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1	Characterization of Disease Course after Intramuscular or Intranasal Exposure to Sin					
2	Nombre virus in Immunosuppressed Syrian Hamsters					
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24 ABSTRACT

25 Syrian hamsters exposed to Sin Nombre virus (SNV) become infected, but do not develop disease. In contrast, hamsters immunosuppressed with dexamethasone (Dex) and 26 cyclophosphamide (CyP) and infected with SNV develop lethal disease resembling hantavirus 27 pulmonary syndrome (HPS) in humans. Here, we provide a detailed analysis regarding the 28 kinetics of virus dissemination after both intramuscular and intranasal challenge. Our findings 29 revealed a ~8 day lag in the spread of virus to the lungs, kidney, liver, spleen, and heart that 30 correlates with a delayed mean day-to-death observed when immunosuppressed hamsters are 31 infected by the intranasal versus the intramuscular route. The length of immunosuppression with 32 Dex and CyP required for lethal disease for intramuscular challenge was 10 days; however 19 33 34 days of treatment were required in the intranasal model. Additionally, we investigated if the steroid-sparing potential of an alternative immunosuppressive drug, mycophenolate mofetil 35 (MMF) could replace the combination of Dex and CyP to produce lethal disease. However, 36 MMF treatment did not increase viral replication in the lung unless it was combined with Dex. 37 38 Furthermore, treatment of SNV-infected hamsters with MMF or Dex/MMF did not result in comparable lethality to hamsters administered Dex/CyP. Taken together, these experiments 39 40 further refine the SNV disease model in hamsters for future use in the evaluation of medical 41 countermeasures.

42 **INTRODUCTION**

43 Sin Nombre virus (SNV, genus Hantavirus, family Bunaviridae) is the predominant hantavirus in North America, and has been associated with sporadic outbreaks of lethal human 44 45 disease, including the 1993 outbreak in the Four Corners region of the United States (Duchin et al., 1994) and the more recent 2012 outbreak in Yosemite National Park (Centers for Disease and 46 47 Prevention, 2012). SNV is an etiological agent of hantavirus pulmonary syndrome (HPS), that is characterized in humans by leukocytosis, thrombocytopenia, and the rapid onset of acute 48 49 respiratory distress (Duchin et al., 1994; Manigold and Vial, 2014). Although HPS outbreaks are isolated and sporadic, unpredictable increases in the rodent vector populations can lead to 50 significant increases in human cases (Oliveira et al., 2016). In addition, the high case fatality rate 51 of approximately 35% (Jonsson et al., 2010) and the absence of specific drugs or vaccines to 52 53 treat or prevent HPS makes medical countermeasure development imperative. Well-

characterized animal models for the study of SNV pathogenesis will facilitate the development
of medical countermeasures.

Currently, there are two SNV animal models that recapitulate human HPS disease. These 56 are a small animal model utilizing immunosuppressed Syrian hamsters (Brocato et al., 2014) and 57 a nonhuman primate model using virus isolated directly from the rodent host (Safronetz et al., 58 2014). The use of these two animal models may represent a pathway to licensure for drugs or 59 biological products using the "Animal Rule" (21 CFR 314.600 and 21 CFR 601.90), respectively 60 (Snoy, 2010). Therefore, continued characterization of these models enhances their potential 61 suitability for use in drug or biological efficacy testing. In this report, we expand on our previous 62 data set by investigating the kinetics of SNV in immunosuppressed hamsters and refine the 63 64 length of immunosuppression required for disease.

Concerns over toxicity observed in lighter hamsters treated with Dex and CyP, along with 65 an effort to reduce daily injections required with Dex/CyP treatment, led us to search for 66 67 alternative immunosuppression methods. MMF (Cellcept, Genentech, Inc.), administered orally, is hydrolyzed to mycophenolic acid, an uncompetitive inhibitor of inosine monophosphate 68 dehydrogenase that inhibits the proliferation of B and T cells (Halloran, 1996). MMF, as with 69 most immunosuppressive drugs, was developed to prevent organ rejection following 70 transplantation. However, MMF is also currently prescribed for the treatment of lupus as a 71 steroid sparing drug (Kapitsinou et al., 2004). MMF is known to have reduced toxicity when 72 compared to CyP treatment (Mok, 2016) and may be able to replicate the leukopenia that is 73 74 necessary for SNV disease in hamsters (Brocato et al., 2014). Therefore, we also investigated the use of orally-administered MMF as an immunosuppressant alone and in combination with the 75 76 steroid Dex in the SNV hamster disease model.

77 **RESULTS**

78 SNV Tissue Burden

We have previously demonstrated that hamsters immunosuppressed with Dex and CyP
and infected with SNV by the intramuscular (i.m.) route develop lethal HPS disease (Brocato et
al., 2014). To refine and expand this proof-of-concept work, a serial sacrifice study was
performed in which immunosuppressed hamsters were infected with 2,000 PFU SNV by the i.m.

route or 4,000 PFU SNV by the intranasal (i.n.) route and then viral and immune response

84 kinetics were analyzed every two days. The kinetics of SNV in lung, kidney, liver, spleen, and

- heart were determined by viral genome detection (**Fig. 1A,B**). In each of the organs tested, there
- 86 was approximately a 6-8 day lag in SNV genome detection from hamsters infected by the i.n.
- 87 route when compared to i.m. challenge (**Fig. 1C**).

We have previously reported on the pathology in lungs from immunosuppressed hamsters 88 89 infected with SNV (Brocato et al., 2014). Here, we expand these pathology findings to the liver, kidney, spleen, and heart. Despite high viral load in these assayed organs, no significant lesions 90 were observed by histology in liver or kidney at any timepoint (Table S1). Neutrophilic 91 granulocytosis was observed in the red pulp of the spleens from immunosuppressed infected and 92 93 uninfected hamsters on day 6 in SNV i.m. hamsters and days 6 and 8 on SNV i.n. hamsters. In 94 addition, mild myocardial degeneration and necrosis was observed in the heart of 8% of infected 95 and uninfected hamsters; this lesion was not specific to treatment group or day postinfection and likely represents a commonly described background lesion in hamsters (McInnes, 2012). Neither 96 97 of these observed lesions were specifically collocalized with SNV antigen. Positive staining for SNV antigen was detected in centrilobular hepatocytes and Kupffer cells in liver tissue sections 98 99 (Fig. 2A). Similarly, glomerular mesangial cells were positive for SNV antigen in kidney tissue 100 sections (Fig. 2B), macrophages and fibroblastic reticular cells were positive in the red pulp of 101 the spleen (Fig. 2C), and cardiac myocytes and capillary endothelial cells were positive in heart tissue sections (Fig. 2D). This observed positive staining was present in animals from both 102 103 challenge routes.

104 The serial pathology data indicate that, regardless of challenge route, the lung is the 105 major site of virus replication. The expression of proinflammatory and immunomodulatory cytokine-related genes and transcription factors were analyzed in lung tissue from 106 immunosuppressed, SNV-infected (i.m.) hamsters (Fig. 3A,B,C). Day 0 represents data points 107 from immunosuppressed, uninfected controls. Increased expression of the proinflammatory 108 109 cytokines IL1β and IL6, and VEGF genes were observed later in infection, and coincided with 110 viremia in the lung (Fig. 1A). Increased expression of IRF2, STAT2, and iNOS were detected 111 throughout viral infection. Expression of the IFN-stimulated genes (ISGs) protein kinase R (PKR), oligoadenylate synthetase 3 (OAS3), and IFN-induced GTP-binding protein (Mx2) were 112

slightly elevated at various times through the acute infection. Increased levels of expression of

- 114 IFNγ and TNFα were not detected in immunosuppressed, SNV-infected hamsters. Levels of
- serum IFN- β were evaluated in immunocompetent and immunosuppressed hamsters infected
- with SNV. There was a significant reduction in IFN- β expression in infected hamsters treated
- with Dex and CyP 2 days postinfection (p<0.0001). However, IFN- β expression levels between
- 118 untreated or Dex/CyP-treated animals were not statistically significant by days 4 and 6
- 119 postinfection (**Fig. 3D**).

120 Optimization of SNV/Hamster Disease Models

121 To evaluate the length of immunosuppression required for SNV-induced HPS, hamsters were pretreated with a combination of Dex and CyP beginning on day -3 and 122 immunosuppression with Dex and CyP was stopped at various time points postinfection. On day 123 0, hamsters were challenged with 2,000 PFU by the i.m. route. Immunosuppression through day 124 7 postinfection resulted in significant lethality (day 7, p<0.0001, day 10, p=0.0024, day 13, 125 126 p=0.0085) (Fig. 4A). In contrast, immunosuppression through day 5 postinfection did not 127 produce uniform disease, but did result in a statistically significant 63% lethality (p=0.0133) compared to untreated, infected hamsters. In surviving hamsters, day 28 (end of study) lung 128 129 tissue was evaluated for SNV genome by RT-PCR. Hamsters treated with Dex and CyP had 130 elevated levels of SNV present in the lungs when compared to untreated, infected controls (Fig. **4B**). 131

132 Using immunosuppression beginning on day -3 through day 7 postinfection, a 50% lethal dose (LD50) experiment was performed to determine the SNV dose necessary for hamsters to 133 134 develop HPS. As little as 2 PFU SNV resulted in lethal disease in 50% of the animals in that group (Fig. 4C) when hamsters were challenged by the i.m. route. Statistical analysis determined 135 136 that the LD50 dosage is 2.5 PFU for this model. Hamster groups challenged with 20, 200, and 2,000 PFU SNV all exhibited statistically significant increases in lethality when compared to 137 138 uninfected hamsters (p=0.0243, p=0.0020, and p=0.0020, respectively). Lung tissue collected on day 28 from surviving animals demonstrate the presence of SNV genome in treated, infected 139 hamsters (Fig. 4D). 140

141 For the SNV i.n. model, the length of immunosuppression with Dex and CyP was determined by truncating immunosuppression to 13 and 16 days postinfection. When challenged 142 143 with 4,000 PFU SNV, only 12.5% of hamsters receiving Dex and CyP through day 13 developed lethal disease. However, 87.5% of hamsters receiving Dex and CyP through day 16 developed 144 lethal disease resulting in a statistically significant reduction in survival (p=0.0012) (Fig. 5A). Of 145 the 7 surviving hamsters on day 28 (end of study), results of a nucleocapsid (N)-ELISA assay 146 indicate that 6 of the hamsters immunosuppressed to day 13 developed an antibody response to 147 SNV and the lone survivor from the Dex/CyP treatment through day 16 did not develop an 148 antibody response to SNV above the limit of detection for the assay (Fig. 5B). Hamsters that 149 developed lethal disease had approximately 10^5 molecules of small (S)-segment genome in the 150 151 lung (Fig. 5C). Hamsters surviving to day 28 had comparable levels of S-segment genome in lung tissue; however, hamsters not receiving Dex and CyP developed an increased antibody 152 response to SNV infection when compared to hamsters receiving Dex and CyP through day 13, 153 although not statistically significant. There is not a direct correlation between the S-segment 154 genome detected and the magnitude of the antibody response as measured by N-ELISA. 155 However, the results of this experiment do support the hypothesis that longer 156 immunosuppression, through day 16 postinfection, is required to allow i.n. instilled SNV to 157

158 develop pathogenic disease in hamsters.

159 Alternative Immunosuppression Approach

The impact of replacing CyP with MMF was evaluated in SNV infection in hamsters. 160 Hamsters were immunosuppressed with either a combination of Dex and CyP, CyP alone, a 161 combination of Dex and MMF, MMF alone, or administered no immunosuppressive treatment 162 163 beginning on day -3 prior to infection through day 10 postinfection. All hamsters treated with MMF were administered a dosage of 30 mg/kg/day. Immunosuppression with the combination of 164 Dex and CyP provided the greatest lethality (86%, p=0.0029 when compared to no treatment 165 controls) (Fig. 6A), followed by immunosuppression with CvP alone (57%, p=0.0346 when 166 167 compared to no treatment controls) thereby confirming our previous report (Brocato et al., 2014). Immunosuppression with the combination of Dex and MMF or MMF alone resulted in 12% and 168 169 0% lethality, respectively. In addition, immunosuppression using solely MMF did not reduce WBCs (Fig. 6B). Lung tissue collected from a subset of animals on day 12 postinfection 170

171 demonstrates that Dex administered in combination with either CyP or MMF is required for a 172 statistically significant increase in SNV genome over No Treatment controls (p=0.0219 and 173 p=0.0089, respectively) (Fig. 6C). Correspondingly, the Dex/CyP and Dex/MMF groups had notable interstitial inflammation in the day 12 lung tissue (3/3 hamsters in both groups), more so 174 than groups receiving CyP or MMF alone, or left untreated (2/3, 2/3, and 0/3 hamsters per group, 175 respectively) (data not shown). RNA analysis of lung homogenates collected from surviving 176 177 animals on day 28 postinfection also supports the requirement for including Dex in a combination treatment (Fig. 6D). Furthermore, results of an N-ELISA conducted with sera from 178 surviving hamsters on day 28 postinfection show that the ability of hamsters to develop SNV-179 specific antibodies is reduced in hamsters immunosuppressed with CyP (alone [p=0.0179 when 180 compared to no treatment controls] or in combination with Dex) but not MMF (alone or in 181 combination with Dex) (Fig. 6E). 182

Increased dosages of MMF were evaluated in uninfected hamsters to determine if a higher concentration could reduce WBCs to levels observed in Dex/CyP treatment. Hamsters were treated with MMF at concentrations ranging from 30-360 mg/kg for two days by the oral route. On the third day, hematology analysis was conducted. The results of this dosing study indicate that even a >10-fold increase in MMF was not able to significantly reduce WBCs when compared to no treatment controls (**Fig. 7**).

189 **DISCUSSION**

190 Animal models are an essential component for elucidating viral pathogenesis, testing and evaluating vaccines, antivirals, and biologicals, and the potential licensing of those products. Our 191 192 first report on the SNV/immunosuppressed hamster model focused mainly on the disease in the target organ (i.e. lung) (Brocato et al., 2014); in the current study we demonstrate that SNV 193 194 disseminates to other organs, namely the kidney, liver, spleen, and heart. Others have demonstrated that dissemination can occur after serially passaging SNV through 195 196 immunocompetent Syrian hamsters (Safronetz et al., 2013b); here we demonstrate that this 197 dissemination can be replicated with low-passage cell culture virus in immunosuppressed hamsters. Similar to immunocompetent hamsters infected by Andes virus (ANDV) by the i.m. 198 route (Wahl-Jensen et al., 2007), there is an approximate 6 day incubation period before viremia 199 200 can be detected in immunosuppressed hamsters infected with SNV by the i.m. route. A 6-8 day

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201 delay in viremia is observed when comparing the SNV/immunosuppressed i.m. and i.n. routes of 202 infection. A similar delay in death is observed between these two routes for ANDV infection in 203 hamsters (Hooper et al., 2008). These ANDV/hamster models have been used to demonstrate that antivirals such as ribavirin, favipiravir, and neutralizing antibodies are only efficacious if 204 205 administered prior to the detection of viremia (Haese et al., 2015; Hooper et al., 2014; Hooper et al., 2008; Ogg et al., 2013; Safronetz et al., 2013a). Efforts to expand this therapeutic window 206 207 have been largely unsuccessful. The SNV/immunosuppressed hamster model provides an alternative small animal model for the evaluation of similar candidate medical countermeasures 208 against another important virus that causes HPS. 209

Viruses have evolved multiple mechanisms for subverting the host immune response 210 211 (Rouse and Horohov, 1986). ANDV modulation of early host innate responses both in vivo (Safronetz et al., 2011) and in vitro (Levine et al., 2010) may contribute to the establishment of 212 213 the infection and the associated pathogenicity observed in the hamster model. The lack of disease associated with SNV infection in immunocompetent hamsters may indicate that, unlike ANDV, 214 215 SNV is unable to modulate early innate responses. We hypothesize that dissemination of SNV and pathogenicity in immunosuppressed hamsters is caused by modulation of early host innate 216 217 responses and a subsequent inability of the adaptive immune response to contain and clear infection. We further speculate that the timing of the immune response to virus infection is 218 219 critical to result in an asymptomatic infection or death of the hamster. Negative regulation of transcription factors AP-1 and NF-kB by Dex treatment suppresses cytokines and chemokines 220 221 such as IL-2, IL-6, IFN- γ , and IL-8 (Karin, 1998). In addition, glucocorticoid treatment has been shown to inhibit signaling by cytokines that utilize the Jak-STAT pathway, namely IL-2 and IL-222 223 12 (Hu et al., 2003). In the current study, hamsters treated with Dex did not have early increased expression of these cytokines and chemokines. Expression of IL-6 later in infection is also 224 observed in human HPS cases (Borges et al., 2008; Morzunov et al., 2015) and represents the 225 226 hamsters' ability to initiate a proinflammatory response despite Dex treatment. VEGF and IL-6 227 promote migration of mononuclear cells to the lung and these cells are observed in the histological analysis of lung sections from SNV-infected, immunosuppressed hamsters (Brocato 228 et al., 2014). The lack of STAT1 upregulation, impacted by Dex treatment (Bhattacharyya et al., 229 2011), contributes to the suppression of IFN- γ (Hu et al., 2003), similar to the expression levels 230 231 of STAT1 and IFN- γ presented herein. Similarly, a reduction in IFN- β expression at short

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timepoints after SNV challenge (i.e. 2 days) likely contributes to increased SNV dissemination
and pathogenicity in immunosuppressed hamsters compared to immunocompetent hamsters. The
ANDV N has been shown to inhibit type I IFN signaling responses whereas SNV N does not
(Cimica et al., 2014). Treatment of hamsters with Dex may allow SNV to replicate in this host in
a manner similar to ANDV infection of immunocompetent hamsters.

SNV readily infects hamsters with a 50% infectious dose (ID50) of less than 2 PFU
(Hooper et al., 2001). When hamsters are immunosuppressed from day -3 to 7 days postinfection
and infected with SNV by the i.m. route, an LD50 dosage of 2.5 PFU was calculated. Thus, the
ability to infect hamsters appears to be very efficient regardless of whether the hamster is
immunosuppressed or not. These optimization experiments demonstrate the balance of the length
of immunosuppression required and challenge dose for a uniformly lethal model for the i.m. and
i.n. routes of exposure.

MMF has recently been accepted as a treatment to patients suffering from lupus nephritis, 244 as an alternative to CyP or CyP combined with a glucocorticoid (Tesar, 2016). By replacing CyP 245 with MMF in the SNV/hamster model, we evaluated the steroid sparing potential of MMF. This 246 experiment was rationalized by the possibility of administering a single drug, and the ability to 247 compound this drug into the feed for future experiments. A hallmark of the Dex/CyP model is 248 pronounced leukopenia resulting in a diminution of adaptive and innate immune responses that 249 allow SNV to replicate and cause acute disease. However, hamsters treated with MMF alone did 250 not exhibit the reduction in WBCs observed with CyP treatment, nor allow SNV to replicate in 251 the lung to levels observed when Dex was incorporated in the treatment regimen. 252 Immunosuppressive drugs were administered through day 10 postinfection. This timepoint was 253 254 selected from the truncated immunosuppression experiment (Fig. 4A) demonstrating that Dex/CyP treatment through day 10 resulted in 100% lethality. A single death was observed in the 255 Dex/MMF treatment group leading us to hypothesize that potentially extending the treatment 256 regimen beyond day 10 may have resulted in increased lethality. Increased concentrations of 257 258 MMF did not reduce WBCs in treated hamsters (Fig. 7); whether this is a species-specific phenomenon or a higher dosage of MMF is needed to induce leukopenia is currently unknown. 259 260 Future efforts will utilize the combination of Dex and CyP in the SNV/hamster model.

The SNV/immunosuppressed hamster model represents an alternative small animal lethal disease model that recapitulates many of the salient features of the ANDV/hamster model and human HPS disease. Further refinement of this model allows the testing of vaccines and medical countermeasures to combat hantavirus disease and may provide an alternative animal model for licensure of products under the FDAs "Animal Rule."

266 MATERIALS AND METHODS

Viruses, cells, and medium. SNV strain CC107 (Schmaljohn et al., 1995) was propagated in
Vero E6 cells (Vero C1008, ATCC CRL 1586). Preparation of twice plaque-purified SNV stock
has been described previously (Hooper et al., 2001). Cells were maintained in Eagle's minimum
essential medium with Earle's salts containing 10% fetal bovine serum, 10 mM HEPES, pH 7.4,
and Penicillin Streptomycin (Invitrogen) at 1X, and gentamicin sulfate (50 µg/ml) at 37°C in a
5% CO₂ incubator.

Dex, CyP, and MMF administration. Water soluble Dex and CyP monohydrate were 273 purchased from Sigma-Aldrich. MMF was purchased from Selleck Chemicals. On the indicated 274 days, anesthetized hamsters were injected intraperitoneally (i.p.) with the indicated dosages per 275 body weight of drug diluted in sterile phosphate-buffered saline (PBS), pH 7.4. Hamsters were 276 treated with a loading dose of 16 mg/kg Dex and 140 mg/kg CyP on day -3, 8 mg/kg Dex on day 277 -2, 8 mg/kg Dex and 100 mg/kg CyP on day -1, 4 mg/kg Dex on days 0, 2, 3, 5, 6, 8, 9, 11, 12, 278 13, 15, 16, 17, 19, 20, 21, and 4 mg/kg Dex and 100 mg/kg CyP on days 1, 4, 7, 10, 14, 18, and 279 22. Each experiment specifies the length of Dex and CyP immunosuppression administered to 280 animals. Hamsters were administered 30 mg/kg MMF by the oral route daily from day -3 to day 281 282 10 postinfection (Fig. 6). Hamsters were administered 30-360 mg/kg MMF by the oral route for 2 days prior to hematology (Fig. 7). 283

Challenge with hantavirus. Female Syrian hamsters 6-8 wks of age and > 100g (Envigo,
Indianapolis, IN) were anesthetized by inhalation of vaporized isoflurane using an IMPAC 6
veterinary anesthesia machine. Once anesthetized, hamsters were injected with the indicated
concentration of virus diluted in PBS. Intramuscular (i.m.) (caudal thigh) injections consisted of
0.2ml delivered with a 1ml syringe with a 25-gauge, 5/8in needle. Intranasal (i.n.) instillation
consisted of 50µl total volume delivered as 25µl per nare with a plastic pipette tip. Groups of 8

290 hamsters were typically used for experimental treatments, unless otherwise stated. All work

involving hamsters was performed in an animal biosafety level 4 (ABSL-4) laboratory.

Euthanasia was performed on animals meeting early endpoint criteria.

ELISA. The enzyme-linked immunosorbent assay (ELISA) used to detect N-specific antibodies

294 (N-ELISA) was described previously (Elgh et al., 1997; Hooper et al., 1999). The endpoint titer

was determined as the highest dilution that had an optical density (OD) greater than the mean

OD for serum samples from negative-control wells plus 3 standard deviations. The Puumala N

antigen was used to detect SNV N-specific antibodies as previously reported (Xiao et al., 1993).

298 Hamster-specific IFN-β ELISA (MyBiosource, San Diego, CA) was run according to

299 manufacturer's published protocols.

Isolation of RNA and real-time PCR. Approximately 250 mg of organ tissue was homogenized 300 in 1.0 ml TRIzol reagent using gentleMACS M tubes and a gentleMACS dissociator on the RNA 301 setting. RNA was extracted from TRIzol samples as recommended by the manufacturer. The 302 303 concentration of the extracted RNA was determined using a NanoDrop 8000 instrument and raised to a final concentration of 10 ng/ul. Real-time PCR was conducted on a BioRad CFX 304 thermal cycler using an Invitrogen Power SYBR Green RNA-to-Ct one-step kit according to the 305 manufacturer's protocols. Primer sequences are SNV S 26F 5'-CTA CGA CTA AAG CTG 306 GAA TGA GC-3' and SNV S 96R 5'-GAG TTG TTG TTC GTG GAG AGT G-3" (Trombley et 307 al., 2010). Cycling conditions were 30 min at 48°C, 10 min at 95°C, followed by 40 cycles of 15 308 sec at 95°C and 1 min at 60°C. Data acquisition occurs following the annealing step. 309

Host responses were monitored using hamster-specific primers for IL1 β , IL2, IL6, IL10,

311 IL12p35, IRF1, IRF2, IFNγ, iNOS, Mx2, OAS3, PKR, STAT1, STAT2, TNFα, and VEGF (Toth

et al., 2015; Zivcec et al., 2011) using HPRT as an internal control. Data was analyzed using the

 $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001) where the Ct was first normalized to the internal

control and then compared to an immunosuppressed, mock-treated animal.

Hematology. Blood samples collected in lithium heparin capillary blood collection tubes were

analyzed using an Advia 120 hematology analyzer using proprietary software version 3.1.8.0-

317 MS. The dog setting was used for the complete blood count and guinea pig setting was used for

the differential.

319 **Preparation of tissues for histology.** Tissues were fixed in 10% neutral-buffered formalin for 320 \geq 21 days. Tissues were then trimmed, processed under vacuum through increasing 321 concentrations of alcohols, and embedded in paraffin. 5-6 µm sections of paraffin embedded tissue were cut and mounted on glass slides, stained with hematoxylin-eosin (H&E), and 322 323 mounted under a glass coverslip for histologic evaluation. Immunolocalization of SNV in tissues was performed with an immunoperoxidase procedure (horseradish peroxidase EnVision system; 324 325 Dako) according to the manufacturer's directions. The primary antibody was an α-SNV nucleocapsid rabbit polyclonal antibody diluted 1:3,000 (provided by Diagnostic Service 326 Division, USAMRIID). Negative controls included naïve hamster tissue incubated with 327 nonimmune rabbit IgG in place of the primary antibody and naïve hamster tissue exposed to the 328 primary antibody and negative serum. After deparaffinization and peroxidase blocking, tissue 329 sections were pretreated with proteinase K for 6 min at room temperature, rinsed, and then 330 covered with primary antibody and incubated at room temperature for 1 hr. They were rinsed, 331 and then the peroxidase-labeled polymer (secondary antibody) was applied for 30 min. Slides 332 were rinsed, and a substrate-chromogen solution (3,3'-diaminobenzidine; Dako) was applied for 333 334 5 min. The substrate-chromogen solution was rinsed off the slides, and the slides were stained with hematoxylin and rinsed. The sections were dehydrated and cleared with xyless, and then a 335 336 coverslip was placed.

337 Statistical analysis. Survival curves were compared with Kaplan-Meier survival analysis with log-rank comparisons and Dunnett correction. A Bayesian probit model was used to estimate 338 339 95% highest posterior density intervals for a 50% lethal dose calculation. Comparison of viral genome results was done using a one-way ANOVA with Dunnett's multiple comparison test. 340 341 Comparison of WBC was done using a paired t test. Comparison of N-ELISA was done using Mann-Whitney test. P values of less than 0.05 were considered significant. Analyses were 342 conducted using GraphPad Prism (version 6); Bayesian analyses were performed using Stan 343 2.1.0. 344

Ethics statement. Animal research was conducted under an IACUC approved protocol at
USAMRIID (USDA Registration Number 51-F-00211728 & OLAW Assurance number A347301) in compliance with the Animal Welfare Act and other federal statutes and regulations
relating to animals and experiments involving animals. The facility where this research was

349 conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory

Animal Care, International and adheres to principles stated in the Guide for the Care and Use of

351 Laboratory Animals, National Research Council, 2011.

FIGURE LEGENDS

Figure 1. SNV kinetics in Syrian hamster organs. Three hamsters from each of the

immunosuppressed, SNV i.m. and i.n. groups were euthanized at each timepoint. Tissues were

excised, homogenized, and RNA isolated for SNV genome detection by RT-PCR. SNV S-

segment kinetics was measured in lungs, kidneys, livers, spleens, and hearts in hamsters infected

by the A) i.m. route and B) i.n. route. Mean values and \pm SD are shown. C) SNV genome organ

burden is shown as the mean of all organs evaluated from all animals at each timepoint. The lag

in SNV genome organ burden between the two routes of infection is shown as the blue arrow. A

single hamster on day 12 from the Dex/CyP SNV i.n. group is shown separately as an outlier

361 depicted by the filled triangle (\blacktriangle).

Figure 2. SNV antigen detection in hamster organs by immunohistochemistry.

363 Immunohistochemistry using an α -nucleocapsid antibody was performed on **A**) liver tissue, **B**)

kidney tissue, **C**) spleen tissue, and **D**) heart tissue from immunosuppressed, SNV-infected

hamsters collected on either day 10 (liver, kidney, and heart) or day 20 (spleen) postinfection. A,

B, **C**, 400X magnification. **D**, 200X magnification. Size bars are indicated in each panel.

367 Figure 3. Normalized fold expression of select cytokines in lung tissue of

368 immunosuppressed, SNV-infected hamsters. A, B, C) RNA from homogenized lung tissue

369 was isolated from immunosuppressed, SNV i.m. hamsters (n=3 per timepoint). Gene regulation

370 was normalized to the reference gene HPRT and compared using the $\Delta\Delta$ Ct method on the

indicated days. Individual values are shown with the horizontal line representing the mean. **D**)

372 Serum IFN- β levels from SNV-infected and immunosuppressed, SNV-infected hamsters

analyzed by ELISA. ***, P < 0.001; ns, not significant.

Figure 4. Optimization of immunosuppressed SNV i.m. model. A) The length of

immunosuppression with Dex and CyP required for the development of lethal HPS was

- determined in groups of 7 or 8 hamsters each. Hamsters were challenged with 2,000 PFU i.m.
- and immunosuppression beginning on day -3 was truncated to days 5, 7, 10, and 13 days

postinfection. Hamsters were monitored for survival. **B**) Lung tissues collected from surviving

- hamsters (day 28) were analyzed for the presence of SNV genome by RT-PCR. C) Groups of 10
- hamsters each were immunosuppressed with Dex and CyP from day -3 through day 7
- postinfection. Hamsters were challenged with the indicated concentration of virus on day 0 and
- monitored for survival. **D**) Lung tissues collected from surviving hamsters (day 28) were
- analyzed for the presence of SNV genome by RT-PCR. *, P < 0.05; **, P < 0.01; ***, P
- **384** 0.001.

Figure 5. Optimization of immunosuppressed SNV i.n. model. A) Groups of 8 hamsters each
were immunosuppressed with Dex and CyP beginning on day -3 through the indicated day. All
hamsters were infected with 4,000 PFU SNV by the i.n. route and monitored for survival. B)
Sera from surviving hamsters on day 35 were analyzed by N-ELISA. C) Lung tissues collected
after the onset of HPS (blue circles indicate hamsters were euthanized or found dead) or on day
postinfection were analyzed for the presence of SNV genome by RT-PCR. **, *P* < 0.01.

391 Figure 6. Alternative immunosuppression of hamsters using MMF. Groups of 6-8 hamsters each were immunosuppressed with CyP, Dex and CyP, MMF, Dex and MMF, or left untreated 392 beginning on day -3 through day 10 postinfection. All hamsters were infected with 2,000 PFU 393 SNV by the i.m. route on day 0. Hamsters were monitored for A) survival and B) WBCs. C) A 394 subset of hamsters not used for survival were euthanized on day 12 and lung homogenates 395 analyzed for SNV genome by RT-PCR. Hamsters surviving to day 28 were euthanized and **D**) 396 lung homogenates analyzed for SNV genome by RT-PCR and E) serum analyzed by N-ELISA. 397 *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ns, not significant. 398

Figure 7. Dose optimization of MMF in hamsters. Groups of 3 hamsters each were
administered increasing concentrations of MMF for two days by the oral route. Whole blood
collected on day 3 was analyzed for WBCs.

402

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- 410

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527

528 Figure 1



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531 Figure 2



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534 Figure 3





537 Figure 4



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540 Figure 5



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543 Figure 6











548 549

550

	Hamster Organ							
	Lung	Kidney	Liver	Spleen	Heart			
Dex/CyP	Histologic	Histologic	Histologic	Histologic	Histologic Finding:			
SNV 1.m.	Finding:	Finding	Finding	Finding: Red	Myocardial			
Day	Interstitial			Pulp	Degeneration/Necrosis			
Postinfection	Inflammation			Granulocytosis				
0	-	-	-	-	-			
2	-	-	-	-	+			
4	-	-	-	-	-			
0	++	-	-	++	-			
8	+	-	-	-	-			
10	++++	-	-	-	-			
12	++	-	_	-	-			
Dex/CyP SNV i.n. Day Postinfection								
0	_	_	_	_	_			
$\frac{3}{2}$	_	-	_	_	+			
4	_	-	_	_	+			
6	_	-	_	+	_			
8	+	-	_	+	-			
10	+	_	_	_	_			
12	+	-	_	_	-			
14	++	-	_	_	-			
16	++	-	_	_	++			
18	++++	-	_	_	_			
20	+++	_	_	_	_			
22	+++	-	-	-	-			
Dex/CyP No Virus Day Postinfection								
0	-	-	-	-	-			
2	-	-	-	-	++			
4	-	-	-	-	-			
6	+	-	_	_	+			
8	ND	_	-	+	-			
10	-	_	_	-	_			
12	+	_	_	+	_			
12	I			I				

551	Table S1.	Histologic fin	dings in Dex.	CyP SNV i.m.	., SNV i.n.,	and uninfected hamsters.
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552 Histopathological findings were scored as follows: +, very mild; ++, mild; +++, moderate; ++++,

553 marked; +++++, severe; -, negative or minimal finding. ND, no data due to autolysis of tissue.