends of arenavirus genomic RNAs are highly conserved

640 and have been shown to be important for successful rescue of recombinant virus using

641 reverse genetics systems and the functionality of the arenavirus RNA polymerase (38, 39,

33

642 124). I isolated viral RNA from infected Vero cells at 96 HPI and subjected it to

643 sequence analysis by the UTMB Genomics Sequencing Facility. The MACV RNA

644 sequence was found identical to the published JN794583 and JN794584 sequences except

645 at the 5' and 3' ends of the UTRs.

Figure 7: Sequence of the MACV 19 Nucleotide 5' and 3' Genomic Termini.
The terminal 19 nucleotide sequences at the 5' and 3' ends of MACV genomic RNAs were determined in this study (experimental), which are identical to the terminal regions of JUNV. Highlighted are the nucleotide differences in two MACV isolates available from GenBank (accession numbers JN794584, JN794583 and AY619643, AY619642, respectively) Copyright Journal of Virology

646   Through RNA ligation and amplification through the region, I found the 19

647 nucleotide sequences at the 5' and 3' termini of the S and L segments identified in this

648 study were identical to that of JUNV (Fig. 7) (38). When compared to the published

649 sequences of MACV S segment (JN794584) and L segment (JN794583), I identified two

650 nucleotide differences at positions 6 and 8 at the 5'-end of the S segment and two

651 nucleotide differences at positions 6 and 8 from the 3'-end of the L segment (Fig. 7). I

652 further identified that there was one extra G present at the 5'-end of L segment for

653 JN794583 and one missing G at the 3'-end of the S segment for JN794584.

34

654   Comparisons of my sequencing results to other reported MACV sequences

655   (AY619643 and AY619642) demonstrated the exact same four nucleotide differences

656   (Fig. 7), which were also reported in the sequences of JUNV (GenBank accession

657   numbers AY619641 and AY619640) by other groups. These alternative nucleotides at the

658   6 and 8 positions were determined not

659   to be viable for the rescue of other

660   hemorrhagic arenaviruses (38, 124).

661   There were two additional nucleotide

662   differences at positions 6 and 8 at the

663   5'-end of L segment between

664   AY619642 and the sequence

665   determined herein (Fig. 7).

Figure 8: Silent Mutation in rMACV. Sequencing of the S segment plasmid and rMACV confirmed the single genetic marker introduced into the GP2 region at nucleotide 1407 (genomic sense) into the original plasmid. This marker allows me to distinguish MACV from rMACV. Copyright Journal of Virology

666   **Construction of Plasmids for MACV Reverse Genetic System**

667   To generate the pBSII-S vector plasmid containing the full-length antigenomic

668   sense MACV S segment, two fragments derived from the S segment with overlapping

669   restriction sites were amplified by PCR. The cDNA fragments were then digested and

670   ligated into the vector pBlueScript (pBSII). The vector pBSII-L plasmid containing L

671   segment was generated in a similar manner using three cDNA fragments. The last two

672   overlapping fragments of cDNA, from ~2400bp to 7200bp, were generated through PCR

673   amplification of the viral RNA. For the first portion of the L segment, I was unable to

35

674 generate a complete insertion of all 3 fragments into a vector plasmid. I ordered the

675 missing 2600 basepair fragment

676 to be synthesized and inserted

677 into a vector plasmid (Gene

678 Synthesis). Upon receiving the

679 synthesized fragment and

680 plasmid, I digested and ligated

681 the two other fragments to

682 generate a complete L segment

683 plasmid.

Figure 9: Bioluminescent Signal From Reporter Plasmids. Bioluminescent signal strength from four reporter plasmids transfected with NP and L expression plasmids into BHK cells. Transfections were completed in triplicate. Blue bars denote signal levels from homologous MACV NP and L along with the respective reporter plasmids. Red bars denote signal from reporter plasmids transfected with MACV NP and JUNV L. Green bars denote signal from reporter plasmids transfected with MACV NP and Candid#1 L. Bioluminescent signal from all transfections were stronger than that of the reporter plasmid transfected without the expression plasmids denoting transcription of fLuc occurred and the NP/L expression plasmids are functional. Copyright Journal of Virology

684 The two full segments

685 were digested from their vector

686 plasmids and inserted in antigenomic orientation into the murine Pol-I driven pRF42

687 mPol-I expressing plasmid, generating pPol-I-MACVSag and pPol-I-MACVLag

688 plasmids, which expressed the full length viral S segment genomic RNA and L segment

689 genomic RNA, respectively (Fig. 5). A silent G to A mutation at nucleotide 1407 within

690 the GPC gene was introduced into the S segment as a genetic marker for the recombinant

691 MACV (Fig. 8).

692 To express the MACV NP and L proteins in *trans*, MACV NP and L genes were

693 cloned into the Pol-II driven mammalian gene expression plasmid pTriEx-1 to generate
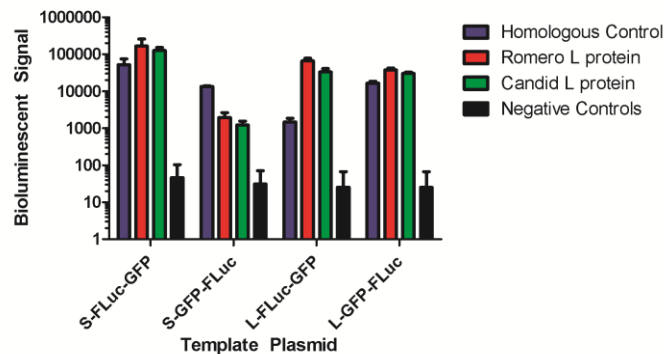
694   plasmids pPol-II-NP and pPol-II-L respectively. An RsrII restriction site was added to

695   the 5' end of primers corresponding to the ATG codon of each gene, and was utilized for

696   digestion and ligation of the gene segments into the plasmids. The L polymerase gene

697   was inserted into pTriEx-1 by three DNA fragment ligation (pPol-II-L) while the NP was

698   directly inserted as a single fragment (pPol-II-NP). Sequences of all plasmids were

699   confirmed by sequence analysis by the UTMB Molecular Sequencing Core.

700   **Development of the Machupo Virus Minigenome**

701   To confirm whether the 5' and 3' UTRs of MACV identified in this study were

702   functional for virus RNA replication and transcription, I developed a MACV minigenome
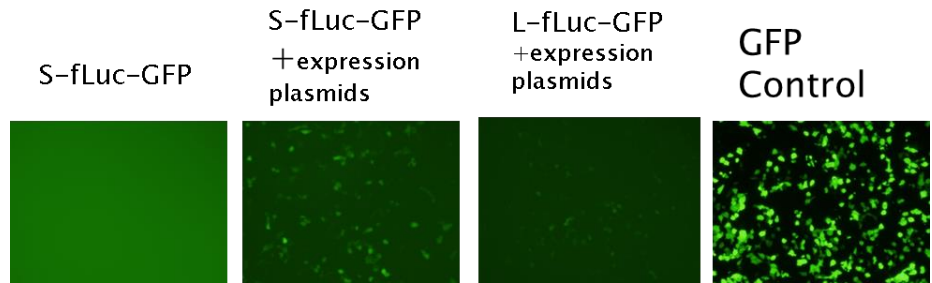
Figure 10: Image of Fluorescent Cells.
Photographs of BHK cells following transfection. Negative control cells were transfected with only the reporter plasmid. The second two slides present BHK cells transfected with the respective reporter plasmid, MACV NP expression plasmid, and MACV L expression plasmid. GFP control represents cells transfected with a GFP expressing plasmid.

703   system. Minigenome reporter plasmids were generated containing the full UTRs and

704   IGRs of MACV S and L segments with viral genes replaced by either fLuc or GFP

705   reporter genes (Fig. 6). Plasmids expressing MACV L and NP in *trans* were generated

706   by inserting viral NP and L protein genes into a Pol-II-based expression vector (pTriEx-1,

707   pPol-II-NP and pPol-II-L). Results from my minigenome experiment (n=3 for each

708   reporter plasmid) confirmed the homologous MACV L and NP provided in *trans* were

709   sufficient to support MACV minigenome RNA replication and transcription (Fig. 9). Cell

710   monolayers visualized under a fluorescent microscope also confirmed the synthesis of

711   GFP in the cells (Fig. 10). These results also confirmed the functionality of the UTR

712   regions I had identified previously. Minigenome-driven fLuc reporter gene expression

713   was substantially stronger for all minigenome constructs in the presence of NP and L

714   protein when compared with samples transfected with the minigenome reporter plasmids

715   only.

716   To further test the compatibility of

717   MACV minigenome template with

718   heterologous L proteins derived from

719   other NWAs, I tested the relative

720   efficiencies of the L protein of JUNV

MACV   **rMACV**

721   Romero and JUNV Candid #1 vaccine

Figure 11: Plaque Morphology of MACV and rMACV.

722   strain in supporting MACV minigenome

Infected Vero-CCL81 cells were fixed 8 DPI and stained with crystal violet. Size and shape of plaques for MACV and rMACV are similar providing

723   replication. I chose the two strains of

evidence that no phenotypic change has occurred in the rMACV. Copyright Journal of Virology

724   JUNV, Romero and Candid#1, as they are genotypically similar to MACV. Sequencing

725   comparison of published JUNV virus sequences (accession numbers AY619640,

726   AY619641, AY746353, and AY746354) to MACV (accession numbers JN794583 and

727   JN794584) identified a 69% and 72% nucleotide similarity for the L and S segments

728 respectively. Amino acid similarity between the virus segments were 73% and 87% for

729 the L and S segments.

730   Analysis of the MACV minigenome-driven fLuc expression confirmed that the L

731 protein of both JUNV and Candid#1 were compatible with MACV NP in supporting

732 MACV minigenome RNA replication and transcription (Fig. 9). These data clearly

733 showed that the L protein of JUNV could replace its MACV counterpart in the

734 minigenome systems, suggesting the feasibility of rational design of a modified rMACV

735 by introducing heterogeneous L gene from the attenuated JUNV into MACV genome.

736   **Rescue of Recombinant Machupo Virus and *In Vitro* Characterization**

737   To generate rMACV

738 from cloned cDNA plasmids, I

739 transfected BHK cells with

740 equimolar concentrations of

741 pPol-I-MACVSag, pPol-I-

742 MACVLag, pPol-II-NP, and

743 pPol-II-L plasmids. I collected

744 TCS at 96 hours post

Figure 12: Infection of A549 Cells With MACV and rMACV. A549 Cells were infected with MACV or rMACV at an MOI =0.01 or 1.0 and cellular supernatant was collected every day for four days. Growth and peak virus titers for both viruses at MOI =0.01 was nearly identical. A significant difference at 1 DPI (***P<.001, two-way ANOVA) and 2 DPI (* P<.05 two-way ANOVA) was observed in cells infected at an MOI=1.0. By 3 and 4 DPI no significant difference was observed. Copyright Journal of Virology

745 transfection and plaque titrations identified p0 of rMACV had a titer of 8×10^4 pfu/mL.

746 To generate a working stock with higher virus titer, I infected Vero cells at an MOI <

747 0.01. The low MOI was to avoid potential formation of defective interfering particles.

748 TCS were harvested at 96 HPI and purified according to manufacturer's protocol

749 (Millipore Amicon Ultra Centrifugal Filters 100K, UFC910096). Whole genomic RNA

750 sequence analysis of rMACV confirmed no additional mutations other than the genetic

751 marker introduced to the rMACV genome RNA (Fig. 8).

752 Plaques formed by both viruses on Vero cells at 8 DPI were similar in their

753 morphology and size (Fig. 11). TCS collected from rMACV and MACV infected IFN

754 competent A549 cells

755 demonstrated very similar

756 growth curve in at MOI=0.01.

757 A549 cells infected at an

758 MOI=1.0 with rMACV and

759 MACV had similar growth

760 pattern and final peak titer but

761 experienced a significant
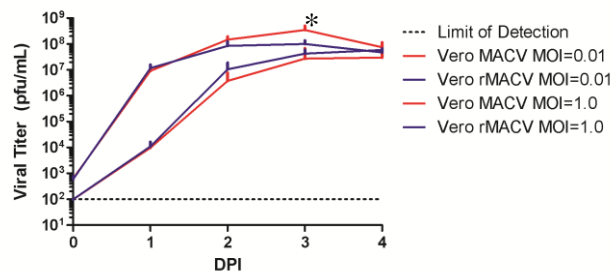
Figure 13: Infection of Vero Cells.
IFN incompetent Vero cells were infected with MACV or rMACV at MOI =0.01 or 1.0 and TCS was collected from 0 to 4 DPI. Cells infected at MOI =.01 had similar growth titers and peak throughout the days observed. TCS from cells infected at MOI=1.0 had a significant difference in viral load at 3 DPI (P<.05, two-way ANOVA) but comparable titers on other days collected. Copyright Journal of Virology

762 difference in titer at 1 and 2 DPI (two-way ANOVA) (Fig. 12). TCS from IFN

763 incompetent Vero-CCL81 cells infected at MOI=0.01 had similar growth and titers for all

764 four days observed while cells infected at MOI=1.0 had a significant difference (p<.05,

765 two-way ANOVA) at 3 DPI but had comparable growth titers at other days observed.

766 (Fig. 13), indicating the rescued rMACV replicated similarly as its parental wild type

767 MACV in cultured cells.

***In vivo* Characterization of Machupo Virus in IFN-αβ/γ R -/- Mice**

769   To examine and compare the disease development and pathogenesis caused by the

770   parental and recombinant MACV *in vivo*, I completed two experiments in which

771   C57BL/6 IFN-αβ/γ R -/- (n=25) and wild type C57BL/6 mice (n=10) were challenged IP

772   with 1×10^4 pfu of MACV or rMACV. Two IFN-αβ/γ R -/- mice were mock challenged

773   with PBS as a negative control. Changes in temperature and bodyweight were observed

774   throughout the study. From 10-15 DPI, significant weight loss (P value from <0.05, two-

775   way ANOVA) was identified in the MACV and rMACV infected IFN-αβ/γ R -/- mice

776   when compared to the infected wild type mice

777   and the uninfected control mice (Fig. 14). At

778   20 DPI, neurological impairment, including

779   partial paralysis, hunched posture, labored

780   breathing, and awkward gait, were observed in

781   IFN- αβ/γ R-/- mice infected with MACV and

782   rMACV. In contrast, the wild type mice

783   infected by either wild type or rMACV did not

784   exhibit any observable symptoms.

Figure 14: Change in Percent Bodyweight of Infected IFN-αβ/γ R -/- mice.
Mice were weighed throughout the study to track disease development. A significant differemce was observed between 10-15 DPI in infected -/- when compared to uninfected controls and infected wild type mice (two-way ANOVA, P value ranging from <.05 to <.001. No difference was observed between the two -/- groups infected with rMACV and MACV. Copyright Journal of Virology

785   From 1 to 20 DPI there was no

786   significant change in temperature observed

787   among any of the mice groups. Starting at 22 DPI the infected IFN-αβγ R-/- mice began

788 succumbing to disease (Fig. 15). Significant weight loss (Fig. 14) and temperature

789 decline (data not shown) were observed 1 to 3 days in all animals prior to death. The

790 mortality rate for both viruses was 93% with an average time to death of ~31 DPI when

791 pooling the data from both studies. There was no significant difference in the MTD

792 between the two viruses (p=0.16,

793 Log Rank test).

Survival curve of MACV and rMACV infected mice

794   Titrations of organ

795 homogenates confirmed similar

796 viral load between rMACV and

797 MACV in infected animals (P<.05,

798 Paired T-Test). A high viral load in

799 the CNS was observed by 24 DPI

800 (~10^8 pfu/gram) which was
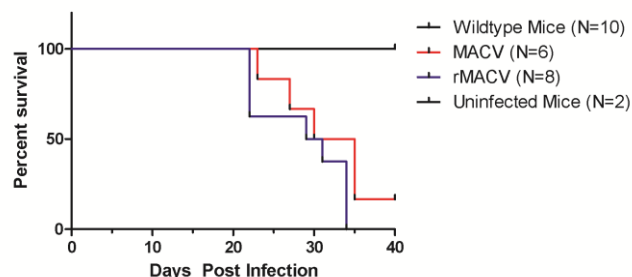
Figure 15: Kaplan Meier Curve of MACV and rMACV Infected Animals.
Graphical representation of survival data from two pooled studies utilizing IFN αβ/γ R -/- mice. Animals were challenged IP with 1x10^4 pfu of MACV or rMACV in 100µL of DPBS and observed for disease development. Animals were euthanized when they reached greater than 20% body weight or became moribund/paralyzed. No significant difference was observed in the time to death (P=.16, Mantel Cox test). One IFN αβ/γ R -/- mice survived challenge with MACV. Copyright Journal of Virology

801 maintained until death (Fig. 16, Appendix IV). The single surviving mouse infected with

802 MACV was euthanized at 40 DPI, and had no detectable viral load in the brain (data not

803 shown). Titrations of kidney homogenates identified a slight increase from 14 DPI to

804 clinical endpoint (Fig. 16, Appendix IV). Liver homogenates had a peak titer at 14 DPI

805 with a generalized downward trend until clinical endpoint (Fig. 16, Appendix IV). Lung

806 homogenates had a peak titer at 24 DPI (Fig. 16, Appendix IV). Spleen samples had a

807 peak titer at 14 DPI with a generalized decrease until clinical endpoint (Fig. 16, Appendix

808   IV). These samples were also most likely to have no viral titer when compared to other

809   organ homogenates. The animals were not perfused prior to necropsy which means blood

810   was collected along with all organs. As virus was identified at 14 and 24 DPI from serum

811   samples, some of these viral loads may be due to viremia and not organ infection as mice

812   were not perfused prior to necropsy (Fig. 16, Appendix IV). Titrations from the organ

813   homogenates of the wild type mice showed no except in a single brain sample from an

814   animal euthanized at 14 DPI confirming previous reports of the resistant nature of the

815   inbred adult mice to MACV (Data not shown). Organ viral loads from mice infected with

816   MACV or rMACV had similar titers (Appendix IV).

817   Histopathology analysis of tissues from infected IFN-αβ/γ R -/- mice

818   demonstrated increasing neuronal damage starting at 14 DPI up to death along with

819   minor vascular and perivascular mononuclear infiltrates in the cortex of the brain

820   (Appendix II). The spleens of IFN-αβ/γ R -/- mice infected with MACV or rMACV

821   showed prominent alterations in microarchitecture with an increase in white pulp volume

822   and expansion of the periarteriolar lymphoid sheath (Appendix 1). The spleen of the

823   uninfected IFN-αβ/γ R -/- mice showed normal white pulp architectures (Appendix II).

824   Immunohistochemical (IHC) staining of the same brain slides confirmed an increasing

825   amount of viral antigen from 14 DPI until death (Appendix III).

826

DISCUSSION:

827 **DISCUSSION:**

828   The sequencing of MACV has clearly shown that the terminal 19 nucleotide

829   sequences at the 5' and 3' UTRs are different from sequences of MACV previously

830   reported in the GenBank. Compared to my new data, the MACV sequences available

831   from GenBank (JN794584 and AY619643) have the C6A and G8U substitutions at the

832   5'-end of the S segment, which predict a perfect base pairing between the 5' and 3' ends

833   of the S segment. My new data suggested two mismatches at positions 6 and 8 in the

834   same region (Fig. 6), which is consistent with the sequences of other arenaviruses such a

835   JUNV, LASV, and Lujo virus (38, 124, 150, 151).

836   The terminal 19 nucleotides at the 5' and 3' ends of L and S genomic RNAs are

837   highly conserved among arenaviruses and play critical roles in viral RNA replication and

838   transcription. Although mutations in positions 6 and 8 are relatively better tolerated than

839   changes in other positions, a previous study using a LASV minireplicon system has

840   demonstrated the same C6A and G8U mutations at the 5'-end of S genomic RNA greatly

841   inhibit LASV viral gene expression by 60% and 90%, respectively (Fig. 17) (150). For

842   JUNV, RNA sequences with the same C6A and G8U substitutions at the 5' terminus of S

843   segment were reported in GenBank (AY619641) and were determined   to be non-

844   functional for the rescue of a recombinant JUNV virus (38).

845   My attempts to rescue MACV with UTRs different from our identified sequence

846   were unsuccessful, highlighting the critical role of these highly conserved sequences at

847   the 5' and 3' termini of viral genomic RNA

848   in viral replication (Data not shown).

849   Additionally, a previous study showed the

850   important role of the 3' terminal sequence of

851   the MACV S segment in MACV RNA

852   polymerase recruitment (39). The difficulty

853   in generating the entire L segment plasmid

854   has been reported in our laboratory for

855   MACV, JUNV, and LASV. The reason for

856   this difficulty is not yet understood. It is

857   possible the complete insertion plasmid is

Figure 16: Titration of Organ Samples.
Brain homogenate samples (red) show an increase in viral titer from 14 DPI. Kidney homogenate (blue) has a gradual decrease in titer from 14 DPI to death. Liver homogenate (green) had a peak titer at 14 and 24 DPI with a decrease at time of death. Lung homogenate (yellow) had a high viral load in a single animal at time of death. Spleen homogenate (purple) had a peak titer at 14 DPI with a decrease until time of death. Modified from an image copyrighted Journal of Virology

858   unstable or has a cytotoxic effect on the competent cell. Once I had the first fragment

859   generated and ligated the entire segment together into the plasmid, I did not identify a

860   loss of the segment within the plasmid. The extended incubation time to grow the

861   plasmid in competent cells when compared to the S segment leads me to believe the

862   insertion has a deleterious effect on the growth of the cell, and that this effect is localized

863   to first 2600 basepairs of the L segment. It is not known if this deleterious effect has an

864   influence in normal host infections.

865   The minigenome was utilized to confirm that the MACV L protein and NP were

866   sufficient to support efficient viral RNA transcription and replication of the MACV

867   minigenome genome. The establishment of a MACV derived minigenome will provide

868   an additional tool to study the molecular biology of new-world arenaviruses. Minigenome

869   assays of other arenaviruses have been proven as useful tools in dissecting the role of

870   viral proteins in arenavirus RNA replication and transcription, identifying the function of the IGR as the transcription terminating signal, studying the role of Z as a matrix protein, and mapping the cap snatching domain of L protein (143-148). The development of a minigenome for
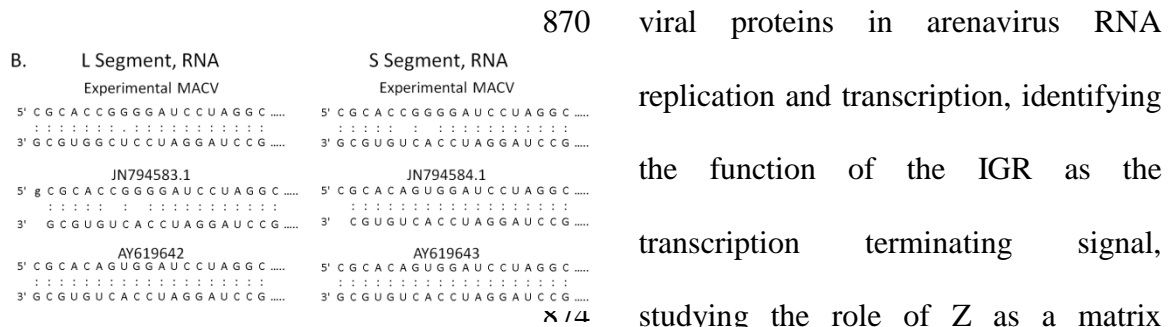
Figure 17: MACV Segment Termini Alignment. The alignment of the 19 bp alignment at the 5' and 3' termini ends of the S and L segment are vital for virus transcription. Sequencing of MACV confirmed a nearly 100% alignment in the L segment while the S segment had two distinct mismatch bps at the 6 and 8 position. When compared to published sequences online of the S segment of MACV, these mismatches are not found. Copyright Journal of Virology .

878   MACV could assist in studying the replication of this virus in the BSL-2 environment or

879   as a tool for testing antivirals as it has been shown for the Lassa minigenome assay (152).

880   My studies with the minigenome also provided preliminary evidence for the

881   compatibility of Romero and Candid#1 L protein with MACV NP for replication of the

882   MACV template. This evidence, in combination with availability of the reverse genetics

883   system, might allow for the generation of chimeric viruses containing genetic sequence

884   from both MACV and Candid#1. The generation of a Lassa/Mopeia reassortment virus as

46

885   a method of attenuation has been proven effective with OWAs, but never with NWAs

886   (153). The introduction of an attenuating sequence or the insertion of entire genes from

887   Candid#1 into the MACV backbone may allow for the rational generation of a live-

888   attenuated vaccine candidate for MACV.

889   My *in vitro* studies have proved similar growth patterns between wild type

890   MACV and rMACV. The similar growth and peak curves helps to confirm the two

891   viruses are phenotypically similar. Furthermore, *in vivo* studies provide additional

892   evidence that rMACV is similar to MACV. Challenge with rMACV and MACV led to

893   similar disease development, weight loss, neurological symptoms, and death in IFN-$\alpha\beta/\gamma$

894   R -/- mice. Viral invasion of the brain appears to have occurred in a similar manner in

895   both groups of mice with comparable peak viral loads. The findings in this study,

896   utilizing the IFN-$\alpha\beta/\gamma$ R -/- mice, identified the development of a biphasic disease with an

897   acute weight loss occurring 10 to 15 DPI and LNS between 20 to 34 DPI. Previously, a

898   lethal model of MACV infection was reported using STAT-1 -/- adult mice, which

899   succumbed rapidly to disease without developing LNS (70).

900   However, the biphasic disease described in my study is similar to some of the

901   reports of BHF in humans as well as in multiple species of NHPs experimentally infected

902   with MACV (53, 72-74). The neurological disease in the late stages of BHF in humans

903   can be correlated to the development of awkward gait, labored breathing, and partial

904   paralysis observed in my murine model (55, 154). Approximately 10% of AHF patients

905 treated with immune plasma develop a late neurological disease which has been lethal in

906 one reported case (61). In NHPs, the development of LNS has been reported in multiple

907 species with mortality near 100%, which is highly comparable to the IFN-αβ/γ R -/-

908 model reported here.

909   One of the most common complications in humans after infection with various

910 hemorrhagic arenaviruses, including Machupo and Junin virus, is the presence of

911 neurological disease, which can be lethal or result in transient or permanent neurological

912 sequelae (61, 140, 155). In addition, a portion of patients who have recovered from

913 LASF develop long term hearing loss showing a potential neurological impact of OWAs.

914 Further investigation into the pathogenesis of arenavirus neurologic disease may provide

915 key insights for multiple diseases. As the animal model I described here also progresses

916 into a lethal neurological disease, it may be an ideal tool for studying this aspect of

917 arenavirus disease compared to other rodent models.

918   The lack of disease development in the wild type C57BL/7 mice corresponds to

919 previously reported data. The gain in weight and the 100% survival rate when compared

920 to the IFN-αβ/γ R -/- mice suggests a potential and important role that the innate immune

921 response plays in BHF progression, which will need to be further elucidated. When

922 compared to the short but fatal disease that has been reported in STAT-1 -/- mice, with a

923 MTD of ~8 days, it may provide a better model for studying the neurological disease

924 which develops in human cases (70). Additionally, the impact of removing either the IFN

48

925 receptors or STAT-1, confirms the importance of the early innate immune response to

926 viral infection.

927   The experimental development of neurological disease for MACV has only been

928 described in NHPs so far, which are costly and not easy to handle in the BSL-4

929 laboratory. Utilizing mice instead of NHPs for neurological disease modeling has

930 multiple benefits. For example, many immunological and imaging tools are available for

931 further elucidation of neurological disease development, and working with mice in the

932 BSL-4 environment is considered to be safer, faster, and more cost effective than

933 research with NHPs.

934

935 **CHAPTER SUMMARY:**

936   My research has identified functional 19-nucleotide sequences at the 5'- and 3'-

937 ends of the MACV S and L genomic RNAs. I have generated a minigenome assay for

938 analyzing the replication and transcription of the S and L segments of the MACV

939 template. This new system was used to demonstrate the compatibility of L and NP

940 between MACV and two different Junin viruses. The establishment of a minigenome for

941 MACV provides a powerful tool for studying the replication kinetics and necessary

942 proteins for MACV replication within a BSL2 environment. It may also allow for future

943 studies investigating antivirals which can impact virus replication. I have successfully

944   rescued rMACV and have reported *in vitro* characterization of its growth in Vero and

945   A549 cells. I also report that MACV and rMACV cause similar disease development *in*

946   *vivo*. Following challenge in IFN-αβ/γ R -/-mice I have identified the first murine model

947   for MACV-induced neurological disease, which is similar to the severe disease reported

948   in human cases and NHPs studies. This model will be useful for future studies on the

949   virulence of MACV *in vivo*.

CHAPTER 3: CHARACTERIZATION OF THE INNATE IMMUNE RESPONSE TO MACV INFECTION *IN VITRO*.

INTRODUCTION:

Background

The innate immune response plays a vital role in early recognition and cellular response to viral infection. Recognition of PAMPs by PRRs initiates downstream pathways leading to the establishment of an antiviral state in infected cells (78, 84, 86, 98, 100). Many viruses have evolved mechanisms for evading this response; prevention of PRR detection, control of downstream pathways, and inhibition of immune modulator synthesis are just a few mechanisms that ensure the virus has the time and environment to replicate (78, 84, 156). The outcome of the back and forth interactions between a virus and the innate immune response can be a helpful predictor in how a disease develops and its severity.

Arenaviruses can cause severe disease through mechanisms not well elucidated. Both NWAs and OWAs have been reported to cause lethal hemorrhagic disease but the clinical presentation can vary drastically between the two complexes (5, 6, 8, 55, 71, 139-

51

966   141, 155, 157). OWAs, such as LASV and LCMV, are associated with low levels of IFN

967   while the NWA JUNV has been associated with extremely high levels of IFN in human

968   cases of AHF (108, 109, 139-141). In addition, a number of arenaviruses cause minimal

969   to no clinical disease in humans but are genotypically similar to those viruses that cause

970   hemorrhagic disease (158-160).

971   A comparison of these distinct viruses can be difficult, especially considering that

972   the research involving hemorrhagic viruses requires a high containment facility. Much of

973   what is known about arenaviruses, MACV in particular, was learned using less virulent

974   arenaviruses, such as LCMV, or through plasmid expressed viral proteins. Without live

975   virus infection modeling or analyzing the effect of a disease can be very difficult.

976   Utilization of these tools has provided a better understanding of the basic interactions

977   with a host system, and important insight into the human innate immune response.

978   However, utilization of these model systems could have provided results that do not

979   represent the entire arenavirus population, especially that of the hemorrhagic NWAs. The

980   expansion of national high security biosafety facilities provides us better opportunities to

981   study the virulent viruses and their pathological effect directly without requiring the

982   utilization of attenuated or non-virulent models.

983   Infection of dendritic cells and macrophages by LASV have been reported to have

984   low levels of type I IFNs and other proinflammatory cytokines corresponding to the

985   clinical description of LASF (105, 106). Arenaviruses have been reported to impact the

986   innate immune response in a number of different ways at different targets along the IFN

987   induction pathway. It has also been reported that LASV infected dendritic cells are

988   unable to activate CD4+ and CD8+ T cells efficiently (107). The NP of LCMV has been

989   shown to bind IRF3 interfering with IFN induction following coinfection with Sendai

990   virus (161). The NP from LCMV, LASV, JUNV, and MACV has been reported to

991   interfere with NF-κB translocation and initiation of gene transcription (118). The 3' to 5'

992   exonuclease activity of LASV and Tacaribe virus of plasmid expressed NP has also been

993   reported as a method of evading viral recognition by the cell (162, 163). The LCMV NP

994   and plasmid expressed NP from JUNV, MACV, LASV, Whitewater Arroyo virus, and

995   Latino virus have all been reported to inhibit IRF-3 phosphorylation and translocation

996   (116, 117, 164). The plasmid expressed Z protein from JUNV, MACV, and other NWAs

997   has been shown to bind with RIG-I, inhibiting signaling with downstream IPS-1 and

998   preventing IFN induction (120).

999   **Gaps in knowledge**

1000   The utilization of plasmid expression systems and arenaviruses other than

1001   hemorrhagic NWAs as prototypical models, has led to the formation of two dogmas

1002   within the field. The first dogma is that pathogenic arenaviruses evade the cellular

1003   immune response and do not induce IFN production. The second dogma is that infection

1004   with pathogenic arenaviruses does not lead to the phosphorylation of PKR or eIF2α. The

1005   data, which led to these dogmas, was acquired primarily through models and not through

53

1006 research with infectious hemorrhagic NWAs such as JUNV and MACV. These dogmas

1007 do not correspond with known clinical data from patients with AHF or animals infected

1008 with JUNV and MACV (111, 165). Further illumination of the impact of MACV

1009 infection on host protein biosynthesis and the corresponding innate immune response can

1010 be an important step in developing therapies and therapeutics that can decrease mortality

1011 rates during an outbreak. In addition, correctly categorizing JUNV and MACV as distinct

1012 modulators of the innate immune response from other arenaviruses is necessary to further

1013 progress within the research field.

1014 **Hypothesis**

1015 Based upon the reports of AHF, the data from animals infected with MACV or

1016 JUNV, and the data recently published from our laboratory on JUNV infection inducing

1017 IFN, I hypothesize that infection by MACV will induce an innate immune response

1018 comparable to that of JUNV. I expect my results to identify an uninhibited IFN induction

1019 and downstream signaling pathway in cells infected with MACV.

1020 **Significance**

1021 Characterization of the impact of MACV infection on the innate immune response

1022 will benefit the field of arenavirus research, as it will provide further evidence for the

1023 separation of pathogenic NWAs from other arenaviruses. It will provide further evidence

1024 that utilizing plasmids expression systems or nonpathogenic arenaviruses as prototypical

54

1025 models does not always provide accurate data. Finally, identification of how MACV

1026 impacts the innate immune response may provide key insights into developing antivirals

1027 which can prevent disease development.

1028 **METHODS:**

1029 **Cells, Viruses, and Biosafety**

1030 Baby hamster kidney (BHK-21) and Vero cells (American Tissue Culture

1031 Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with

1032 10% fetal calf serum and L-glutamine. The wild-type Carvallo strain of MACV

1033 (Genbank accession no. JN794583.1 and JN794584.1) was obtained from Dr. Thomas G.

1034 Ksiazek (University of Texas Medical Branch [UTMB]). Viral working stocks of the

1035 wild type and recombinant virus was generated by infecting Vero cells (multiplicity of

1036 infection [MOI] = 0.01 plaque forming unit (PFU)/cell) and collecting virus containing

1037 tissue culture supernatant (TCS) at 96 hours post infection (HPI). Cellular debris was

1038 eliminated from the TCS through centrifugation and the viruses were concentrated and

1039 purified through Ultra 100K Filter Devices (Ultracell 100K centrifugation filter, Amicon)

1040 to remove cellular factors, which may affect the immune response. All work with

1041 infectious MACV and rMACV was performed in the UTMB BSL-4 facility in

1042 accordance with institutional and safety guidelines.

1043

55

1044   **Knockdown of RIG-I in A549 Cells**

1045   A549 cells with a knockdown of RIG-I gene expression were provided by Dr.

1046   Tseng (UTMB) and the description on how they were generated can be found in a recent

1047   publication from our laboratory (114). In brief, ON-TARGET plus SMART pool siRNA

1048   targeting RIG-I or a Non-targeting Pool (Thermo Fisher Scientific Inc.), were transfected

1049   into A549 cells by electroporation Amaxa Cell Line Nucleofector Kit T (Lonza

1050   Walkersville, Inc.) according to manufacturer's protocols.

1051   **Plaque Titrations**

1052   All plaque titrations were completed on Vero-CCL81 cell lines, seeded on 12-well

1053   plates ~16 hours prior to infection. Samples were serially diluted in DMEM with 2% FBS

1054   and .5% P/S. Cells were infected with 100μl of diluent and incubated for ~1 hour with

1055   agitation every 15 minutes. Wells were then overlayed with a 50:50 warmed mixture of

1056   2% agarose in H2O and 2X MEM with 10% FBS and .5% P/S. The plates were incubated

1057   for 8 days post infection (DPI) and fixed with 10% formalin solution. Cells were stained

1058   with crystal violet and plaques were counted to determine viral load.

1059   **Western Blots and Antibodies:**

1060   Cellular lysate samples were collected by removing supernatant, washing the cells

1061   with PBS, and incubating the cells for ~3 minutes with sample lysate buffer (BioRad, 2x

1062   Laemmli Sample Buffer). Samples were frozen at -80˚C until they were removed from

1063   the BSL-4 following GNL sample removal guidelines. Samples were heated to 95°C for 5

1064   minutes prior to being loading onto the gel. Samples were resolved on 4-20% SDS-PAGE

1065   gels (Bio-Rad Precast gels, Catalog #4561093 and 4561096) using a Mini Trans-Blot

1066   Electrophoretic Transfer Cell apparatus (Bio-Rad, Catalog #170-3930). Samples were

1067   then transferred to PVDF membranes using a Trans-Blot SD Semi-Dry Transfer Cell

1068   apparatus (Bio-Rad, Catalog #170-3940) according to manufacturer's recommended

1069   protocol. Membranes were incubated overnight at 4°C with the primary antibody.

1070   Membranes were washed and the appropriate secondary antibody was incubated for 1

1071   hour at room temperature.

1072   Proteins were visualized using ECL Western blotting Detection Reagents (GE,

1073   NJ) according to the manufacturer's instruction. Primary antibodies used for Western

1074   blotting analysis were rabbit anti-phosphorylated STAT1 antibody (#9171, Cell

1075   Signaling), mouse anti-STAT-1 antibody (WH0006772M1, Sigma), rabbit anti-IRF 3

1076   antibody (ab76409, Abcam), rabbit anti-PKR (#3079, Cell Signaling), rabbit anti-

1077   phosphorylated PKR (#2283-1, Epitomics), rabbit anti-eIF2α (#9722, Cell Signaling),

1078   rabbit anti-phosphorylated eIF2α (#9721, Cell Signaling), monoclonal anti-Junin

1079   immunoglobulin G targeting NP of MACV (BEI Resources, NA05-AG12), and goat anti-

1080   human b actin antibody (sc-1616, Santa Cruz Biotechnology). Secondary antibodies used

1081   were HRP conjugated goat anti-rabbit IgG (#7074, Cell Signaling), HRP conjugated Goat

1082   anti-mouse IgG (115-035-146, Jackson Immunology) and HRP-conjugated donkey anti-

57

1083 goat IgG (sc-2020, Santa Cruz). These methods are described in previous publications

1084 from our laboratory (114).

1085 **Statistics:**

1086 GraphPad Prism V5.0 was used for all statistical calculations. Viral growth curve

1087 analysis was completed utilizing a two-way ANOVA test comparing all virus and cell

1088 culture TCS over each day.

1089 **RESULTS:**

1090 **Effect of RIG-I Knockdown on MACV Growth**

1091 The cytoplasmic PRR

1092 RIG-I plays a pivotal role in

1093 recognizing JUNV infection and

1094 initiating induction of IFN

1095 production (114). To identify if

1096 RIG-I also has an effect on

1097 MACV infection, I seeded

1098 twelve well plates ~16 hours

Figure 18: Effect of RIG-I Knockdown on MACV Growth in A549 Cells.
The TCS from A549 cells, either K/D or control transfected, infected at an MOI=0.01 with MACV was collected for 4 DPI. While virus growth followed a similar pattern a significant difference was observed at 1, 2 and 4 DPI (P<.001, <.01, <.01 respectively, two-way ANOVA). Cell monolayer by 4 DPI was badly damaged in wells infected with both viruses.

1099 prior to infection with either A549 Control cells, cells containing an inserted Non-

1100 Targeting siRNA, or A549 RIG-I-K/D cells with siRNA targeting RIG-I. I infected cells

1101 with MACV (MOI=0.01) and collected TCS at 0, 24, 48, 72, and 96 HPI. Titration of
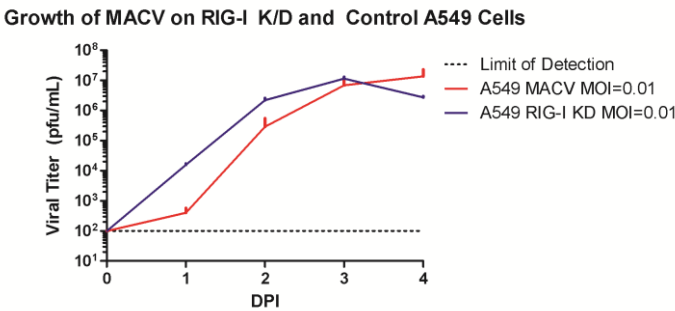
58

1102   samples confirmed significant impact on virus growth at 1(P<.001), 2(P<.01), and

1103   4(P<.01) DPI (two-way ANOVA) (Fig. 18).

1104   **Effect of Infection on IFN Competent Cells**

1105   To elucidate the impact of MACV infection on IFN competent A549 cells, I

1106   infected A549 cells with MACV (MOI=0.01) and collected cellular lysate at 24, 48, and

1107   72 HPI. Uninfected control A549 samples

1108   were collected concurrently with infected

1109   lysates for the Western blot analysis.

1110   Phosphorylation of STAT-1 (pSTAT-1)

1111   occurs following induction of interferon

1112   production, which activates JAK leading to

1113   phosphorylation of STAT-1. In the case of

1114   type I IFNs, pSTAT-1 complexes with

1115   pSTAT-2 and IRF-9. The complex

1116   translocates into the nucleus, binding to

1117   interferon stimulated response element and

1118   inducing transcription of ISGs, such as

1119   ISG15 and STAT-1. Cellular lysate samples

1120   were collected from IFN competent A549

Figure 19: Activation of the JAK/STAT Pathway in IFN Competent A549 Cells Infected With MACV. A549 cells were mock-infected or infected with MACV at MOI=1.0. Cellular lysates were prepared and resolved by Western blot. Analysis identified increasing levels of the ISG STAT-1 for all time points resolved when compared to uninfected controls. The presence of pSTAT-1 was confirmed in all infected cellular lysates with a peak at 48 HPI. ISG15 production was also confirmed with a peak at 48 HPI. A positive control of cells infected with Candid#1 was utilized as it has been previously shown to induce IFN production in A549 cells within my laboratory.

1121   cells at 24, 48, and 72 HPI and a notable difference was detected in protein concentration

1122   of pSTAT-1 when compared to uninfected A549 cells. The increase in pSTAT-1

1123   provides direct evidence of type I IFN signaling (Fig. 19) with high levels of pSTAT-1

1124   when compared to uninfected controls through 72 HPI.

1125   To provide further evidence of type I IFN synthesis, I analyzed downstream

1126   protein production linked to IFN signaling, specifically interferon stimulated genes

1127   (ISGs). I detected an increase in STAT-1 concentration when compared to the controls in

1128   all time points collected with a peak

1129   concentration at 48 HPI (Fig. 19).

1130   Additionally, total ISG15 concentration was

1131   increased when compared to control samples

1132   with a peak at 48 HPI (Fig. 19). To confirm

1133   that MACV had generated a viable infection

1134   in the A549 cells, I utilized an anti-NP

1135   MACV/JUNV monoclonal antibody to detect

1136   production of this viral protein. As a loading

1137   control, I analyzed concentration levels of

1138   actin in all samples. My positive control was

1139   a cellular lysate sample of A549 cells

1140   infected with Candid#1 collected at 48 HPI

1141   (Fig. 19).
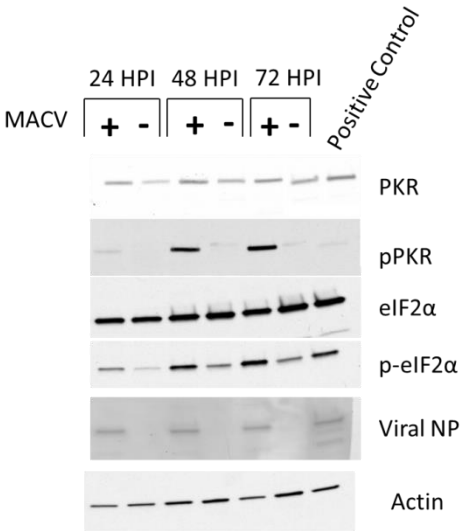
Figure 20: Recognition of MACV Infection by PKR in A549 Cells.
A549 cells were mock-infected or infected with MACV at MOI=1.0. Cellular lysates were prepared and resolved by Western blot. Analysis identified increasing and higher levels of the ISG PKR when compared to mock-infected cells. Resolution also identified high levels of peIF2α when compared to mock infected cells. Interestingly A549 cells infected with Candid#1 as a positive control experienced increased levels of peIF2α but not pPKR.

1142 **Effect of MACV Infection on Cellular Protein Biosynthesis**

1143   To investigate the impact of MACV infection on cellular protein biosynthesis, I

1144 looked at phosphorylation of PKR and eIF2α. Samples collected from IFN competent

1145 A549 cells at 24, 48, and 72 hours were resolved by Western blot. I detected an increase

1146 in PKR and phosphorylated PKR (p-PKR) concentrations starting at 24 HPI and peaking

1147 at 72 HPI (Fig. 20). I identified no discernable change in eIF2α levels at any time point

1148 between infected or uninfected cells (Fig. 20). Levels of phosphorylated eIF2α (p-eIF2α)

1149 in infected cells were detected at all time points collected. Uninfected cells also showed

1150 an increasing level of p-eIF2α but with markedly lower concentrations than infected cells

1151 (Fig. 20).

1152 **DISCUSSION:**

1153   The paucity of clinical data from patients infected with MACV has made it very

1154 difficult to describe the host innate immune response. Unlike JUNV, where high levels of

1155 IFN in clinical cases has been well described, the only published data relevant to MACV

1156 and IFN production is a NHP model which investigated the potential of Polyinosinic:

1157 polycytidylic acid (poly I:C), a synthetic dsRNA analog and toll-like receptor 3 agonist,

1158 treatment (108, 111). This study reported detectable IFN by 3 to 5 DPI with a peak level

1159 of around 700 units of IFN in infected untreated animals. Stephen *et al.* also showed that

1160 treatment with poly I:C lead to earlier detection of viremia and higher titers in treated

1161 NHPs (111). There was no significant difference in late stage disease, or a decrease in

1162   mortality rate when compared to untreated animals. These published results correspond to

1163   my *in vitro* data presented in Chapter 2 in which peak titer of MACV in IFN competent

1164   cells was not significantly affected, as seen with MACV growth in IFN incompetent Vero

1165   cells. While a significant difference in the virus growth curve was identified at 1 DPI, no

1166   significance was identified at any other time point collected (Fig. 18).

1167   While no clinical data exists from MACV infected patients, recent studies

1168   utilizing plasmid over expression of MACV or JUNV NP *in vitro* identified an inhibition

1169   of IRF-3 translocation (116). In contrast, recent work by Cheng *et al.* reported strong IFN

1170   production in cells infected with JUNV (114). My data further supports the distinction

1171   between NWA vs OWA and infectious virus vs plasmid protein expression.

1172   My results support that MACV infection in IFN competent cells induces

1173   upregulation of interferon production leading to expression of ISGs (Fig. 19). Previous

1174   publications that reported an inhibition of IFN induction in Vero cells could be correct as

1175   Vero cells are IFN deficient (166-168). Additionally, overexpression of NP by plasmid

1176   instead of through viral replication, as was used in these studies, could result in higher

1177   concentrations of NP than what is seen during an infection. The higher levels of NP may

1178   exaggerate the proteins capability to control the innate immune response. While clinical

1179   data and *in vitro* data supports the rational that low levels of IFN are induced during

1180   LASV infection, reports human cases of AHF identify high levels of endogenous IFN-α

1181   (109). Due to the variances reported in human cases of AHF and LASF, a case can be

1182 made that a distinction between NWAs and OWAs should be made, especially as it

1183 relates to induction of innate immune response in human cells. However, considering the

1184 extended incubation period of both MACV and JUNV, some mechanism of controlling

1185 the innate immune response is reasonable and must be addressed.

1186   A correlation between high levels of IFN with severe outcome in patients with

1187 AHF has been reported (109). My data also clearly demonstrates that the infection with

1188 MACV can affect cellular protein biosynthesis, which is in contrast with the model of

1189 LCMV cell infection. When compared to the extended incubations and persistent

1190 infections, which are reported in JUNV, MACV, and LCMV, this data is intriguing.

1191 Many viruses that cause persistent infection have evolved mechanisms to avoid

1192 impacting cellular protein biosynthesis (156). Infection by LCMV does not induce eIF2α

1193 phosphorylation in Huh 7 cells, but does lead to disassociation of BiP from ATF6, an

1194 essential mediator of endoplasmic reticulum (ER) stress (169). This disassociation

1195 commonly occurs in the presence of high concentrations of misfolded proteins (16, 169,

1196 170). The paper by Pasqual *et al.* also reported that the disassociation of ATF6 played an

1197 important role in viral titers during acute but not persistent infection of LCMV.

1198   Further investigations on the effect of MACV on the innate immune response can

1199 follow a number of paths. First, the cytoplasmic PRR responsible for recognizing MACV

1200 infection must be confirmed. While MACV is very similar to JUNV and it is reasonable

63

1201   to assume that the RIG-I is the primary PRR for recognizing MACV, it must be

1202   confirmed.

1203   The next step to further elucidate the impact of MACV and mechanism of

1204   immune modulation on cell protein biosynthesis is to identify activation of PKR. There

1205   are multiple mechanisms for PKR activation; recognition of dsRNA, interaction with the

1206   protein PACT (PKR activating protein), or overexpression of ISG15, all of which can

1207   lead to phosphorylation of PKR (171, 172). The primary method of activation of PKR is

1208   recognition of viral dsRNA, which is believed to be generated during the viral replication

1209   cycle. Interestingly, arenaviruses have been reported to induce discrete cytosolic

1210   structures where replication can take place potentially sequestering any dsRNA

1211   intermediates from PKR recognition (173). While PACT has been shown to activate

1212   PKR, it also has been shown that this activation does not play a role in an antiviral

1213   response (174). As my results confirm upregulation of ISG15 following infection, I

1214   would propose investigating if it plays a role in PKR activation. This can be

1215   accomplished utilizing the siRNA-transfected cell line targeting the PRRs described

1216   previously or utilizing siRNA targeting ISG15 specifically. If PKR activation continues

1217   without expressed ISG15, it would be reasonable to assume dsRNA is the primary

1218   instigator of activation.

1219   In addition to reports of LCMV induction of ER stress through ATF6, and in

1220   contrast to previous reports of negligible eIF2α phosphorylation, my data supports that

1221   MACV infection leads to strong phosphorylation of eIF2α in IFN competent cells. My

1222   data suggests the phosphorylation of eIF2α does not affect virus growth. While inhibition

1223   of PKR activation by viral proteins is well described, descriptions of how viruses

1224   overcome phosphorylation of eIF2α appears to be less common. Poliovirus has been

1225   shown to cleave eIF5B to rescue biosynthesis through an eIF2α independent mechanism

1226   (175). Alternatively, human T-cell leukemia virus type 1 has been shown to upregulate

1227   and stabilize the suppressor of cytokine signaling (SOCS) 1 that acts as a negative

1228   regulator of JAK/STAT signaling (176). MACV infection of A549 cells causes the

1229   phosphorylation of eIF2α, yet the cell continues to produce infectious virus. The

1230   mechanism of virus replication must be identified as it appears MACV can still grow in

1231   cells with by phosphorylated eIF2α.

1232   Interestingly, the Western blot data (Fig. 19 and 20) also confirmed there was no

1233   significant change in concentration of actin in the cellular lysate, even though eIF2α had

1234   been phosphorylated for more than 48 HPI. Further investigation is necessary to

1235   determine if MACV is capable of restoring cell protein biosynthesis completely. This

1236   could be accomplished utilizing radiolabeled S^{35} or P^{32} in a Pulse-Chase experiment

1237   model in future experiments. A variant of pulse-chase experimentation, utilizing mass

1238   spectrometry with radio labeled amino acids, could also be utilized to identify a change in

1239   whole cell protein synthesis (177). This methodology would allow us to determine at

1240 different time points when synthesis is being impacted and if there is a specific period at

1241 which MACV is able to overcome eIF2α phosphorylation.

1242 **CHAPTER SUMMARY:**

1243   In this chapter I present data demonstrating that the MACV infection can induce

1244 an innate immune response in IFN competent cells. I showed that STAT-1

1245 phosphorylation and ISG upregulation occurs within 1 DPI in A549 cells. Finally, I

1246 showed that both PKR and eIF2α are phosphorylated, which commonly occurs in cells

1247 entering an antiviral state leading to downregulation of cellular protein biosynthesis. This

1248 data contradicts commonly held dogma of arenavirus innate immune evasion and their

1249 impact on cellular biosynthesis. These dogmas have been established utilizing plasmid

1250 expression systems or nonpathogenic NWAs. My data is similar to IFN induction in

1251 A549 cells following JUNV infection and corresponds with clinical samples of IFN

1252 induction in patients with AHF. This data is significant as it provides evidence that

1253 nonpathogenic models and expression systems may not be accurate representations of

1254 hemorrhagic NWAs and this must be considered for all future research within the

1255 arenavirus field. Additionally, further elucidation of how cells detect MACV and the

1256 mechanisms of evading biosynthesis shutdown MACV utilizes are necessary to form a

1257 more complete description of BHF, and to potentially develop novel therapeutics.

1258   # CHAPTER 4: RATIONAL ATTENUATION OF MACV*

1259   **INTRODUCTION:**

1260   **Background**

1261   With the reemergence of MACV in Bolivia and the lack of proven

1262   countermeasures, additional research must investigate methods of controlling future

1263   outbreaks. Transfusion of immune plasma has proven effective in preventing lethal AHF,

1264   if administered within eight days of disease development (65). Successful treatment

1265   through transfusion of immune plasma has been reported in a limited number of BHF

1266   cases, but there have been no clinical trials to confirm efficacy (55). Utilization of

1267   plasma requires rapid recognition of disease, available health professionals in the region,

1268   consistent collection and storage of the plasma, and the infrastructure to support these

1269   endeavors. This would be potentially difficult to achieve in the remote Beni district of

1270   Bolivia (178, 179). Preliminary studies utilizing ribavirin have had positive results in

1271   cases of both BHF and AHF, but there has been no clinical efficacy testing (67, 180,

1272   181). With the limitations of infrastructure and cost of therapeutics, a protective vaccine

1273   may prove the most effective method of preventing further outbreaks of BHF, as has been

1274   reported with Candid#1 in Argentina.

*The work and figures covered in this chapter will soon be submitted as a
manuscript to the Journal of Virology.

1275   The generation of a novel arenavirus vaccine candidate through reassortment has

1276   been reported for LASV (182, 183). The novel virus, ML29, contains the L segment of

1277   Mopeia virus (MOPV) and the S segment of LASV. It was generated through co-

1278   infection of Vero cells with both LASV and MOPV and was selected following

1279   identification of plaque phenotype change in cell culture. ML29 protects against lethal

1280   LASV challenge in both guinea pig and NHP animal models providing strong evidence

1281   for its potential as a vaccine candidate (153). However, the mechanism of reassortment

1282   and selection brings up questions of stock purity and safety. One risk factor is that

1283   through   the

1284   reassortment

1285   process,   it   is

Figure 21: Passage History of Candid#1.
Candid#1 has had 65 passages in different cells. The F437I mutation occurred in the last

1286   possible a small   19 passages in FRhL cells. GP-Guinea Pig, MB-Mouse Brain, FRhL- Fetal rhesus lung
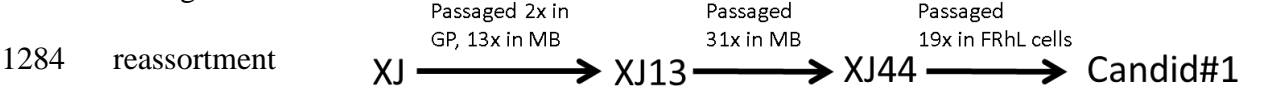
1287   portion of the virus population maintains the L segment of LASV. With the L segment

1288   still present, it is possible to generate virulent LASV from a vaccine seed stock. These

1289   small virus populations would need to be completely eliminated prior to clinical trials.

1290   The generation of the attenuated vaccine strain for JUNV, Candid#1, was

1291   accomplished through passaging the virulent XJ strain in guinea pigs, mouse brains, and

1292   a fetal rhesus monkey lung cell line (FRhL-2) for a total of 65 different passages (Fig. 21)

1293   (155, 184). Comparison of the amino acid sequence of Candid#1 NP, GPC, and Z

1294   proteins to the XJ13 strain proteins has identified a number of genetic changes which

1295   have occurred following these passages (185). Recent analysis of these mutations has

1296 identified the F427I mutation in the GP2 transmembrane region as having an attenuating

1297 role in murine neurovirulence following challenge with the single mutant JUNV (121,

1298 122). This region is highly conserved between MACV and JUNV (Fig. 22). Additional

1299 research is required to better elucidate the genetic mechanisms of attenuation and stability

1300 of these mutations prior to licensing within the U.S. or Bolivia.

1301 **Gaps in knowledge**

1302 While unsubstantiated reports have identified Candid#1 as protecting NHPs

1303 against MACV challenge, there is no published evidence of protection. Additionally, the

1304 reports of an increase in liver enzymes in challenged guinea pigs vaccinated with MOPV,

1305 when compared to those vaccinated with ML29, indicated that the presence of

1306 homologous antigens against the target virus may be necessary for complete protection

1307 (153). This report of

1308 improved protection through

1309 heterologous vaccination

1310 provides evidence for the

1311 importance of a MACV

1312 derived vaccine instead of

1313 utilizing Candid#1 within

1314 Bolivia (153). The

1315 availability of MACV

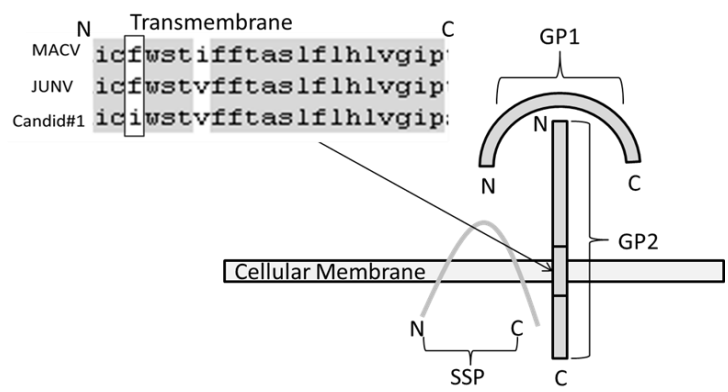Figure 22: Transmembrane Region of MACV and JUNV.
Textual and graphical representation of the transmembrane region of GPC. The alignment of amino acids making up the transmembrane region for MACV, JUNV, and Candid#1 show significant similarity with only one amino acid difference a the MACV I441 and JUNV/Candid#1 V43I residues. The highlighted residue identifies the single mutation found in Candid#1.

1316   isolates is extremely limited and there have been no reports of an attenuated strain of

1317   MACV from which to identify the genetic determinants of attenuation.

1318   **Hypothesis**

1319   The reverse genetics system developed in Aim 1 of this dissertation is an ideal

1320   tool to investigate the impact of a single mutation in MACV at the F437 amino acid

1321   residue. I hypothesize that utilizing my reverse genetics system I can introduce a

1322   mutation at the F437 amino acid position of the transmembrane region of GP2 to

1323   attenuate MACV neurovirulence in a mouse model. The lethal murine model I

1324   characterized in Aim 1 can be used to study changes in MACV virulence to determine if

1325   the mutation attenuates the virus. I expect from my results that rMACV-F437I will be

1326   attenuating *in vivo*.

1327   **Significance**

1328   The proposed modification of rMACV would be the first report of an attenuated

1329   MACV. It would provide useful insight into how NWAs other than JUNV can be

1330   rationally attenuated. It would also be the first step towards the development of a design

1331   vaccine for MACV.

1332

1333

70

METHODS:

Cells, viruses, and biosafety

Baby hamster kidney (BHK-21) and Vero cells (American Tissue Culture

Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with

10% fetal calf serum and L-glutamine. Viral working stocks of rMACV-F437I were

generated by infecting Vero cells (MOI =0.01 PFU/cell) and collecting virus containing

TCS at 96 HPI. Cellular debris was eliminated from the TCS through centrifugation and

the viruses were concentrated and purified through Ultra 100K Filter Devices to remove

cellular factors, which may affect the immune response. All work with infectious virus

was performed in the UTMB BSL-4 facility in accordance with institutional and safety

guidelines.

Construction of the F437I Mutant S Plasmid

To generate an S segment plasmid containing the identical amino acid change

found in Candid#1, I modified the wild-type MACV S segment plasmid generated in

Chapter 2 through PCR mutagenesis. To accomplish this, I designed two overlapping and

reverse direction primers which contained the nucleotide change. This base pair change

would cause a change from phenylalanine at residue 437 to an isoleucine (GP2-F437I-

R1v2,   CACATTAGTTGA**T**ATTTGTATCTGGAGCACAATTTTCTTC   and   GP2-

F437I-F2v2,   GAAGAAAATTGTGCTCCAGATACAAAT**A**TCAACTAATGTG).

1353   Additionally I generated two primers located downstream and upstream of the

1354   transmembrane region (GP2-F437I-F1v2 and GP2-F437I-R2v2) that would be used to
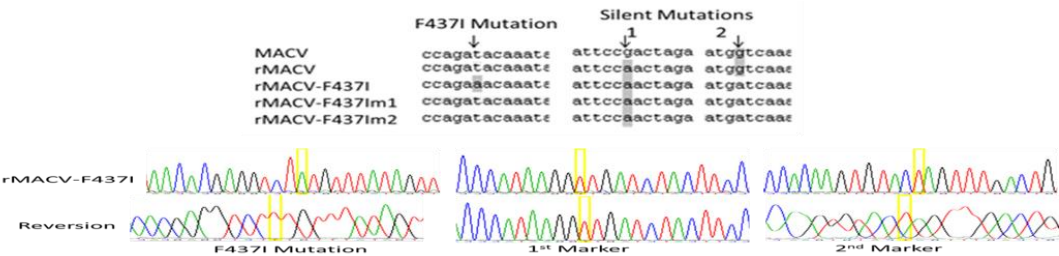

Figure 23: Sequence Analysis of rMACV-F437I.
Graphical sequence analysis and comparison of cDNA generated from MACV, rMACV, and rMACV-F437I isolates. rMACV and MACV maintain the wild type genotype at the nucleotide coding for F437. The nucleotide change coding for I437 is present in the stock of rMACV-F437I while a reversion has occurred in the viral RNA isolated from mouse 1 and 2. The presence of the second silent marker confirms that both animals were challenged with rMACV-F437I. Chromatography analysis of sequenced viral cDNA confirming the reversion of the mutation in the consensus sequence and the presence of both silent markers.

1355   amplify the two fragments.

1356   Utilizing these four primers, I synthesized two cDNA fragments, 1.2kb and .9kb

1357   each, utilizing a high fidelity polymerase (NEB, Q5 Hot Start # M0493S).   I then

1358   generated a single cDNA fragment from both smaller fragments by amplifying through

1359   the overlapping region utilizing the external forward and reverse primers. I digested the

1360   whole cDNA fragment and the original S segment plasmid inclusive of the F437I

1361   mutation and second gene marker to distinguish rMACV-F437I from rMACV, ligated the

1362   insert into the plasmid, and transfected the new plasmid into competent cells to generate a

1363   working stock of plasmid. In order to   differentiate rMACV from rMACV-F437I, I

1364   introduced an additional silent mutation at the 808 nucleotide, G to A (Fig. 23). The new

1365 plasmid was sequenced by the UTMB Molecular Genetics Core to ensure the single

1366 mutation and two gene markers were present within the plasmid with no additional

1367 mutations.

1368 **Rescue of rMACV-F437I**

1369 The rescue of rMACV-F437I was completed in a similar manner as described

1370 previously by my laboratory and in Chapter 2 of this dissertation (38). Equimolar

1371 amounts of the wild type L full segment MACV plasmid and the S segment single mutant

1372 plasmid and the two expression plasmids were transfected into BHK21 cells.

1373 Supernatant from these cells was collected at 4 days post transfection. A single passage in

1374 Vero cells was performed to generate a high titer stock of rMACV-F437I. The rMACV-

1375 F437I sequence, including the introduced G808A and G1447A gene tags within the GP

1376 gene which were inserted to distinguish rMACV-F437I from rMACV and MACV, were

1377 confirmed by whole genomic sequence analysis.

1378 **Sequencing of Full Length S and L Genomic RNAs from rMACV-F437I**

1379 RNA (0.5 to 1.0 mg) was isolated by a RNA purification kit (Zymo Research,

1380 DNA-Free RNA kit, R1014) at 96 HPI from rMACV-F437I infected Vero cells. Viral

1381 cDNA was synthesized by reverse transcription (RT) using random primers. Viral

1382 specific primers complementary to S and L genome RNAs were used to generate cDNA

1383 fragments of each segment. The entire S and L segments were amplified in three and five

1384 DNA fragments, respectively. PCR products were gel purified (Zymo Research,

1385 Zymoclean Gel DNA recovery kit, D4001) and directly sequenced to obtain the

1386 corresponding master sequences for the MACV S and L genome RNAs. Sequencing data

1387 was analyzed using the program Clone Manager V9. The sequencing of RNA isolated

1388 from tissue samples follows this same procedure except Trizol sample (Zymo Research,

1389 DNA-Free RNA kit, R1014) is utilized to homogenize samples.

1390   **Plaque Titrations**

1391   All plaque titrations were completed on Vero-CCL81 cell lines, seeded on 12-well

1392 plates ~16 hours prior to infection. Samples were serially diluted in DMEM with 2% FBS

1393 and .5% P/S. Cells were infected with 100μl of diluted virus and incubated for ~1 hour

1394 with shaking every 15 minutes. Wells were then overlayed with a 50:50 warmed mixture

1395 of 2% agarose in sterile H2O and 2X MEM with 10% FBS and .5% P/S. The plates were

1396 incubated for 8 DPI and fixed with 10% formalin solution. Cells were stained with crystal

1397 violet and plaques counted to determine viral load. When calculating the viral load of

1398 tissue samples, tissues were weighed prior to homogenization and media was added to the

1399 samples consistent to the measured weight. Utilizing these values, viral loads per gram of

1400 tissue was calculated for each sample.

1401

1402

1403   **Animal Experiments**

1404   Six-to-eight-week old IFN-αβ/γ R -/- mice on a C57BL/6 background were

1405   utilized for all studies. All animals were housed in a pathogen-free environment. All

1406   virus infections were performed in the BSL-4 in the GNL, UTMB. All animal studies

1407   were reviewed and approved by the UTMB Institutional Animal Care and Use

1408   Committee, and were completed according to the National Institutes of Health guidelines.

1409   Animals were anesthetized using an isoflurane precision variable-bypass vaporizer prior

1410   to virus inoculation by the IP route with 10^4 PFU. Telemetric monitoring of body

1411   temperature was accomplished throughout the studies. A BMDS IPTT-300 transponder

1412   (Bio Medic Data Systems, Inc.) was implanted subcutaneously using a trocar needle

1413   assembly. Transponders were read with a DAS-6007 reader (Bio Medic Data Systems,

1414   Inc.) and downloaded in accordance with manufacturer's protocol. Body weight

1415   measurements were performed throughout the studies by anesthetizing the animals and

1416   weighing them, weights were compared to baseline collected at 0 DPI (38, 149). The

1417   experimental endpoints of the studies were ~40 DPI, where surviving animals were

1418   humanely euthanized and necropsied.

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1422 **Statistical Analysis**

1423   GraphPad Prism v5 was utilized for all data analysis. To determine significance

1424 in weight and temperature change, two-way ANOVA tests were performed comparing

1425 pooled data from the infected rMACV or rMACV-F437I IFN-αβ/γ R -/-mice. Viral

1426 growth curve analysis was

1427 completed utilizing a two-way

1428 ANOVA test comparing all virus

1429 and cell culture types over each

1430 day. A Kaplan Meier survival

1431 curve was generated and

1432 statistical Mantel Cox test was

1433 completed to determine

1434 significance between rMACV

1435 and rMACV-F437I.
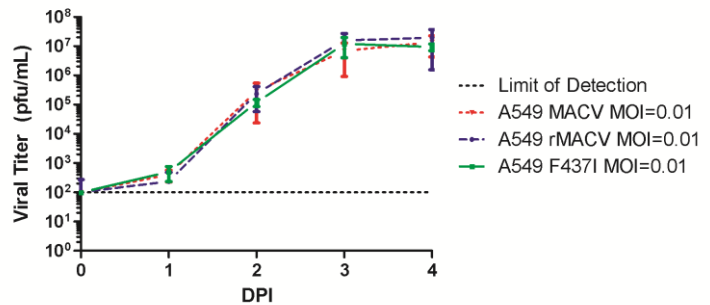
Figure 24: Infection of IFN Competent A549 Cells with MACV, rMACV, and rMACV-F437I.
A549 cells were infected at an MOI=0.01 with MACV, rMACV, and rMACV-F437I. TCS was collected from 0 to 4 DPI in triplicate from infected cells and plaque titered. No significant difference (P>.05, two-way ANOVA) was observed on any day between the three viruses. All three viruses had similar growth kinetics and peak titers.

1436 **RESULTS:**

1437   *In Vitro* **Characterization of rMACV-F437I**

1438   The rescue of rMACV-F437I was accomplished in a manner similar to rMACV

1439 presented in Chapter 2 and as described in the methods of this chapter. A working stock

1440 of rMACV-F437I with a titer of 4e7pfu/mL was generated following a single passage of

1441   Vero cells. Sequencing confirmed no additional mutations were generated during the

1442   passage. To characterize the *in vitro* growth kinetics of rMACV-F437I, I infected IFN

1443   competent A549 cells at an MOI = 0.01. I collected TCS at 0, 1, 2, 3, and 4 DPI in

1444   triplicate and completed plaque titrations on all samples (Fig. 24). Additionally, I infected

1445   IFN incompetent Vero cells at an MOI=0.01 and collected TCS at 0, 1, 2, 3, and 4 DPI

1446   (Fig. 25). Both growth curves corresponded closely with MACV and rMACV in the same

1447   cell lines.

1448   ### *In Vivo* Characterization of rMACV-F437I

1449   To investigate the impact of the single mutation on rMACV virulence, I utilized

1450   the IFN-$\alpha\beta/\gamma$ R -/- murine model I characterized previously in Chapter 2. I hypothesized

1451   that the introduction of

1452   the single mutation would

1453   alter the virulence and

1454   disease development in

1455   infected animals. I

1456   completed two

1457   experiments from which

1458   the pooled data is

1459   presented. C57BL/6 IFN-
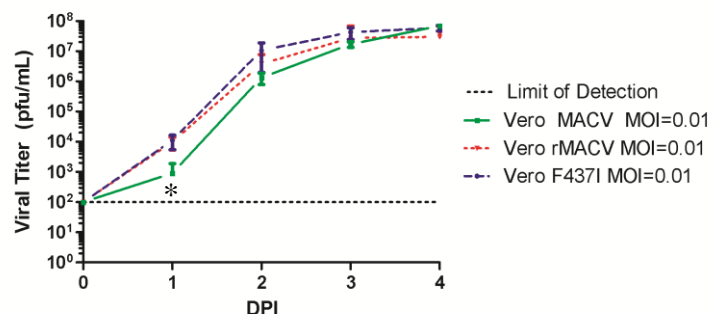
Figure 25: Infection of IFN Incompetent Vero-CLL81 Cells with MACV, rMACV, and rMACV-F437I.
Vero cells were infected at an MOI=0.01 and TCS was collected from 0 to 4 DPI. A significant difference was observed at 1 DPI between the titers of MACV and rMACV/rMACV-F437I (P<.01, two-way ANOVA). No significant difference was observed from 2 to 4 DPI (P>.05, two-way ANOVA). All three viruses grew similarly with comparable titers after 2 DPI.

1460   αβ/γ R -/- mice were challenged IP with 1×10^4 pfu of rMACV (N=9) or rMACV-F437I

1461   (N=7).

1462   Mice infected with rMACV became hunched-but-active as early as 9 DPI and I

1463   observed progressive weight loss from 10-15 DPI. Between 15 to 20 DPI all animals had

1464   no discernable disease. Starting at 20 DPI, rMACV infected mice began to develop

1465   neurological impairments. These impairments included partial to full paralysis, hunched

1466   posture, labored breathing, and ataxia. All rMACV infected animals developed lethal

1467   disease or were humanely euthanized after reaching disease endpoints defined within the

1468   study protocols (Fig. 26).

Figure 26: Kaplan Meier Curve of rMACV and rMACV-F437I Infected IFN-αβ/γ R -/- Mice.
Data is representative of two pooled studies. Mice were challenged with rMACV and rMACV-F437I through the IP route with 1×10^4 pfu/mL and observed for clinical disease. All animals challenged with rMACV reached endpoints defined by the study or were found dead with a MTD of ~27 DPI. Two of the seven animals challenged with rMACV-F437I develop endpoints defined by the study protocol, the other five developed no observable disease symptoms and were euthanized at the end of the study. A significant difference (P=.02, Mantel Cox test) was observed between the two groups of animals survival rates. The two mice infected with rMACV-F437I which reached disease endpoints had a MTD of 20.5 DPI.

1469   In   contrast,

1470   rMACV-F437I   infected

1471   mice could be classified

1472   into two separate groups

1473   based upon their clinical

1474   outcome. The first group,

1475   five of the seven mice,

1476   developed no discernable

1477   disease   throughout   the
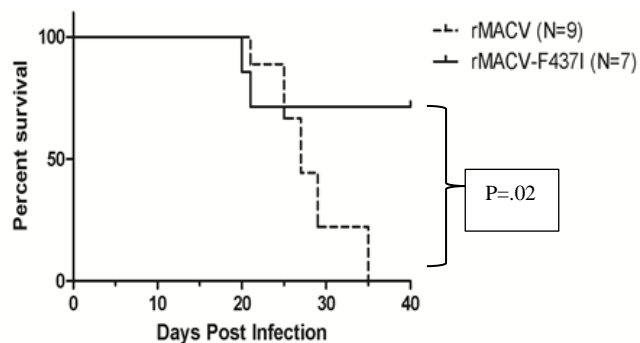
1478   period of the two studies.

1479   No significant changes in temperature or weight were reported in these animals. The

1480   second group was comparable to the first group for the first 18 DPI. Starting at 18 DPI

1481   the second group became hunched-but-active and/or ataxic. By 20 DPI both animals in

1482   the second group were losing body weight and were paralyzed or moribund. One animal

1483   was euthanized at 20 DPI, and the other at 21 DPI (Fig. 26).

1484   Temperature and bodyweight data was collected throughout the two studies. At

1485   10, 14, and 15 DPI I identified significant weight loss ($p < 0.05$, Two-way ANOVA) in

1486   the rMACV infected IFN-$\alpha\beta/\gamma$ R -/- mice when compared to the infected rMACV-F437I

1487   mice (Fig. 27). Additionally, a significant difference in weight change was identified at

1488   22, 24, and 27 DPI, corresponding to 1-3 days prior to individual animals succumbing to

1489   disease. There was minimal change in body temperature throughout most of the study

1490   between the rMACV and rMACV-F437I infected mice. These days correspond to the

1491   death of two animals in the rMACV pool, both of which developed hypothermia at the

1492   final temperature collection prior to death/euthanizations (Fig. 28).

1493   Organ samples were collected and homogenized for titration from infected

1494   animals. Animals challenged with rMACV-F437I which survived until the end of the

1495   study had no detectable virus in their brains, kidneys, lungs, livers, or spleen (Data not

1496   shown). Interestingly, only the brain homogenates from the two mice which develop

1497   lethal infection showed titerable plaques with viral loads at 2×10^6 and 2×10^5 pfu/mL

1498   respectively, there was no sign of infectious virus within the peripheral organs.

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Isolation of Viral RNA from Two rMACV-F437I Infected Mice

1502   To investigate if the two

1503 distinct outcomes in IFN-αβ/γ R -/-

1504 mice infected by rMACV-F437I was

1505 driven by a change in the virus, I

1506 isolated and sequenced viral RNA

1507 collected from organ homogenate

1508 from the two animals. Brains, livers,
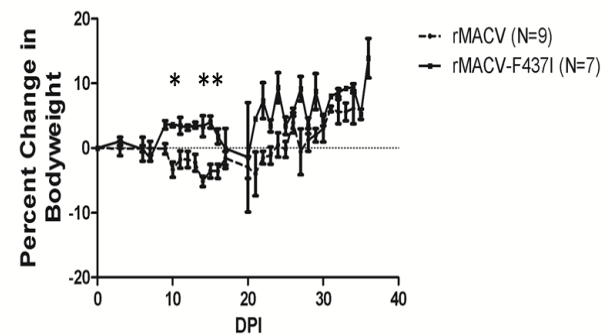
1509 lungs, spleens, and kidneys were

1510 collected in thirds during necropsy

1511 with one part, around 0.1 grams added

1512 to 1mL of Trizol (Life Technologies,

Figure 27: Weight Change of IFN-αβ/γ R -/- Mice infected with rMACV and rMACV-F437I.
Observations of weight change of rMACV and rMACV-F437I infect mice were collected throughout the study. A significant difference (P<.05, two-way ANOVA) was observed during the acute phase of disease between the two groups, at 10, 14, and 15 DPI. Weight loss was observed at 20 DPI and later 1 to 2 days prior to death or the animal reaching the defined study end point.

1513 #15596-026). Samples were homogenized and centrifuged with the aqueous layer

1514 transferred to a clean vial. Fresh Trizol was added to the aqueous layer and the samples

1515 were removed from the BSL-4 following approved GNL protocols.

1516   RNA was isolated from the Trizol lysate according to the manufacturer's protocol

1517 as described in the methods section. I completed RT-PCR utilizing random primers on

1518 the samples and amplified the entire MACV genome utilizing the primers I had

1519 previously designed to sequence MACV. Amplified fragments from the brains, lungs,

80

1520 and kidneys were sequenced by the UTMB Sequencing Core. Analysis of the sequence

1521 identified the two silent mutations, confirming the virus as rMACV-F437I. However, at

1522 the single mutation F437I, there was a reversion to the original phenylalanine 437 amino

1523 acid (Fig. 23). This residue reversion was confirmed in viral RNA isolated from the

1524 organs collected from both mice.

1525 **DISCUSSION:**

1526 The data presented in this chapter confirms the reverse genetics system described

1527 in Chapter 2 as a powerful tool for studying rMACV biology. The highly conserved

1528 nature of the GP2

1529 transmembrane region of

1530 MACV and JUNV makes

1531 targeting the single F437

1532 residue an ideal target for

1533 modification of MACV.
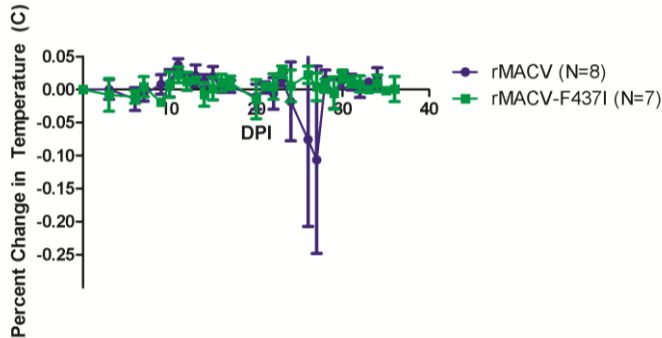
1534 Introduction and subsequent

Figure 28: Change in Temperature in rMACV and rMACV-F437I Infected Mice.

Collection of body temperature from rMACV and rMACV-F437I

1535 rescue of the virus with the

infected animals was completed throughout the study through the subcutaneous implantation of a BMDS transponder chip. No development of febrile disease was observed throughout the

1536 single mutation confirms the

studies. Drastic loss in temperature was observed in animals 1 to 3 days prior to death or reaching defined study end point.

1537 viability of the virus with this single mutation, as reported with LASV and JUNV (122).

1538 This is the first report of the attenuation of MACV *in vivo*.

1539   Initial rescue and concentration of rMACV-F437I resulted in a similar peak titer

1540   as seen with rMACV. Characterization of viral growth kinetics in IFN incompetent Vero

1541   cells confirmed similar growth curves and peaks as the wild type virus. Similar growth

1542   analysis in IFN competent A549 cells confirmed that the single mutant virus grew and

1543   peaked at similar titers as MACV and rMACV. This data is comparable to results

1544   reported from the single mutant rXJ13-F427I, a passaged strain of JUNV with the single

1545   mutation (121). rXJ13-F427I also exhibited similar growth characteristics when

1546   compared to wild type XJ13 (121). In comparison, the addition of the single mutation in

1547   rLASV greatly reduced viral growth when compared to the wild-type virus (121).

1548   Following two passages in Vero cells, additional spontaneous mutations developed in the

1549   rLASV single mutant virus that allowed for robust growth of the single mutant virus

1550   comparable to that of the wild-type virus. The characteristics identified from *in vitro*

1551   analysis of rMACV provide further evidence of the distinct natures of NWAs when

1552   compared to OWAs.

1553   Challenge of IFN-αβ/γ R -/- mice with rMACV-F437I resulted in a significant

1554   difference (p=.02) in mortality rate when compared to mice infected with rMACV.

1555   Disease presentation also was drastically distinct between the majority of the rMACV-

1556   F437I infected and rMACV infected animals. The periods of weight loss and

1557   neurological disease present in rMACV infected animals in these two studies were

1558   identical to what I previously reported in Chapter 2. The period of acute disease and

1559   weight loss was not present in mice infected with rMACV-F437I, even when looking at

1560   the two mice that did succumb following viral challenge.

1561   Interestingly, both mice which developed lethal disease following challenge with

1562   rMACV-F437I had a gain in weight at 10 to14 DPI comparative to the rMACV infected

1563   animals. Both animals developed neurological disease, albeit 2-3 days earlier than

1564   previously reported in rMACV-and MACV-infected animals. The mortality rate I

1565   identified in the murine model challenged with rMACV-F437I is comparable to the

1566   reported mortality rate of mice challenged with XJ13-F427I but it is not known if a

1567   reversion occurred in the animals challenged with XJ13-F427I (121). The MTD for the

1568   two mice which developed lethal disease following rMACV-F437I infection was 20.5

1569   DPI. When compared to the ~28 DPI MTD reported in animals challenged with rMACV,

1570   this initial data suggests the reversion may result in more rapid disease progression than

1571   infection with the wild type virus. Without further study it is not possible to confirm this

1572   phenomenon statistically.

1573   In an attempt to determine why infection with rMACV-F437I resulted in such

1574   variable outcomes for a significant portion of the experimental animals, I investigated if

1575   any genetic mutations had occurred in rMACV-F437I following challenge. The

1576   sequencing I completed from viral RNA isolated from different organs of the two mice

1577   confirmed that the mice had been infected with the single mutant virus, but that the single

1578   mutation was no longer present. All RNA isolates from organ homogenates presented

1579   with an identical reversion of the single nucleotide at the I437F residue resulting in a

1580   wild-type genotype.

1581   The F427I mutation appeared in the final series of passaging during the

1582   generation of Candid#1; it was not present in XJ44 or any previous passage strain (121).

1583   It has been proposed that this mutation alters the pH requirements for fusion allowing for

1584   more relaxed environmental requirements for fusogenic transformation and entry into

1585   cells (122). This alteration of fusion phenotype could have a number of different effects

1586   on the virus. It has been shown that Candid#1 has a greater dependence upon human

1587   transferrin receptor (hTfR) 1 than wild-type JUNV but this is linked to mutations in GP1

1588   and not GP2 or F437I (121).

1589   A change in MACV ability to fuse to the early or late endosome following

1590   invagination caused by the F437I mutation may lead to a change in cell tropism. With the

1591   fusion to the endosome occurring earlier, it may be possible the virus is injecting into a

1592   different location within the cytoplasm compared to where it normally occurs, impacting

1593   the replication of the virus. This could greatly alter disease progression and severity. As

1594   my murine model has shown, late-stage disease and death occurs when the titer of

1595   MACV in the brain is at its peak. It is possible the reversion of rMACV-F437I and

1596   subsequent rapid decline of the animals is due to a more rapid dissemination leading to

1597   faster invasion of the central nervous system by the single mutant followed by the

1598   reversion to a more virulent genotype. The *in vitro* characterization of the single mutant

84

1599   virus showed no significant change in growth kinetics compared to rMACV, but this is in

1600   cell culture with only a single cell type. Further characterization in different cell lines and

1601   *in vivo* studies are warranted to determine if the presence of the single mutation changes

1602   the rate of infection or type of cell normally targeted by MACV. It would also be

1603   interesting to identify if the virus enters the cytoplasm earlier, fusing through the early

1604   endosome instead of the late endosome, forcing the virus to replicate in a different

1605   manner or location within the cell.

1606   An alternative method of attenuation could be that F437I mutation causes a

1607   decrease in virus infectivity due to a change in virion stability. The mutation might not

1608   change the cellular tropism but could change the rate or efficiency of cellular entry by

1609   MACV. This was not seen in the *in vitro* characterization but may be cell type dependent.

1610   As reported previously, this mutation could lead to a conformational change at neutral pH

1611   prior to the low pH reported to be necessary for virus fusion (186, 187). It has been

1612   shown that the GP of LASV undergoes irreversible conformational changes at pH 6.0,

1613   potentially shedding GP1 following binding to the cell receptor and endocytosis. A

1614   conformational change at neutral pH might lead to the shedding of GP1, preventing

1615   rMACV-F437I from binding the cellular receptor correctly and entering the cell. The

1616   mutation might also lead to early shedding or a conformational change within the

1617   endosome prior to reaching the low pH required for normal fusion; this could expose the

1618   viral proteins and RNA to a low pH environment earlier than normal. To determine if this

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1619   is occurring I recommend investigating if conformational changes occur in the GP-1/2

1620   spike in different pH environments, potentially utilizing the expression plasmids

1621   generated in Chapter 2. If changes occur, I would investigate at what pH to determine if it

1622   is occurring prior to binding the receptor, within the early endosome, or at the late

1623   endosome. Finally, further characterization of cellular tropism is necessary to understand

1624   the route and method of infection, not just for MACV but for many arenaviruses as

1625   knowledge of primary cell targets is extremely limited for hemorrhagic NWAs.

1626   **CHAPTER SUMMARY:**

1627   The research presented in this chapter describes the rational modification and

1628   attenuation of rMACV. The modification and generation of an attenuated rMACV proves

1629   the usefulness of the tools developed in Chapter 2, and demonstrates areas of interest for

1630   further studies to elucidate the genetic mechanisms of attenuation. I have presented the *in*

1631   *vitro* characterization of rMACV-F437I on two different cell lines, IFN competent and

1632   incompetent, showing that the mutation in the transmembrane region does not greatly

1633   affect virus growth kinetics in cell culture. I have also shown *in vivo* that rMACV-F437I

1634   is attenuated in the IFN-$\alpha\beta/\gamma$ R -/- mouse model when compared to rMACV. Finally, I

1635   have shown that the single F437I mutation, on its own, appears to be unstable with a

1636   reversion rate of 28% in the two studies completed. Further studies are necessary to

1637   determine how this single mutation is causing attenuation and what pressures are leading

1638   to the reversion to wild-type genotype.

CHAPTER 5: DISSERTATION SUMMARY

1639

1640   In Chapter 1, I provide a comprehensive review of MACV based upon published

1641   articles and conference presentations dating back to the original outbreak in 1959. I also

1642   provide the three aims from my original dissertation proposal, my hypotheses, and a brief

1643   rationale for my hypotheses. I provide a summary of all the published information on

1644   MACV, including details on all reported outbreaks, the genomic makeup of arenaviruses,

1645   a clinical description of BHF, and a list of all published animal models utilized for

1646   studying BHF. I also detail the recent discoveries made from models utilizing plasmid

1647   expression of MACV protein or other arenaviruses which have been ascribed to what can

1648   occur with infectious MACV.

1649   In Chapter 2, I discuss the development of the reverse genetics system I utilized

1650   for rescuing rMACV. To ensure the viability of the virus I completed a full sequence of

1651   the viral genome, including the UTRs, and identified the correct 5' and 3' terminal

1652   regions of the S and L segments. During this process I generated a minigenome assay that

1653   allowed me to identify the functional NP and L proteins in the BSL-2. Additionally, I

1654   showed that the L protein from Candid#1 and Romero were able to replicate the MACV

1655   genome. Following rescue of rMACV, I completed growth curve analysis at different

1656   MOIs in different cell lines. This analysis allowed me to compare the wild-type virus to

1657   the recombinant virus, ensuring they were similar. I characterized a novel murine model,

1658   IFN-αβ/γ R -/- mice, which developed a biphasic disease following IP challenge with

1659   MACV or rMACV.

1660   The identification of the 5' and 3' termini regions of MACV is an important step

1661   in the development of the reverse genetics system and it also confirms the conservation of

1662   this region across multiple arenaviruses. This is the fourth report of a hemorrhagic

1663   arenavirus with identical termini sequence, the other three being Lujo virus, JUNV, and

1664   LASV (38, 124, 150). Confirmation of this sequence may assist in future attempts at

1665   rescuing hemorrhagic arenaviruses. The development of the novel minigenome assay was

1666   essential for confirming the functionality of the plasmid-expressed viral proteins and the

1667   termini regions of MACV. Other groups have utilized similar assays to elucidate viral

1668   replication kinetics and to test antivirals (122, 143-148, 152). I utilized this assay to show

1669   that two different JUNV L proteins could replicate the reporter genes, providing evidence

1670   that a chimeric virus with JUNV and MACV genes could be generated. The development

1671   of the reverse genetics system is the tool necessary for such a project. Unlike ML29,

1672   which was generated through *in vitro* co-infection and reassortment of the two viruses,

1673   my reverse genetics system could be used to rationally generate chimeric viruses (153,

1674   182). Additionally, my system can be utilized to modify specific regions of the MACV

1675   genome to better elucidate the genetic determinants of attenuation as comparable systems

1676   have been utilized for other arenaviruses (38, 124-126, 151, 188, 189).

88

1677   In Chapter 3, I provide evidence that RIG-I knockdown in A549 cells had

1678   minimal impact on viral growth or peak titer at 3 and 4 DPI when compared to control

1679   cells. The reduced growth at 1 and 2 DPI may imply that MACV is inhibited by IFN

1680   induction early in infection but is able to overcome the inhibiting factor at later stages of

1681   infection. I show that MACV infection of A549 cells leads to activation of STAT-1,

1682   induction of STAT-1, and induction of ISG15. All three of these steps are correlated with

1683   PRR recognition of the virus infection leading to synthesis of IFN and IFN signaling. The

1684   evidence I provide identifies activation of PKR and phosphorylation of eIF2α, which is

1685   also contradictory to the commonly held dogma within the arenavirus field, that these

1686   viruses do not impact cellular biosynthesis.

1687   The indication that MACV is affected by but may overcome the activity of IFN is

1688   very intriguing especially as early tests utilizing therapeutic poly I:C in MACV

1689   challenged NHPs reported no clinical benefit. However, it did identify a more rapid

1690   detection of viremia in treated compared to untreated animals (111). While poly I:C

1691   failed to elicit a beneficial response, Ribavirin has been reported to reduce the NWA

1692   virulence in infected animals and, in a very limited number of cases, humans (67, 70,

1693   112, 179-181). The identification that MACV infection of A549 induces an IFN signaling

1694   response is contradictory to the accepted arenavirus dogma, which is primarily based

1695   upon plasmid-expressed proteins and non-pathogenic arenaviruses (117, 118, 120, 164).

1696   Previous research identified no phosphorylation of eIF2α following infection of cells Huh

1697 7 cells with the prototypical arenavirus LCMV (169). The existence of these dogmas can

1698 be a detriment to the field as it may encourage scientific pursuits based upon incorrect

1699 evidence. Clinical data from JUNV-infected patients have clearly identified IFN

1700 induction, as have reports from MACV infected NHPs and JUNV infected guinea pigs

1701 (108, 111, 165). The clinical data from LASV infected patients clearly identify a

1702 suppression of IFN induction (139, 140, 190). The distinctions between pathogenic

1703 NWAs and OWAs must be clarified to allow further unconstrained progress within the

1704 field. Based upon this data, I propose MACV and JUNV both induce IFN early in

1705 infection and the production of IFN is maintained throughout the infection within the

1706 host. The ability of MACV to propagate within an IFN inducing cell and the role of IFN

1707 in MACV infection must be further elucidated, especially if, at late stages of severe

1708 disease, IFN is pathogenic instead of protective as seen with Severe acute respiratory

1709 syndrome and Influenza A (191-193).

1710   In Chapter 4 I utilized the reverse genetics system and mouse model I

1711 characterized in Chapter 2 to study the impact of a single mutation in the glycoprotein

1712 membrane of MACV at residue F437. This mutation has been identified in Candid#1 and

1713 shown to play a significant role in attenuating JUNV in a murine neurovirulence model.

1714 After generating the rMACV-F437I, I confirmed that the single mutant virus had similar

1715 growth curves and peaks compared to the wild type virus. After challenging IFN-αβ/γ R

1716 -/- mice with rMACV and rMACV-F437I, I identified a significant reduction in

1717   morbidity and mortality between the two viruses. Reversion to wild type sequence was

1718   identified in the two of seven rMACV-F437I-infected animals, both of which succumbed

1719   to disease with a MTD of 20.5 DPI.

1720   The role of the F437I substitution, while significant in its attenuation, is not well

1721   characterized. The mutation has been shown to play a role in cell-to-cell fusion at more

1722   neutral pH, reducing infectivity in cell culture (122). The mutation also plays a critical

1723   role in attenuating the neurovirulence of JUNV in a murine model (121). My data further

1724   exemplify the attenuating role, but does not characterize the molecular mechanism of

1725   attenuation. The identical reversion of the single mutation confirms the importance of the

1726   residue, but does not identify the role of the residue.

1727   Looking towards the future of this project, further investigation needs to be

1728   completed to determine the role of IFN induction on hemorrhagic NWAs to determine if

1729   the IFN response plays an antiviral or pathogenic role in the outcome of the patient. As a

1730   correlation of high IFN levels to more severe outcomes has already been published

1731   relating to JUNV, early identification of high levels of endogenous IFN may provide an

1732   ideal marker to identify critical cases of BHF prior to severe disease development (109). I

1733   also propose investigating the role of phosphorylated PKR to identify if there is an impact

1734   on cellular and viral protein biosynthesis or if MACV has a method of evading cap-

1735   dependent translation shutdown. If MACV is able to overcome cap-dependent translation

1736   shutdown within an infected cell, it avoids an essential antiviral cell response. If the

1737   mechanistic target can be identified, there are two distinct opportunities for antiviral

1738   development. The first would be to identify which MACV protein plays a critical role in

1739   the process and possibly target the protein-protein interaction restoring the shutdown of

1740   translation. The second would be to identify which part of the translation pathway is

1741   being affected and develop therapeutics, which could possibly restore the antiviral state.

1742   Both of these targets would aim to restore the shutdown of translation within infected

1743   cells, inhibiting MACV replication and slowing infection.

1744   Another direction is to investigate the single mutation F437I, to determine if it can

1745   be stabilized similar to Candid#1 and the role it plays in attenuating the MACV.

1746   Elucidation of this mutation may play a key role in the development of a future vaccine,

1747   especially if attenuation of other hemorrhagic NWAs can be achieved in a similar

1748   manner. The reverse genetics system I developed and the lethal animal model I

1749   characterized in this dissertation will play a vital role in further elucidation of MACV

1750   virulence and the future development of an attenuated rMACV.

1751

Appendix:

I: TABLE OF MACV ANIMAL EXPERIMENTS

Animal Models		Route of Challenge (Dose)	Disease Symptoms	Mean time to death	Late Neurological Syndrome (Mortality %)	Neutralizing Antibodies
Non-Human Primates	Adult Marmosets (Saguinus geoffroyi)	SC, SS, Cr, IN, Or	1-3 days prior to death refuse food, huddled, inactive, weakness, hypothermia	11-21 DPI	N/A	N/A
	Adult Rhesus macaques (Macaca mulatta)	SC (10^5 pfu, 10^3 pfu)	Biphasic disease identified ~7 DPI. Skin petechiae, facial rash, nasal discharge fever, and anorexia Moribund ~2 before death.	13-17, 17-25 (50%)	Yes (100%)	Yes
	Young Rhesus macaques (Macaca mulatta)	SC (10^3 pfu)	Biphasic disease identified ~7 DPI. Skin petechiae, facial rash, nasal discharge fever, and anorexia Moribund ~2 before death.	19 DPI (86%)	Yes (50%)	Yes
	Cynomolgus monkeys (Macaca fascicularis)	SC (10^3 pfu)	Sudden death with disease reported 1-3 days prior to death	17 DPI (71%)	Yes (50%)	Yes
	African green monkey (Cercopithecus aethiops)	SC (10^3 pfu)	Fever, Anorexia, Shock, Hemorrhage, Pnuemonia	15 DPI (83%)	N/A	N/A
Murine	Adult Inbred white mice	IC, IP, IN, Or	No Disease		N/A	Yes
	Young (<2 days old) Inbred white mice	IC, IP	Growth retardation, tremors, convulsions	9-16 DPI	N/A	N/A
	STAT-1-/-	IP (10^3 pfu), SC (10^3 pfu)	Ruffled, hunched, and lethargic	7.3 DPI, 10.5 DPI	N/A	N/A
	IFN αβ/γ R-/-	IP (10^3 pfu)	Biphasic disease- acute weight loss; Neurological- ataxia, awkward gait, loss of balance, paralysis, death	30 DPI	Yes (100%)	N/A
Other Animals	Adult Guinea Pigs	IP	N/A	N/A	N/A	Yes
	C-13 Guinea Pigs	IP	N/A	N/A	N/A	Yes
	Young Guinea Pigs	IP, IC	N/A	N/A (87%)	N/A	N/A
	Adult Hamsters	IC, IP, IN, Or	No Disease	N/A	N/A	Yes
	Young Hamsters (<5 days)	IC, IP	Neurological	N/A	N/A	N/A
	Cats	IC, IP, IV, Or	No Disease	N/A	N/A	Yes
	Chickens	N/A	No Disease	N/A	N/A	No
	Swine	N/A	No Disease	N/A	N/A	No
	Equine	N/A	No Disease	N/A	N/A	Yes
	Rabbit	N/A	No Disease	N/A	N/A	Yes

Table 1: Table of published animal models from the 1960s to present.
When available, data of clinical development, routes of exposure, and doses are reported. Routes of exposure which are underlined represent lethal challenges. Acronyms: Sub-cutaneous (SC), Scarified skin (SS), Corneal instillation (Cr), Intra-nasal (IN), Oral (Or), Plaque forming unit (pfu), Intracranial (IC), Intravenous (IV), and intraperitoneal (IP). Copyright Current Opinions in Virology
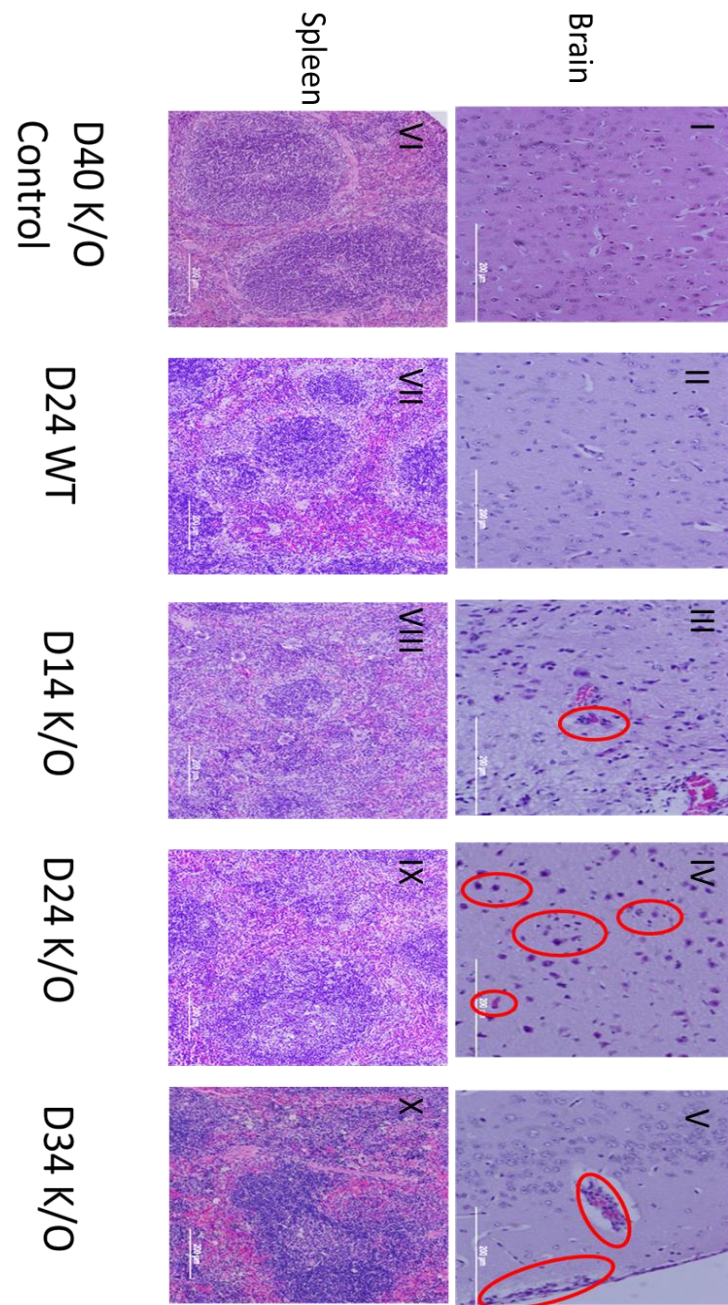
II: Brain and Spleen Histology from Infected Mice

Figure 29: Histopathology Staining of Brain and Spleen Tissues.
Sections I and VI are from an uninfected IFN-αβ/γ R /mouse showing no neuronal death and good spleen structure. Sections II and VII are from a C57BL/6 mouse infected with MACV with no visible neuron death or inflammation and good spleen structure. Sections III and VIII are from an IFN-αβ/γ R /mouse euthanized at 14 dpi, the red circle identifying vascular infiltrates. Sections IV and IX are from IFN-αβ/γ R /mice euthanized at 24 dpi; red circles identify microglial cells and cellular debris from dead neuronal cells. Sections V and X are from an IFN-αβ/γ R /mouse that died at 34 dpi; red circles identify increased vascular and perivascular cellular infiltrates.
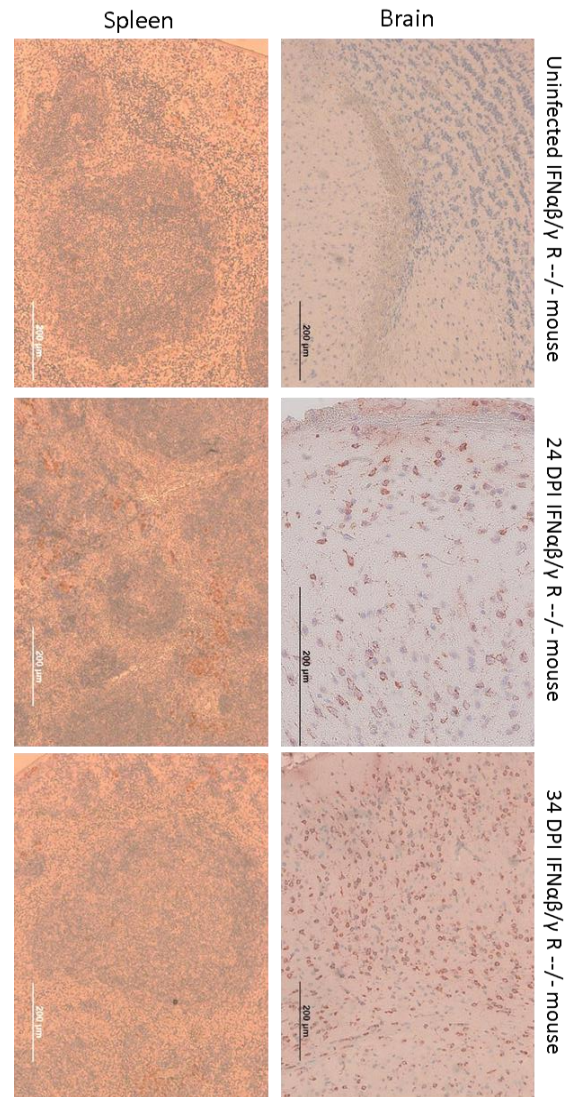
III: IMMUNOHISTOCHEMISTRY FROM INFECTED MICE

Figure 30: Immunohistochemistry of Brain and Spleen Tissue Slides from IFN αβ/γ R -/- Mice.
Tissue slides made from Brain and Spleen organs collected at 24 and 34 DPI with accompanying control. By 34 DPI a large percentage of neuronal cells are infected while spleens have minimal signs of viral antigen and have started to regain normal architecture.

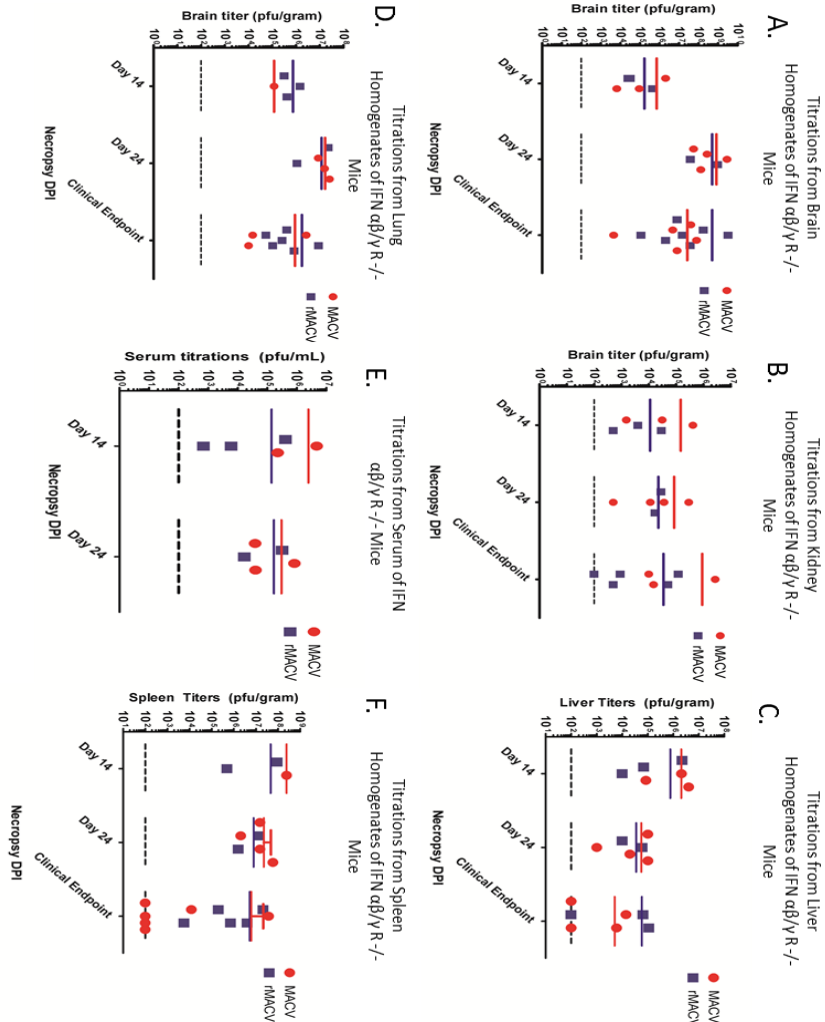# IV: Viral Load of Different Organs from Infected Mice

Figure 31: Titrations From Organ Homogenates of Infected IFN αβ/γ R -/- Mice. Organs from infected animals were weighed and homogenized in 2% FBS DMEM. Plaque titrations on Vero-CCL81 cells were completed and corresponding titrations are shown above. No significant difference (P>.05, two-way ANOVA) was identified between the two viruses in any homogenate sample. A high viral load in the CNS was observed by 24 DPI (~10^8 pfu/gram) and was maintained until death. The single surviving mouse infected with MACV was euthanized at 40 DPI, and had no detectable viral load in the brain (data not shown). Titrations of kidney homogenates identified a slight increase from 14 DPI to clinical endpoint. Liver homogenates had a peak titer at 14 DPI with a generalized downward trend until clinical endpoint. Lung homogenates had a peak titer at 24 DPI. Serum samples confirmed the presence of viremia at 14 and 24 DPI. Spleen samples had a peak titer at 14 DPI with a generalized decrease until clinical endpoint.

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lung tissues from fatal cases of 2009 pandemic influenza A (H1N1): role of the host immune response in pathogenesis. Am J Pathol **183:**1258-1268.

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Education:

2014	Doctor of Philosophy: Experimental Pathology, University of Texas Medical Branch, Galveston, TX, USA
2013	Master of Public Health: Infectious Disease Epidemiology, University of Texas Medical Branch, Galveston, TX, USA
2008	B.A.: Biochemistry, Biophysics and Molecular Biology. Whitman College, Walla Walla, WA, USA
2007	National University of Galway, Galway, Ireland

Professional Experience:

2008-Present	Graduate Assistant at UTMB, Galveston, TX
2008	Organic Chemistry laboratory assistant, Whitman College, Walla Walla, WA: Assisted in preparing experiments and teaching classes of 30+ students bi-weekly
2007	Summer Undergraduate Research Program (SURP), UTMB, Galveston, TX: Studied the effects of drug and exercise treatment on protein expression in recovering burn patients.
2006	Summer Intern at PASCO Electronics, Roseville CA: Worked with the experiment design team developing high school and undergraduate level protocols and documentation. Additional work included troubleshooting newly designed equipment and software.

Peer Reviewed Publications:

1. **Patterson M,** Grant A, Paessler S**.** 2014. Epidemiology and pathogenesis of Bolivian hemorrhagic fever. Curr Opin Virol **5c:**82-90.

2. **Patterson M,** Seregin A, Huang C, Kolokoltsova O, Smith J, Miller M, Yun N, Poussard A, Grant A, Tigabu B, Walker A, Paessler S. 2014. Rescue of a Recombinant Machupo Virus from Cloned cDNAs and In Vivo Characterization in Interferon (alphabeta/gamma) Receptor Double Knockout Mice. J Virol **88:**1914-1923

3. Poussard, A.*, **Patterson, M**.*, et al., In vivo imaging systems (IVIS) detection of a neuro-invasive encephalitic virus. J Vis Exp, 2012(70): p. e4429.*co-first authors

4. **Patterson, M.**, Poussard, A., Taylor, K., et al. Rapid, non-invasive imaging of alphaviral brain infection: Reducing animal numbers and morbidity to identify efficacy of potential vaccines and antivirals. Vaccine 2011;29:9345-51.

5. Salazar, M., Yun, N., Poussard, A., et al. Effect of Ribavirin on Junin Virus Infection in Guinea Pigs. Zoonoses and Public Health 2012;59:278-85.

6. Taylor, K., Kolokoltsova, O., **Patterson, M.**, et al. Natural killer cell mediated pathogenesis determines outcome of central nervous system infection with Venezuelan equine encephalitis virus in C3H/HeN mice. Vaccine 2012;30:4095-105.

Other:

1. **Patterson, M**. Dissertation for Doctor in Philosophy: The Development of a Reverse Genetics System for Machupo virus. March 2014. University of Texas Medical Branch, Galveston Texas 77555

2. **Patterson, M**. Masters of Public Health Capstone: *A Review of the Government Sponsored Offensive Biological Programs, Weaponized Biological Pathogens and their Countermeasures.* August 2013. University of Texas Medical Branch, Galveston Texas 77555

Research:

Research Fields/Key Words:

Molecular biology, biochemistry, immunology, viral pathogenesis, disease modeling, vaccine development, recombinant genetics, *in vivo* imaging, infectious disease epidemiology, biodefense, biosecurity, bioweapons

Clearance:

CDC and USDA security risk assessment approved for work with Tier 1 Select Agents

Fellowships:

2013-2014   Emerging Leaders in Biosecurity Fellowship awarded by the
Center for

Biosecurity at the University of Pittsburgh Medical Center: Competitive fellowship with the aim of bringing together a diverse group of individuals interested in a career in biosecurity.

2011   UTMB-Fogarty Fellowship: Worked through the Naval Medical Research Unit -6 in Lima, Peru and the Peruvian Health Department in Puerto Maldonado. Research consisted of house-to-house visits, collection of samples from febrile patients, and administration of a questionnaire with the goal of tracking Influenza in the region. Additionally, assisted with the local health department with childhood and young-adult vaccination programs.

Grant Support:

2010-2011   1UL1RR029876-01 NIH/Institute of Translational Sciences UTMB, *Imaging of CNS invasion by Venezuelan Equine Encephalitis Virus*.

Competitive Awards and Scholarships:

2013   Edwards S. Reynolds award- Annual Pathology Trainee Day
2013   ASV 2013 Annual Conference Travel award
2013   ICAAC 2013 Annual Conference Travel award
2013   Robert Shope PhD Endowed Scholarship award
2013   Christina Fleischmann GSBS Travel award
2012   Betty Williams, PhD General Scholarship
2012   Sealy Center for Tropical Diseases Scholarship
2011   Field Epidemiology Course Fellowship-Naval Medical Research
            Unit-6 and UTMB
2011   Zhou Sisters Great Expectations Scholarship

2011	ASV 2011 Annual Conference Travel award
2010	James E. Beall II Memorial Best Poster Presentation in Neuroscience-NSRF
2010	Sealy Center for Vaccine Development travel award
2007	UTMB SURP Best Poster Presentation award

Committee Responsibilities:
2009-Present	Student and Post-Doctoral Organization for Committee for Career Development
2010-2012	Graduate Student Government Webmaster
2011-2012	Graduate Student Government Treasurer- Organized the annual UTMB schoolwide event
2011-2013	Public Health Organization Treasurer
2012-2013	Public Health Organization Outreach Coordinator-Organized multiple presentations with the community including elementary students focusing on the importance of vaccination and herd immunity.

Extra-Curricular
2009-Present	Founder and Organizer of UTMB Volleyball: Scheduling, organizing, and communicating tri-weekly an organization consisting of more than 30 students, professors and members of the surrounding community.

Society Memberships:
2012	American Public Health Association
2010	American Society for Virology
2009	American Association for the Advancement of Science
2007	Sigma Xi

Certified Training Experience:
UTMB Biosafety Level 4, 3, and 2 access

UTMB Animal Biosafety Level 3 and 2 (Murine, Guinea Pig, Ferret, Non-Human Primate)

Naval Medical Research Unit 6 Field Epidemiology training, Lima and Tumbes, Peru

Equipment and Technical Skills:

Laboratory Techniques**:** PCR, real-time PCR, quantitative PCR, Western blots, Southern

blots, tissue culture, virus propagation, recombinant genetics, *in vivo* imaging

systems (IVIS), histology, immunohistochemistry, ELISA, high
containment research, cloning

Technical Skills: GraphPad Prism, Clone Manager, Word, Powerpoint, Excel, GIS,
Dreamweaver, Movie Maker

Published Abstracts, Posters, and Presentations:

Patterson, M., et al. *The Development Of A Reverse Genetics System For The Rescue Of A Recombinant Machupo Virus*. Oral presentation at: 32nd Annual American Society for Virology; 2013 July 20-24. State College, PA.

Patterson, M., et al. *The Development Of A Reverse Genetics System For The Rescue Of A Recombinant Machupo Virus.* Poster presented at: McLaughlin Colloquium; 2013 April 12. Galveston, TX.

Patterson, M., et al. *The Development Of A Reverse Genetics System For The Rescue Of A Recombinant Machupo Virus*. Poster presented at: UTMB Pathology Department Trainee Research Day; 2013 April 1. Galveston, TX.

Patterson, M., Poussard, A.*, et al. *Utilizing in vivo imaging systems in neuroinvasive alphavirus infection: rapidly identifying potential vaccines and therapeutics.* Oral presentation at: American Society for Virology annual meeting; 2011 July 15. Minneapolis, MI 2011. *Submitted by Patterson, Presented by Poussard

Patterson, M., et al. *In VIVO Imaging of Alphavirus Infection: Visualizing the Early CNS Infection Using Non-Invasive Technique*. Poster presented at: 2010 Texas-UK Symposium 'Controlling Emerging Infectious Diseases in the 21st Century'; 2010, Feb. 26-28. Galveston TX

Patterson, M., et al. *In VIVO Imaging of Alphavirus Infection: Visualizing the Early CNS Infection Using Non-Invasive Technique.* Poster presented at: National Foundation of Infectious Diseases: Vaccine; 2011 May 16-18. Boston, MA.

Patterson, M., et al. *In VIVO Imaging of Alphavirus Infection: Visualizing the Early CNS Infection Using Non-Invasive Technique.* Poster presented at: National Student Research Forum; 2010 April 22-23. Galveston, TX.

Patterson, M., et al. *Effects of Exercise and Oxandrolone Treatment on the Proteomic Biomarkers in Skeletal Muscle (PRISM) after Severe Burn Injury in Children*. Poster presented at: Whitman College Undergraduate Conference; 2008 April 14. Walla Walla, WA

Patterson, M., et al. *Effects of Exercise and Oxandrolone Treatment on the Proteomic Biomarkers in Skeletal Muscle (PRISM) after Severe Burn Injury in Children*. Poster presented at: UTMB SURP Poster Presentation; 2007 August. Galveston, TX.