

1 **Depletion of Alveolar Macrophages Does Not Prevent Hantavirus Disease Pathogenesis in**
2 **Golden Syrian Hamsters**

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12 Running Head: Depletion of AM θ in ANDV-infected hamsters

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20 ABSTRACT

21 Andes virus (ANDV) is associated with a lethal vascular leak syndrome in humans termed
22 hantavirus pulmonary syndrome (HPS). The mechanism for the massive vascular leakage
23 associated with HPS is poorly understood, however dysregulation of components of the
24 immune response is often suggested as a possible cause. Alveolar macrophages are found in
25 the alveoli of the lung and represent the first line of defense to many airborne pathogens. To
26 determine whether alveolar macrophages play a role in HPS pathogenesis, alveolar
27 macrophages were depleted in an adult rodent model of HPS that closely resembles human
28 HPS. Syrian hamsters were treated, intratracheally, with clodronate-encapsulated liposomes or
29 control liposomes and were then challenged with ANDV. Treatment with clodronate-
30 encapsulated liposomes resulted in significant reduction in alveolar macrophages but depletion
31 did not prevent pathogenesis or prolong disease. Depletion also did not significantly reduce the
32 amount of virus in the lung of ANDV-infected hamsters but altered neutrophil recruitment,
33 MIP-1 α and MIP-2 chemokine expression and VEGF levels in hamster BAL early after intranasal
34 challenge. These data demonstrate that alveolar macrophages may play a limited protective
35 role early after exposure to aerosolized ANDV, but do not directly contribute to hantavirus
36 disease pathogenesis in the hamster model of HPS.

37 IMPORTANCE

38 Hantaviruses continue to cause disease worldwide for which there are no FDA licensed
39 vaccines, effective post-exposure prophylactics or therapeutics. Much of this can be attributed
40 to a poor understanding of the mechanism of hantavirus disease pathogenesis. Hantavirus

41 disease has long been considered an immune-mediated disease; however, by directly
42 manipulating the Syrian hamster model, we continue to eliminate individual immune cell types.
43 As the most numerous immune cell present in the respiratory tract, alveolar macrophages are
44 poised to defend against hantavirus infection; but, those antiviral responses may also
45 contribute to hantavirus disease. Here, we demonstrate that, like our prior T and B cell studies,
46 alveolar macrophages neither prevent hantavirus infection nor cause hantavirus disease. While
47 these studies reflect pathogenesis in the hamster model, they should help us rule-out specific
48 cell types and prompt us to consider other potential mechanisms of disease in an effort to
49 improve the outcome of human HPS.

50

51 INTRODUCTION

52 Hantaviruses are enveloped members of the family *Bunyaviridae* that contain a tri-
53 segmented, negative-sense, single-strand RNA genome. The three gene segments, L, S, and M
54 encode the RNA polymerase, nucleoprotein, and envelope glycoproteins (G1 and G2),
55 respectively. While these pathogens are carried chronically and asymptotically in rodent
56 hosts, in humans, hantaviruses cause two unique vascular-leak syndromes that cover a
57 spectrum of severity ranging from proteinuria to pulmonary edema and frank hemorrhage(1-4).
58 Old-World hantaviruses, including Puumala virus (PUUV), Dobrava virus (DOBV), Seoul virus
59 (SEOV), and Hantaan virus (HTNV), have been associated with a mild-to-severe disease,
60 hemorrhagic fever with renal syndrome (HFRS). HFRS has a case-fatality rate between <0.1% to
61 15% and is characterized by fever, vascular leakage resulting in hemorrhagic manifestations and

62 renal failure. New-World hantaviruses have been associated with a highly lethal disease,
63 hantavirus pulmonary syndrome (HPS). HPS caused by the most prevalent North American and
64 South American hantaviruses, Sin Nombre virus (SNV) and Andes virus (ANDV), respectively, has
65 a case-fatality rate of 30-50% and is characterized by fever and vascular leakage resulting in
66 non-cardiogenic pulmonary edema followed by shock. Hantaviruses alter the barrier properties
67 of the microvascular endothelial cells that they infect, causing vascular leakage in the kidneys or
68 lungs (5). The specific mechanism underlying this endothelium dysfunction remains unknown,
69 but hantavirus infection of endothelial cells is nonlytic, suggesting that other, possibly host
70 derived factors, renders the endothelium unable to regulate barrier integrity, leading to
71 pulmonary edema (6).

72 While hantaviruses are known to cause disease by multiple routes of infection(5), the
73 predominant route of human exposure is thought to be inhalation of excreta from infected
74 rodent hosts (reviewed in references (6) and (7)) suggesting that cells in the alveoli may play an
75 important role in clearing, or alternatively, contributing to disease caused by aerosolized
76 hantaviruses. Alveolar macrophages (AM θ) are found in the alveoli and alveolar ducts of the
77 lung and represent the first line of defense to many airborne pathogens(8). Not only are they
78 crucial regulators of immune system activity through their secretion of either pro- or anti-
79 inflammatory cytokines, but they are vitally important in the maintenance and remodeling of
80 lung tissue via the production of growth factors, cytokines and proteinases. Activated AM θ are
81 known to provide a critical element of protection against pathogens(9, 10) by releasing
82 chemokines that recruit other innate immune cell types to areas of infection and secreting
83 antiviral cytokines. However, activation of AM θ , can also contribute to pathology by releasing

84 the same cytokines that are important in providing protection from pathogens(11-14). Alveolar
85 macrophages secrete multiple cytokines when activated including IL-1, IL-6, IL-8, TGF- β ,
86 inducible nitric oxide synthase (iNOS), and TNF α . Notably, the production of TNF α further
87 upregulates the release of other proinflammatory cytokines such as IL-1 β , IL-6, and IL-8 which
88 contribute to the initiation of adaptive immune responses(15). While these cytokines and
89 chemokines act locally to choreograph immune responses that are important for protection
90 against pulmonary pathogens, a number of these cytokines have been shown to promote
91 vascular permeability and pulmonary edema that are the hallmarks of pathogenic hantavirus
92 infection (16-20). Correspondingly, studies of humans infected with hantavirus have detected
93 high titers of proinflammatory and vasoactive cytokines in lung tissue of hantavirus pulmonary
94 syndrome (HPS) patients and high numbers of cytokine producing cells correlated with the
95 severity of HPS pathology (21). Moreover, systemic levels of inflammatory cytokines have also
96 been reported in plasma of patients with hemorrhagic fever with renal symptoms (HFRS) (22)
97 suggesting a role for these cytokines in disease pathogenesis.

98 Alveolar macrophages are known to be permissive to hantavirus infection(23, 24) but do
99 not appear to be primary targets of infection as hantavirus replication in alveolar macrophages
100 is less efficient than in endothelial cells. Furthermore, AM θ have been found to be associated
101 with hantavirus antigen in cases of human HPS(25) caused by SNV or in cases of "European
102 HPS" following PUUV infection(26) but it isn't clear if that is a result of direct infection of
103 alveolar macrophages or as a result of phagocytosis. Despite these associations, hantavirus
104 infection of human AM θ induced only modest antiviral responses and cell culture supernatants
105 from SNV infected AM θ failed to cause increased permeability of endothelial cell

106 monolayers(24) suggesting that soluble mediators secreted by infected AM θ do not contribute
107 to hantavirus disease.

108 ANDV causes a lethal disease in adult Syrian hamsters (27) that resembles HPS in humans
109 including the clinical signs including severe dyspnea, rapid progression from first signs to death,
110 fluid in the pleural cavity; the histopathology in the lungs and spleen; and the viral incubation
111 period(28). To determine if AM θ contribute to hantavirus disease in hamsters, we depleted
112 AM θ using clodronate-encapsulated liposomes, delivered prior to ANDV challenge. Clodronate
113 treatment significantly reduced the percent and number of AM θ in hamster bronchial alveolar
114 lavage (BAL) during intramuscular and intranasal ANDV challenge but had little effect on disease
115 pathogenesis. Depletion did result in a slightly more rapid and uniform disease course during
116 intranasal infection suggesting that AM θ may provide some protection against exposure to
117 airborne ANDV but overall, these data suggest that AM θ do not directly contribute to
118 hantavirus disease pathogenesis in the Syrian hamster model of human hantavirus pulmonary
119 syndrome.

120

121 **MATERIALS AND METHODS**

122 **Virus, cells, and medium.** ANDV strain Chile-9717869 (27) was propagated in Vero E6 cells
123 (Vero C1008, ATCC CRL 1586). Preparation of twice-plaque-purified ANDV stock has been
124 described previously(27). Cells were maintained in Eagle's minimum essential medium with
125 Earle's salts containing 10% fetal bovine serum, 10 mM HEPES, pH 7.4, penicillin-streptomycin
126 (Invitrogen) at 1 \times , and gentamicin sulfate (50 μ g/ml) at 37°C in a 5% CO₂ incubator.

127 **Challenge with hantavirus.** Female Syrian hamsters 6 to 8 weeks of age (Harlan, Indianapolis,
128 IN) were anesthetized by inhalation of vaporized isoflurane using an IMPAC 6 veterinary
129 anesthesia machine. For intramuscular (i.m.) challenges, anesthetized hamsters were injected
130 with 80 PFU (10 LD₅₀) of virus diluted in PBS (0.2 ml, caudal thigh) delivered with a 1-ml syringe
131 with a 25-gauge, five-eighths-inch needle. For intranasal (i.n.) challenges, anesthetized
132 hamsters were administered 50 µl delivered as 25 µl per nare with a plastic pipette tip (4,000
133 PFU ANDV total, 42 LD₅₀). Groups of 8 hamsters were typically used for experimental
134 treatments, unless otherwise stated. All work involving hamsters was performed in an animal
135 biosafety level 4 (ABSL-4) laboratory. Hamsters were observed two to three times daily.

136 **Macrophage depletion.** Clodronate-encapsulated liposomes (Clodrosome – 5mg/ml
137 clodronate) and control PBS-encapsulated liposomes (Encapsome) were purchased from
138 Encapsula Nano Sciences. Hamsters were anesthetized using 0.2 ml / 100g rat KAX (ketamine-
139 acepromazin-xylazine) administered by i.m. injection. Each animal was then placed in a dorsal
140 recumbent position and an otoscope (Welch Allyn) was used visualize the vocal folds. The vocal
141 folds were numbed by topically administering a 2% Lidocaine HCl jelly (Akorn) and then a 16G x
142 1¼” Surflo catheter (Terumo) was passed between the vocal folds. Hamsters were then
143 treated with either 0.2 ml Clodrosome or 0.2 ml Encapsome by attaching a loaded syringe to
144 the catheter and aspirating the contents into the lung.

145 **Flow cytometry analysis.** Hamsters were deeply anesthetized (0.4 ml Rat KAX / 100g) and then
146 extensively perfused with sterile saline (Baxter) before being euthanized. To isolate alveolar
147 macrophages, animals were placed in a dorsal recumbent position, then a midline neck incision

148 was made and dissected down carefully so that the trachea was exposed. A second incision
149 was made near the xyphoid process then scissors were used to carefully remove the rib cage
150 exposing the lungs ensuring not to damage the lungs in the process. A 16g x 1 ¼" catheter was
151 inserted into the trachea and the lungs were lavaged 3 times using 1 ml of a 0.02% EDTA
152 solution. BAL samples were then centrifuged at 514 x g for 5 minutes. Cells were then
153 collected and washed twice in PBS containing 2% FBS. In some experiments, cells were
154 incubated at 4° C for 15 min in a blocking buffer consisting of PBS containing 2% FBS and 2%
155 normal rat serum (Sigma–Aldrich) prior to staining with antibody. Approximately 10⁶ cells were
156 stained with mouse-anti-hamster MARCO(29) (clone PAL-1; 10µg/100µl; AbD Serotec) followed
157 by anti-mouse IgM (clone RMM-1; 0.4µg/ml; BioLegend) for 15-20 min at 4° C. Stained cells
158 were then were fixed in Cytofix buffer (BD Biosciences) for 15 min at 4°C before being analyzed
159 on a FACSCalibur™ flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences)
160 or FACSCanto™ II flow cytometer (BD Biosciences) using FACSDiva software (BD Biosciences).
161 AM and neutrophil cell numbers in BAL preparations were mathematically determined by
162 comparing cell numbers to numbers of PKH26 reference microbeads (Sigma) using the formula:
163 # cells/ml = (# cell events x dilution factor/# bead events x dilution factor) x # beads/ml. Data
164 were analyzed using FlowJo software (Treestar).

165

166 **Plaque assay.** Hantavirus plaque assays were performed as previously described(30).

167 **Isolation of RNA and real-time PCR.** Approximately 250 mg of lung tissue was homogenized in
168 1.0 ml TRIzol reagent using gentleMACS M tubes and a gentleMACS dissociator on the RNA

169 setting. RNA was extracted from TRIzol samples as recommended by the manufacturer. The
170 concentration of the extracted RNA was determined using a NanoDrop 8000 instrument and
171 raised to a final concentration of 10 ng/ μ l. Real-time PCR was conducted on a Bio-Rad CFX
172 thermal cycler using an Invitrogen Power SYBR green RNA-to- C_T one-step kit according to the
173 manufacturer's protocols. Primer sequences are as follows (26): ANDV S 41F, 5' - GAA TGA GCA
174 CCC TCC AAG AAT TG - 3'; ANDV S 107R, 5' - CGA GCA GTC ACG AGC TGT TG - 3'. Cycling
175 conditions were 30 min at 48°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C.
176 Data acquisition occurred following the annealing step.

177 **Hamster cytokine ELISAs.**

178 Anti-hamster MIP-1 α (MBS033532), MIP-2 (MBS006761), TNF α (MBS046042) and VEGF-A
179 (MBS024541) ELISA ELISA kits were purchased from MyBioSource and were used according to
180 the manufacturer's recommendations.

181 **Preparation of tissues for histology.** Tissues were fixed in 10% neutral buffered formalin,
182 trimmed, processed, embedded in paraffin, cut at 5 to 6 μ m, and stained with hematoxylin and
183 eosin (H&E) for histopathology analysis. To determine the presence of ANDV antigens in
184 association with alveolar macrophages or colocalized with endothelial cells, serial sections were
185 then stained as follows. For ANDV immunohistochemistry, a monoclonal antibody
186 (USAMRIID#1244) against ANDES virus was used on all tissue slides. Normal mouse IgG was
187 used as the negative serum control for the control slides. Briefly, the unstained sections were
188 deparaffinized, rehydrated, and pretreated with Tris/EDTA buffer for 30 minutes at 95-100°C.
189 Slides were rinsed and a serum free protein block with 5% horse serum was applied for 30

190 minutes. The monoclonal antibody was then applied to the tissue at a dilution of 1:1200 and
191 incubated for 1 hour at room temperature. The slides were then treated with alkaline
192 phosphatase labeled secondary mouse IgG antibody (Vector Labs, Burlingame, CA. cat# MP-
193 5402) for 30 minutes at room temperature. All slides were exposed to ImmPACT Vector® Red
194 (Vector Labs, Burlingame, CA. cat# SK-5105) substrate-chromagen for 30 minutes, rinsed,
195 counterstained with hematoxylin, dehydrated and cover-slipped with Permount®(Fisher,cat#
196 SP15-500). For CD31 immunohistochemistry, an immunoperoxidase assay was performed using
197 a rabbit anti-CD31 polyclonal antibody (Abcam; cat# ab28364). A normal rabbit IgG was used as
198 the negative serum control for the control slides. Briefly, the unstained sections were
199 deparaffinized, rehydrated, subjected to a methanol hydrogen peroxide block, rinsed and
200 pretreated with Tris/EDTA buffer for 30 minutes at 95-100°C. Slides were rinsed and a serum
201 free protein block with 5% goat serum was applied for 30 minutes. The polyclonal antibody was
202 then applied to the tissue at a dilution of 1:75 and incubated overnight at room temperature.
203 The slides were then treated with the EnVision horseradish peroxidase labeled secondary
204 antibody (Dako, Carpinteria, CA, cat# K4007) for 30 minutes at room temperature. All sections
205 were exposed to a DAB (3,3-diaminobenzidine) substrate-chromagen for 5 minutes, rinsed,
206 counterstained with hematoxylin, dehydrated and cover-slipped with Permount.

207

208 **Statistical analysis.** Survival curves were compared with Kaplan-Meier survival analysis with
209 log-rank comparisons and Dunnett's correction. Comparisons of viral genome, infectious virus,
210 alveolar macrophage and neutrophil percent and number, and ELISA cytokine titers were done
211 using a one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test. P values

212 of less than 0.05 were considered significant. Analyses were conducted using GraphPad Prism
213 (version 5).

214 **Ethics statement.** Research at U.S. Army Medical Research Institute of Infectious Diseases
215 (USAMRIID) was conducted under an Institutional Animal Care and Use Committee (IACUC)
216 approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other federal
217 statutes and regulations relating to animals and experiments involving animals. The facility
218 where this research was conducted is accredited by the Association for Assessment and
219 Accreditation of Laboratory Animal Care, International and adheres to principles stated in the
220 Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

221

222 **RESULTS**

223 **Depletion of AM θ does not prevent disease following intramuscular ANDV challenge.**

224 Clodronate has been used extensively to deplete AM θ in many animal models including the
225 Syrian hamster (31). In the Syrian hamster, AM θ were identified as FSC^{hi}SSC^{hi}, MARCO
226 expressing cells in hamster BAL fluid (Fig. 1A) and intratracheal administration of clodronate
227 encapsulated liposomes was found to effectively reduce the number of AM θ in hamsters during
228 ANDV infection as determined by a reduction in either MARCO⁺ cells (Fig. 1B and 1D) or
229 FSC^{hi}SSC^{hi} cells (Fig. 1C and 1D) and histopathologic analysis of hamster lung tissue (Fig. 1E)
230 during ANDV infection of hamsters. To begin to understand the role that AM θ play during
231 ANDV disease pathogenesis, hamsters were treated intratracheally with clodronate

232 encapsulated liposomes (Clodrosome) or control PBS encapsulated liposomes (Encapsomes) on
233 days -3 and -1. One group of hamsters was left untreated. Hamsters were then challenged
234 with 80 pfu (10 LD₅₀) ANDV i.m. 10 days later, the number and percent of AM θ in hamster BAL
235 were determined. Encapsome treatment did induce an increase in the number of alveolar
236 macrophages (**Fig. 2A**) but by comparison, Clodrosome treatment resulted in a significant
237 reduction in the total number of alveolar macrophages (**Fig. 2A**). Macrophage depletion did not
238 prevent disease in hamsters (**Fig. 2B**) or significantly alter the mean time to death (Clodrosome
239 = 12.88 days, Encapsome = 13.13 days, untreated = 11.38 days) The mean time to death
240 following Encapsome treatment was significantly longer compared to untreated animals (13.13
241 days vs. 11.38 days; p=0.05) but not when compared to Clodrosome treated animals. Depletion
242 of AM θ also did not result in increased ANDV titers in the lung as measured by PCR (**Fig. 2C**).
243 These data suggest that despite becoming activated, AM θ are not important for protection
244 against an intramuscular ANDV challenge nor do they contribute to disease pathogenesis
245 following intramuscular challenge.

246 Serial sections of lung tissue from these groups further revealed the presence of ANDV antigen
247 co-localized to CD31 positive endothelial cells in both capillaries and larger vessels (**Fig. 3A-3D**).
248 Regardless of treatment, no differences were observed in the pathogenesis of HPS-like disease
249 in ANDV-infected hamsters. Hamsters in all groups exhibited signs of mild to moderate
250 inflammation, interstitial pneumonia, alveolar fibrin deposition and edema characteristic of
251 ANDV infection.

252 Also noticed were multifocal foci of neutrophilic inflammation, along with mesothelial
253 hypertrophy and atelectasis. The presence of necrotic/apoptotic debris was rare. Consistent

254 with the detection of viral genome by PCR, immunohistochemistry analysis revealed little to no
255 difference in overall viral load within endothelial cells following clodrosome treatment.

256 Interestingly, all identifiable alveolar macrophages found in ANDV infected hamsters were
257 negative for Andes virus although positive cytoplasmic CD31 staining (**Fig. 3E**).

258

259 **Depletion of AM θ does not prevent disease following intranasal ANDV challenge**

260 Alveolar macrophages are more likely to be involved in the defense against airborne pathogens.

261 To understand the protective or pathogenic responses that AM θ elicit to inhaled hantaviruses,

262 AM θ were depleted prior to intranasal ANDV challenge. Groups of hamsters were treated

263 intratracheally with Clodrosomes or control Encapsomes on days -3 and -1. One group of

264 hamsters was left untreated. Hamsters were then challenged with 4000 pfu (42 LD₅₀) ANDV i.n.

265 10 and 17 days later, the number and percent of AM θ in hamster BAL were determined. Similar

266 to what was seen after intramuscular challenge, 10 days after intranasal challenge the

267 percentage and total number of AM θ in untreated or Encapsome treated animals were

268 comparable (**Fig. 4A**). Encapsome treatment resulted in a trend towards increased numbers of

269 AM θ compared to untreated animals but this difference was not significant. By comparison,

270 Clodrosome treatment resulted in a significant reduction in both the percent and total number

271 of AM θ . The percent of AM θ remained significantly reduced following clodrosome treatment

272 17 days after intranasal challenge (**Fig. 4B**). Surprisingly, though, was the observation that the

273 number of AM θ at day 17 in the untreated group was significantly lower than the number of

274 AM θ in the untreated group at day 10 (**Fig. 4C**). This phenomenon was only seen in the

275 untreated groups as the number of AM θ in the Encapsome treated animals remained similar

276 between days 10 and 17. Although the total number of AM θ in the Clodrosome treated animals
277 was lower on day 17 than on day 10, the difference between number of AM θ in the Clodrosome
278 treated animals and untreated animals on day 17 was not significant. However, the difference
279 in the number of AM θ in the Encapsome treated animals and untreated animals on day 17 was
280 significant. The reduction in AM θ did not prevent disease in hamsters (**Fig. 4D**) but depletion
281 did result in a more uniform and slightly more rapid disease course (mean time to death:
282 Clodrosome = 14.75 days, Encapsome = 21.43 days, untreated = 19.13 days). ANDV titers in the
283 lung were not significantly different in Clodrosome-treated hamster either day 10 or day 17 as
284 determined by the presence of viral genome measured by PCR (**Fig. 4E**). However, there was a
285 trend towards increased ANDV M copy number in the lungs of Clodrosome-treated animals 10
286 days post challenge.

287 TNF α levels in hamster BAL samples were significantly higher at the peak of disease
288 compared to earlier time points (**Fig. 5A**; day 17 versus day 10) but AM θ depletion only affected
289 TNF α expression early after infection. Interestingly, treatment with either Clodrosomes or
290 control Encapsomes resulted in an increase in TNF α detected compared to untreated hamsters
291 which could reflect the higher numbers of neutrophils and AM θ present in the BAL samples
292 from these groups, respectively (**Fig. 4B and 6D**). The reduction in AM θ did not reduce the
293 amount of TNF α detected in BAL samples 10 days after intramuscular challenge (**Fig. 5B**) which
294 was similar to the amount of TNF α detected in BAL samples 10 day after intranasal challenge.
295 Remarkably, TNF α levels at the peak of disease following intranasal challenge (day 17) were
296 nearly twice that detected at the peak of disease following intramuscular challenge possibly
297 reflecting the differences in AM θ activation when virus is administered directly to the lung.

298 These data suggest that AM θ do not contribute to disease pathogenesis but may contribute
299 some degree of protection against following intranasal ANDV challenge.

300 **Depletion of AM θ alters neutrophil recruitment early, but not late, after intranasal ANDV**
301 **challenge**

302 Alveolar macrophages coordinate many aspects of immune responses to airborne
303 pathogens, including the recruitment of other immune cell types such as neutrophils. At the
304 peak of hantavirus disease following intramuscular infection with ANDV (day 10), we observed
305 a decrease in the neutrophil chemoattractant MIP-1 α in BAL samples from AM θ depleted
306 hamsters compared to untreated ANDV infected hamsters suggesting AM θ may be an
307 important source of MIP-1 α during hantavirus infection in the lung(**Fig. 6A**). However, the
308 expression of the neutrophil chemoattractant MIP-2 in BAL samples from all hamsters
309 remained unchanged(**Fig. 6B**), and, correspondingly, there was also little significant change in
310 the number of neutrophils found in hamster BAL samples 10 days after intramuscular challenge
311 (**Fig. 6C**). By comparison, when we investigated the role of AM θ in recruitment of neutrophils
312 following intranasal challenge, we found that early in disease pathogenesis (day 10), MIP-1 α
313 and MIP-2 expression were reduced in both Clodrosome and control Encapsome treated
314 hamsters compared to untreated ANDV infected hamsters (**Fig. 6D and 6E**). We also found that
315 Encapsome treatment resulted in increased numbers of recruited AM θ (**Fig. 4B**) while
316 Clodrosome treatment resulted in an increased number of neutrophils (**Fig. 6F**). At the peak of
317 disease following intranasal ANDV challenge (day 17), the expression of MIP-1 α and MIP-2 was
318 reduced approximately 50% compared to the expression seen on day 10. Moreover, the

319 expression of MIP-1 α and MIP-2 was not dependent on treatment as equivalent amount of
320 MIP-1 α and MIP-2 were detected in BAL samples from all hamster (**Fig. 6D and 6E**). Neutrophil
321 numbers were reduced in clodronate-treated animals by day 17 compared to numbers
322 observed on day 10 but neutrophil numbers in the control Encapsome-treated and untreated
323 hamsters remained virtually unchanged between days 10 and 17(**Fig. 6G**). In contrast, AM θ
324 numbers in Encapsome-treated hamsters remained elevated on days 10 and 17 despite an
325 overall drop in AM θ numbers in untreated hamsters (**Fig.4a and 4B**). These data suggest that
326 AM θ may regulate neutrophil recruitment to the lung early after hantavirus infection but do not
327 contribute significantly to neutrophil recruitment towards the peak of disease pathogenesis.

328 **Depletion of AM θ alters VEGF-A expression early after intranasal ANDV challenge**

329 Recently, vascular endothelial growth factor (VEGF) has been hypothesized to play a role
330 in hantavirus disease pathogenesis. Moreover, AM θ are known sources of VEGF. We therefore
331 asked whether VEGF expression in the lungs of hamsters infected with ANDV was dependent on
332 the presence of AM θ . Compared to normal uninfected hamsters, VEGF-A expression in the BAL
333 of hamsters 10 days after intranasal ANDV challenge was only slightly, but not significantly
334 elevated (**Fig. 7**). Interestingly, at the time of peak disease on day 17, there was almost a two-
335 fold increase in VEGF-A protein. Macrophage depletion did not further enhance VEGF-A in BAL
336 samples late into infection (day17) as we observed no difference in VEGF titers in ANDV
337 infected hamster BAL samples in the presence or absence of AM θ . However, when AM θ were
338 depleted, VEGF-A expression in the BAL on day 10 was equivalent to the amount of VEGF-A
339 detected in all hamster BAL samples on day 17. A similar increase in VEGF was observed in

340 Encapsome-treated hamsters. These data demonstrate that VEGF expression is enhanced in
341 hamsters infected with ANDV but suggest that while AM θ may regulate the expression of VEGF
342 by other cell types in the lung, they are not a major source of VEGF during hantavirus disease
343 pathogenesis.

344

345 **DISCUSSION**

346 The human lung has a surface area of approximately 70m², and contains, on average,
347 480 million alveoli (32) which are in constant contact with the outside world. In this
348 environment, homeostasis along the endothelial/epithelial boarder must be maintained to
349 allow oxygen and carbon dioxide to freely exchange; however, these homeostatic mechanisms
350 must remain pliable enough to allow immune responses to clear invading pathogens. Often
351 considered the first line of defense against respiratory pathogens, the estimated 2 billion AM θ
352 residing in the alveoli of the human lung(33) are uniquely juxtaposed to maintain lung
353 homeostasis as well as orchestrate protection against airborne viruses and bacteria(34). In the
354 case of hantaviruses, the predominant route of human exposure is thought to be inhalation of
355 excreta from infected rodent hosts (reviewed in references (6) and (7)) suggesting that alveolar
356 macrophages may play an important role in clearing, or alternatively, contributing to disease
357 caused by aerosolized hantaviruses. Here, we demonstrate that alveolar macrophages play
358 only a marginal role in protecting hamsters from lethal hantavirus infection but do not
359 contribute to the disease caused by hantaviruses.

360 Alveolar macrophages contribute to the defense against many aerosolized pathogens,
361 and in many cases, these responses are critical for host protection. In models of vaccinia virus,
362 RSV and influenza virus infection, the depletion of alveolar macrophages results in greater viral
363 replication and dissemination and an overall increase in the severity of infection (35-38). In
364 some cases, the reduced levels of protection in the absence of alveolar macrophages is likely
365 due to the impaired initiation of antiviral responses that result in abolished early cytokine and
366 chemokine release and inhibited immune cell activation and recruitment (38, 39). In addition,
367 Schneider et al (40) demonstrated that when alveolar macrophages were depleted in mice prior
368 to infection with influenza virus, mice exhibited lower percent sO₂ and pO₂ pressure arguing
369 that AM θ are important for maintaining lung function during infection. However, the same
370 mechanisms that alveolar macrophages use to protect against pathogens have also been
371 implicated in causing disease and increased vascular permeability in models of acute lung injury
372 caused by infectious disease agents, such as human metapneumovirus (hMPV) (41) and
373 *Pseudomonas aeruginosa* (42) as well as chronic obstructive pulmonary disease (COPD)(43) and
374 nonischemic inflammatory lung injury (44). Alveolar macrophages may also serve as a reservoir
375 for pathogens such as human metapneumovirus (hMPV) (41), measles virus(45) and *Legionella*
376 *pneumophila*(46) and may indirectly contribute to the pathogenesis diseases caused by these
377 pathogens by allowing for their replication and dissemination. Still, the enhancement of
378 disease in the presence of alveolar macrophages may not always reflect a direct contribution by
379 AM θ by way of proinflammatory cytokines or angiogenic factors but may indirectly be the result
380 of increased immune cell recruitment by AM θ as seen in mouse models of mouse hepatitis virus
381 type 1 (MHV-1) infection (47).

382 The role that AM θ play in disease caused by classical hemorrhagic fever viruses is less
383 well understood. Alveolar macrophages express the primary and secondary receptors(48) for
384 both hantaviruses ($\alpha\beta$ 3 integrin(49) and complement receptor 3 and 4 (CR3 / CR4) (50)) and
385 Ebola virus (DC-SIGN and DC-SIGNR(51)) and, corresponding, are known to be permissive to
386 hantavirus (23, 24) and Ebola virus infection(52). However, in these cases, infection is less
387 efficient or fails to induce a sustained inflammatory response compared to the primary targets
388 of infection for these viruses and there is no evidence that hantavirus infection of AM θ induces
389 apoptosis. Antigens of Yellow Fever virus, which is transmitted by the bite of the *Aedes aegypti*
390 *mosquito*, can be found inside the rough endoplasmic reticulum and Golgi complex of AM θ ,
391 suggesting that viral replication can occur in these cells(53, 54) but it is unknown whether AM θ
392 play any role in disease pathogenesis other than acting as a virus reservoir. Still, it is unknown
393 whether other hemorrhagic fever viruses that commonly target monocyte lineage cells such as
394 Dengue virus and Crimean Congo hemorrhagic fever virus target AM θ in a way that contributes
395 to human disease. We failed to detect the presence of ANDV NP associated with hamster AM θ
396 (Fig. 3E) suggesting that AM θ may only constitute a relatively minor target for hantaviruses and
397 AM θ dysfunction due to direct hantavirus infection is unlikely. Our analysis of ANDV NP
398 associated with AM θ was limited to intramuscular challenge so it is possible that a greater
399 associated may be seen following intranasal challenge. However, we see no difference in HPS
400 pathology in hamsters following either i.n. or i.m. challenge. Moreover, our analysis was done
401 at the peak of disease pathogenesis following i.m. challenge (Day 10) when ANDV NP staining of
402 the lung endothelium was nearly continuous suggesting that the likelihood of ANDV / AM θ
403 interactions would be as high as that following intranasal challenge.

404 How hamster AM θ respond to ANDV infection isn't entirely clear. Alveolar
405 macrophages, in general, need to walk a fine line between homeostasis and host defense to
406 protect the host while preventing catastrophic inflammation. One way AM θ contribute to lung
407 protection is by phagocytizing most of the particulate matter that enters the lungs. This
408 suggested that they may also contribute to the clearance of ANDV from the lungs of hamsters.
409 Early after intranasal ANDV challenge we did see a trend toward increased detection of viral
410 genome in hamsters that were depleted of AM θ although depletion of AM θ does not
411 significantly alter the amount of live virus or viral genome detected in lung tissue late after
412 infection (**Fig. 3E and 3F**). Hamsters devoid of AM θ also developed disease faster than
413 untreated or control treated ANDV-infected hamsters. Interestingly, the highest number of
414 surviving animals was found in encapsome-treated animals (**Fig. 3D**). Correspondingly, this
415 group also had greater numbers of AM θ than clodronate-treated or untreated animals(**Fig. 3A-**
416 **C**). Like other models, this could suggest that early after infection, AM θ help prevent the
417 spread of infection by reducing infectious virus in the lung and by doing so may help control the
418 rate at which disease pathogenesis progresses. At later times after infection, similar levels of
419 viral genome and/or infectious virus was found in the lung of all hamsters arguing that AM θ
420 contribute more substantially to the immune response against ANDV early after infection but
421 less so at later times once ANDV is primarily replicating in endothelial cells. This is also
422 consistent with the reduced numbers of AM θ detected on day 17 compared to day 10.

423 A second way AM θ contribute to lung protect is by modulating immune responses in
424 the lung(34, 55). In the presence of harmless particulates such as dust, they may go as far as
425 suppressing antigen-specific adaptive immune responses by either directly suppressing tissue-

426 resident T cells(56, 57) or by suppressing lung-resident dendritic cells(58), thus preventing them
427 from migrating to draining lymph nodes and initiating immune responses. However, in the case
428 of aerosolized pathogens, activation of AM θ results in a change in phenotype from a regulator
429 of lung homeostasis to that of a cell capable of coordinating and participating in inflammatory
430 immune responses (59, 60). Human AM θ make little TNF α when exposed to SNV compared to
431 LPS (24) suggesting that hantavirus may be ineffective at activating AM θ . Correspondingly, we
432 noticed little difference in the amount of TNF α in the BAL of hamsters in the presence or
433 absence of AM θ following infection with ANDV intramuscularly (**Fig. 4A**) or at late times after
434 intranasal infection (**Fig. 4B**). In the case of intramuscular infection, it's not clear whether AM θ
435 would be effectively stimulated since it is assumed that infection of the endothelium would
436 occur directly via the blood and not by inhalation. We did observe an increase in the amount of
437 TNF α in BAL samples between day 10 and day 17 post-intranasal challenge indicating that an
438 inflammatory response was occurring. However, at late times, depletion of macrophages did
439 not reduce the amount of TNF α detected suggesting that AM θ are not a major source of TNF α
440 that late in infection. Somewhat surprisingly, at early times (day 10) after intranasal challenge,
441 the depletion of AM θ resulted in an increase in TNF α in BAL samples. One explanation is that in
442 hamsters, AM θ are more prone to an immunosuppressive phenotype and by depleting them,
443 other cell types including neutrophils, endothelial cells, T cells and epithelial cells are no longer
444 prevented from producing TNF α . The fact that control Encapsome treated animals also had
445 increased levels of TNF α may be reflected in the increased numbers of AM θ induced by
446 Encapsome treatment. Resident AM θ at the time of Encapsome may retain their

447 immunosuppressive phenotype but any newly recruited AM θ could be expected to have a
448 markedly different phenotype prior to adopting suppressive functions (61).

449 As immune sentinels, one of the primary roles of AM θ is to orchestrate immune
450 responses by recruiting other immune cell types to the lung. In some models of acute lung
451 injury(62, 63) (44)) including pneumonia induced by LPS or *P. aeruginosa pneumonia* infection,
452 depletion of AM θ attenuates the recruitment of neutrophils to the lung indicating that AM θ can
453 be a key source of neutrophil chemoattractants. Conversely, in other models, AM θ appear to
454 play a greater role in the negative regulation of neutrophil migration in that the depletion of
455 AM θ amplifies neutrophil recruitment (35, 42, 64-68). Neutrophils migrate in response to a
456 number of chemoattractants(69) including CXCL2 (MIP-2 [mouse] / GRO β [human]) and CCL3
457 (MIP-1 α), of which AM θ can be a major source(38, 70-72). Clodronate treatment resulted in a
458 decrease in MIP-1 α detected in BAL samples on day 10 following intramuscular ANDV challenge
459 (**Fig. 5A**) and also resulted in a decrease in MIP-1 α and MIP-2 on day 10 following intranasal
460 challenge (**Fig.5B**). Surprisingly, the decrease in MIP-1 α and MIP-2 in clodronate treated
461 animals following intranasal challenge was accompanied by an increase in BAL neutrophils (**Fig.**
462 **5D**). Moreover, control encapsome treatment also resulted in decreases in MIP-1 α and MIP-2
463 but recruitment of new AM θ rather than neutrophils. One possible explanation for this
464 apparent paradox is that AM θ are an important source of MIP-1 α and MIP-2 in the hamster but
465 hamster neutrophils preferentially respond to other chemokines, such as monocyte
466 chemoattractant protein-1 (MCP-1) or KC, that may be more abundant in the absence of MIP-
467 1 α and MIP-2. Alternatively, MIP-1 α and MIP-2 are preferentially secreted by cell types other
468 than neutrophils in the hamster and the presence of AM θ suppresses neutrophil migration.

469 When alveolar macrophages are depleted, neutrophils freely migrate to the lung and act as a
470 MIP-1 α /MIP-2 “sponge” which soaks up free chemokine and reduces overall levels of
471 bioavailable MIP-1 α /MIP-2. A similar explanation could hold true for the decreased abundance
472 of MIP-1 α and MIP-2 following control encapsome treatment in which newly arrived AM θ act as
473 the chemokine sponge. A closer analysis of the kinetics of MIP-1 α and MIP-2 expression
474 following alveolar macrophage depletion would be necessary to elucidate these possibilities.
475 The expression of MIP-1 α and MIP-2 on day 17 following intranasal ANDV challenge was similar
476 across all treatment groups and substantially lower than that detected in ANDV alone hamsters
477 on day 10. Whether this reflects decreased numbers of AM θ at day 17 versus day 10 or
478 whether this is a natural attempt by the hamster to downregulate lung inflammation has yet to
479 be determined. We believe this is first report of hamster-specific ELISA kits from a commercial
480 vendor to be used with the Syrian hamster animal model. Given the brevity between the
481 publishing of the Syrian hamster genome and the commercial availability of these ELISA kits ,
482 we can not discount the possibility that the cytokine expression patterns we see following
483 clodrosome/encapsome treatments of ANDV-infected hamsters are related to the specificity of
484 these kits. As such, while these measurements are reproducible, they should be interpreted
485 within the context of the other parameters measured in these experiments (e.g. survival and
486 AM θ numbers) until verified by other independent reports.

487 The potential role of VEGF in hantavirus disease pathogenesis has recently received a
488 great deal of attention. In vitro, hantavirus infection sensitizes endothelial cells to VEGF
489 rendering them hyperpermeable(73) in a process involving VE-cadherin(74, 75) and potentially
490 β 3 integrin(76). Similarly, increased levels of VEGF can be detected in pleural effluent from

491 patients with acute HPS(77) or in serum samples of acute HFRS patients(78). Alveolar
492 macrophages are known to express VEGF during pulmonary infection and other forms of acute
493 lung injury (79-84)suggesting that AM θ may respond similarly to hantavirus infection. Still, the
494 fact that all hamsters infected with ANDV developed disease (**Fig. 2E and 3D**) suggested that
495 AM θ do not significantly influence the expression of VEGF in hamsters. A significant increase in
496 VEGF expression in the BAL of ANDV infected hamsters in late after infection (Day 17) was
497 observed compared to normal hamsters but, as expected, the level of VEGF detected in the BAL
498 of ANDV infected animals was nearly identical at later times after infection (Day 17) regardless
499 of the presence or absence of AM θ (**Fig. 6**). However, contrary to our expectations, the
500 depletion of AM θ resulted in a significant increase in the level of VEGF in the BAL of ANDV-
501 infected hamsters at early times after intranasal challenge. Interestingly, when macrophages
502 were depleted in animals prior to intranasal challenge, those animals developed disease faster
503 than either untreated ANDV-infected animals or animals receiving control liposome treatments.
504 This would seem to support the suggestion that VEGF contributes to hantavirus disease but it
505 would argue that AM θ are not the sole source of VEGF. Pulmonary epithelial cells (85, 86) and
506 neutrophils (87, 88) are other known sources of VEGF in the lung and could be contributing to
507 the increased levels of VEGF seen in ANDV-infected hamsters. Epithelial cells are not the
508 primary targets of hantaviruses and are thus unlikely to be expressing VEGF as a result of direct
509 infection, but expression could be induced by inflammatory cytokines produced by other cells
510 during infection or by hypoxia caused during HPS(89). Recently, a role for neutrophils in
511 vascular leakage caused by HTNV infection of SCID mice has been suggested(90).
512 Correspondingly, clodrosome treatment resulted in both a significant increase in BAL VEGF and

513 neutrophil numbers early after infection (Day10). Whether the depletion of neutrophils in
514 hamsters alters hantavirus disease pathogenesis and the expression of VEGF in hamsters
515 remains to be determined.

516 Small animal models are invaluable tools to study the pathogenesis of diseases caused
517 by neglected infectious disease agents such as Andes virus. However, the utility of the hamster
518 model, as well as the role of the immune response in hantavirus disease pathogenesis is
519 contentious. Previously, we and others have demonstrated that the ablation of adaptive T and
520 B cell responses to ANDV infection in hamsters does not alter the course of disease (91, 92).
521 Here, using the Syrian hamster / Andes virus lethal disease model we demonstrate that another
522 component of the immune system is not directly responsible for the HPS-like disease cause by
523 ANDV in hamsters. The mechanism by which hantaviruses cause disease in humans and
524 hamsters alike is not clear and many mechanisms of disease, both immune related and virus
525 intrinsic, have been proposed. Making this more difficult is that aspects of the immune
526 response to hantavirus infection are likely to be important for protection and viral clearance,
527 even as they are viewed as contributing to disease. Still, it will be necessary to continue to
528 evaluate other immune cell types, as we seek to understand their role in contributing to disease
529 or protection following hantavirus infection.

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543 **FIGURE LEGENDS**

544 **Figure 1. Identification and depletion of AM θ in Syrian hamsters.** (A) AM θ from Syrian
545 hamster BAL fluid were analyzed for the expression of the MARCO scavenger receptor by flow
546 cytometry. Hamsters were treated intratracheally with Clodrosomes or control Encapsomes on
547 days -3 and -1 prior to an 80 pfu ANDV i.m. challenge . 10 days post ANDV challenge, the ability
548 of Encapsome or Clodrosome treatment to deplete AM θ was determined by analyzing the
549 percent of MARCO⁺ cells (B) or FSC^{hi}/SSC^{hi} cells (C) in hamster BAL samples. The percent of
550 MARCO⁺ cells or FSC^{hi}/SSC^{hi} cells was then quantified (D) (*, $P < 0.05$; ***, $P < 0.001$). (E)
551 Staining of lungs of day 10 ANDV-infected hamsters with H&E (total magnification, $\times 400$). 5 to 6
552 μm sections of one of the cranial lung lobes was stained with H&E to visually determine if the

553 number of alveolar macrophages is affected when treated with Clodrosomes, Encapsomes or
554 when left untreated. Black arrows indicate alveolar macrophages.

555 **Figure 2. Depletion of AM θ does not prevent disease following intramuscular ANDV**

556 **challenge.** Hamsters were treated intratracheally with Clodrosomes or control Encapsomes on
557 days -3 and -1 or were left untreated. On day 0, all hamsters were challenged with 80 pfu
558 ANDV by intramuscular infection. **(A)** 10 days post ANDV challenge, the number of AM θ was
559 determined by flow cytometry by gating on FSC^{hi}/SSC^{hi} cells (as described in Fig. 1) in hamster
560 BAL samples (**, $P < 0.01$; ****, $P < 0.0001$). **(B)** The depletion of AM θ did not prevent disease
561 in hamsters. Lung tissue isolated from all hamsters 10 days post-challenge were evaluated for
562 viral genome **(C)** by RT-PCR (not significant).

563 **Figure 3. Depletion of AM θ does not alter the localization of ANDV NP to CD31 positive**

564 **endothelial cells.** 5 to 6 μ m serial sections from one of the cranial lobes of Day 10 i.m. ANDV-
565 infected hamsters treated with Clodrosomes **(A)**, or Encapsomes **(B)**, untreated ANDV-infected
566 hamsters **(C)**, or normal uninfected hamsters **(D)** were stained with antibodies specific for CD31
567 (DAB - brown) or ANDV NP (Alkaline phosphatase - red). ANDV NP staining colocalized to CD31
568 positive cells in adjacent serial sections. Normal hamster tissue remained negative for ANDV
569 NP. No differences were seen in the pattern of CD31 and/or ANDV NP staining across
570 treatment groups. **(E)** Alveolar macrophages from untreated ANDV-infected hamsters were
571 evaluated for the presence of ANDV NP and CD31. All were found to be CD31 positive but no
572 evidence of positive staining for ANDV NP was detected.

573 **Figure 4. Depletion of alveolar macrophages does not prevent disease following intranasal**
574 **ANDV challenge.** Hamsters were treated intratracheally with Clodrosomes or control
575 Encapsomes on days -3 and -1 or were left untreated. On day 0, all hamsters were challenged
576 with 4000 pfu ANDV by intranasal infection. **(A)** 10 days and **(B)** 17 days post ANDV challenge,
577 the number of AM θ was determined by flow cytometry by gating on FSC^{hi}/SSC^{hi} cells (as
578 described in Fig. 1) in hamster BAL samples (**, $P < 0.01$; ***, $P < 0.001$; ns, not significant). **(C)**
579 The number of AM θ on days 10 and 17 in untreated, ANDV-challenged hamsters was directly
580 compared (**, $P < 0.01$). **(D)** The depletion of AM θ did not prevent disease in hamsters. All
581 surviving animals seroconverted indicating that they had been exposed to virus (data not
582 shown). Lung tissue isolated from all hamsters 10 and 17 post-challenge were evaluated for
583 viral genome (E) by RT-PCR.

584 **Figure 5. Depletion AM θ alters TNF α expression but only early after intranasal ANDV**
585 **challenge.** Hamsters were treated intratracheally with Clodrosomes or control Encapsomes on
586 days -3 and -1 or were left untreated. On day 0, all hamsters were challenged with either 4000
587 pfu ANDV by intranasal infection or with 80 pfu ANDV by intramuscular infection. BAL samples
588 were collected from all hamsters 10 and 17 days after intranasal challenge **(A)** or 10 days after
589 intramuscular challenge **(B)** and TNF α expression was analyzed by ELISA. The depletion of AM θ
590 resulted in increased TNF α levels 10 days after intranasal ANDV challenge **(A)** (**, $P < 0.01$) but
591 did not affect TNF α levels 17 days after intranasal challenge or 10 days after intramuscular
592 ANDV challenge **(B)**. TNF α expression in BAL samples from untreated ANDV-challenged
593 hamsters following either intranasal or intramuscular virus challenge were directly compared
594 **(C)** (****, $P < 0.0001$).

595 **Figure 6. Depletion of AM θ alters neutrophil recruitment and neutrophil chemoattractant**
596 **expression early after intranasal ANDV challenge.** BAL samples were collected from all
597 hamsters 10 days after intramuscular challenge (**A-C**) or 10 and 17 days after 4000 pfu
598 intranasal ANDV challenge (**D-G**) and were analyzed for the presence of neutrophils by flow
599 cytometry and MIP-1 α and MIP-2 by ELISA. Both Clodrosome and Encapsome treatments
600 resulted in a decrease in MIP-1 α (**A**) but did not alter MIP-2 expression (**B**) or the number of
601 neutrophils (**C**) compared to untreated hamsters after intramuscular challenge. Clodrosome
602 and Encapsome treatments resulted in a decrease in both MIP-1 α (**D**) and MIP-2 expression (**E**).
603 Clodrosome treatment resulted in an increase in the number of neutrophils in hamster BAL
604 fluid on day 10 (**F**) but not on day 17 (**G**) after intranasal challenge (*, $P < 0.05$; **, $P < 0.01$; ***,
605 $P < 0.001$; ****, $P < 0.0001$; ns, not significant).

606 **Figure 7. Depletion of AM θ alters VEGF-A expression early after intranasal ANDV challenge.**
607 BAL samples were collected from all hamsters 10 and 17 days after 4000 pfu intranasal ANDV
608 challenge and were analyzed for the presence of VEGF-A by ELISA. Both Clodrosome and
609 Encapsome treatments resulted in increased VEGF-A expression 10 days post ANDV challenge
610 comparable to VEGF-A levels found in all hamsters 17 days post challenge (**, $P < 0.01$; ***, $P <$
611 0.001 ; ****, $P < 0.0001$; ns, not significant).

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615 **References**

616

- 617 1. **Schmaljohn C, Hjelle B.** 1997. Hantaviruses: a global disease problem. *Emerging infectious*
618 *diseases* **3**:95-104.
- 619 2. **Peters CJ, Simpson GL, Levy H.** 1999. Spectrum of hantavirus infection: hemorrhagic fever with
620 renal syndrome and hantavirus pulmonary syndrome. *Annual review of medicine* **50**:531-545.
- 621 3. **Khan A, Khan AS.** 2003. Hantaviruses: a tale of two hemispheres. *Panminerva medica* **45**:43-51.
- 622 4. **Jonsson CB, Hooper J, Mertz G.** 2008. Treatment of hantavirus pulmonary syndrome. *Antiviral*
623 *research* **78**:162-169.
- 624 5. **Hooper JW, Ferro AM, Wahl-Jensen V.** 2008. Immune serum produced by DNA vaccination
625 protects hamsters against lethal respiratory challenge with Andes virus. *Journal of virology*
626 **82**:1332-1338.
- 627 6. **Hooper JW, Li D.** 2001. Vaccines against hantaviruses. *Current topics in microbiology and*
628 *immunology* **256**:171-191.
- 629 7. **Schmaljohn CSaJWH.** 2001. Bunyaviridae: The Viruses and Their Replication, p. 1581-1602,
630 *Fields Virology*, 4 ed. Lippincott, Williams, and Wilkins, Philadelphia, PA.
- 631 8. **Smith MR, Standiford TJ, Reddy RC.** 2007. PPARs in Alveolar Macrophage Biology. *PPAR*
632 *research* **2007**:23812.
- 633 9. **Kim HM, Lee YW, Lee KJ, Kim HS, Cho SW, van Rooijen N, Guan Y, Seo SH.** 2008. Alveolar
634 macrophages are indispensable for controlling influenza viruses in lungs of pigs. *Journal of*
635 *virology* **82**:4265-4274.
- 636 10. **Tumpey TM, Garcia-Sastre A, Taubenberger JK, Palese P, Swayne DE, Pantin-Jackwood MJ,**
637 **Schultz-Cherry S, Solorzano A, Van Rooijen N, Katz JM, Basler CF.** 2005. Pathogenicity of
638 influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar
639 macrophages and neutrophils in limiting virus replication and mortality in mice. *Journal of*
640 *virology* **79**:14933-14944.
- 641 11. **Farley KS, Wang L, Mehta S.** 2009. Septic pulmonary microvascular endothelial cell injury: role
642 of alveolar macrophage NADPH oxidase. *American journal of physiology* **296**:L480-488.
- 643 12. **Naidu BV, Woolley SM, Farivar AS, Thomas R, Fraga CH, Goss CH, Mulligan MS.** 2004. Early
644 tumor necrosis factor-alpha release from the pulmonary macrophage in lung ischemia-
645 reperfusion injury. *The Journal of thoracic and cardiovascular surgery* **127**:1502-1508.
- 646 13. **Sakamaki F, Ishizaka A, Urano T, Sayama K, Nakamura H, Terashima T, Waki Y, Soejima K,**
647 **Tasaka S, Sawafuji M, Kobayashi K, Yamaguchi K, Kanazawa M.** 2003. Attenuation by
648 intravenous 2-chloroadenosine of acute lung injury induced by live escherichia coli or latex
649 particles added to endotoxin in the neutropenic state. *The Journal of laboratory and clinical*
650 *medicine* **142**:128-135.
- 651 14. **Wallaert B, Bart F, Aerts C, Ouaisi A, Hatron PY, Tonnel AB, Voisin C.** 1988. Activated alveolar
652 macrophages in subclinical pulmonary inflammation in collagen vascular diseases. *Thorax* **43**:24-
653 30.
- 654 15. **Raychaudhuri SP, Nguyen CT, Raychaudhuri SK, Gershwin ME.** 2009. Incidence and nature of
655 infectious disease in patients treated with anti-TNF agents. *Autoimmunity reviews*.
- 656 16. **Beynon HL, Haskard DO, Davies KA, Haroutunian R, Walport MJ.** 1993. Combinations of low
657 concentrations of cytokines and acute agonists synergize in increasing the permeability of
658 endothelial monolayers. *Clinical and experimental immunology* **91**:314-319.

- 659 17. **Kerkar S, Williams M, Blocksom JM, Wilson RF, Tyburski JG, Steffes CP.** 2006. TNF-alpha and IL-
660 1beta increase pericyte/endothelial cell co-culture permeability. *J Surg Res* **132**:40-45.
- 661 18. **Kotowicz K, Callard RE, Klein NJ, Jacobs MG.** 2004. Interleukin-4 increases the permeability of
662 human endothelial cells in culture. *Clin Exp Allergy* **34**:445-449.
- 663 19. **Maruo N, Morita I, Shirao M, Murota S.** 1992. IL-6 increases endothelial permeability in vitro.
664 *Endocrinology* **131**:710-714.
- 665 20. **Nooteboom A, Bleichrodt RP, Hendriks T.** 2006. Modulation of endothelial monolayer
666 permeability induced by plasma obtained from lipopolysaccharide-stimulated whole blood.
667 *Clinical and experimental immunology* **144**:362-369.
- 668 21. **Mori M, Rothman AL, Kurane I, Montoya JM, Nolte KB, Norman JE, Waite DC, Koster FT, Ennis
669 FA.** 1999. High levels of cytokine-producing cells in the lung tissues of patients with fatal
670 hantavirus pulmonary syndrome. *The Journal of infectious diseases* **179**:295-302.
- 671 22. **Linderholm M, Ahlm C, Settergren B, Waage A, Tarnvik A.** 1996. Elevated plasma levels of
672 tumor necrosis factor (TNF)-alpha, soluble TNF receptors, interleukin (IL)-6, and IL-10 in patients
673 with hemorrhagic fever with renal syndrome. *The Journal of infectious diseases* **173**:38-43.
- 674 23. **Li W, Klein SL.** 2012. Seoul virus-infected rat lung endothelial cells and alveolar macrophages
675 differ in their ability to support virus replication and induce regulatory T cell phenotypes. *Journal
676 of virology* **86**:11845-11855.
- 677 24. **Khaiboullina SF, Netski DM, Krumpe P, St Jeor SC.** 2000. Effects of tumor necrosis factor alpha
678 on sin nombre virus infection in vitro. *Journal of virology* **74**:11966-11971.
- 679 25. **Zaki SR, Greer PW, Coffield LM, Goldsmith CS, Nolte KB, Foucar K, Feddersen RM, Zumwalt RE,
680 Miller GL, Khan AS, et al.** 1995. Hantavirus pulmonary syndrome. Pathogenesis of an emerging
681 infectious disease. *The American journal of pathology* **146**:552-579.
- 682 26. **Gizzi M, Delaere B, Weynand B, Clement J, Maes P, Vergote V, Laenen L, Hjelle B, Verroken A,
683 Dive A, Michaux I, Evrard P, Creytens D, Bulpa P.** 2013. Another case of "European hantavirus
684 pulmonary syndrome" with severe lung, prior to kidney, involvement, and diagnosed by viral
685 inclusions in lung macrophages. *European journal of clinical microbiology & infectious diseases* :
686 official publication of the European Society of Clinical Microbiology **32**:1341-1345.
- 687 27. **Hooper JW, Larsen T, Custer DM, Schmaljohn CS.** 2001. A lethal disease model for hantavirus
688 pulmonary syndrome. *Virology* **289**:6-14.
- 689 28. **Padula PJ, Colavecchia SB, Martinez VP, Gonzalez Della Valle MO, Edelstein A, Miguel SD, Russi
690 J, Riquelme JM, Colucci N, Almiron M, Rabinovich RD.** 2000. Genetic diversity, distribution, and
691 serological features of hantavirus infection in five countries in South America. *Journal of clinical
692 microbiology* **38**:3029-3035.
- 693 29. **Palecanda A, Paulauskis J, Al-Mutairi E, Imrich A, Qin G, Suzuki H, Kodama T, Tryggvason K,
694 Koziel H, Kobzik L.** 1999. Role of the scavenger receptor MARCO in alveolar macrophage binding
695 of unopsonized environmental particles. *The Journal of experimental medicine* **189**:1497-1506.
- 696 30. **Wahl-Jensen V, Chapman J, Asher L, Fisher R, Zimmerman M, Larsen T, Hooper JW.** 2007.
697 Temporal analysis of Andes virus and Sin Nombre virus infections of Syrian hamsters. *Journal of
698 virology* **81**:7449-7462.
- 699 31. **Nemmar A, Nemery B, Hoet PH, Van Rooijen N, Hoylaerts MF.** 2005. Silica particles enhance
700 peripheral thrombosis: key role of lung macrophage-neutrophil cross-talk. *American journal of
701 respiratory and critical care medicine* **171**:872-879.
- 702 32. **Ochs M, Nyengaard JR, Jung A, Knudsen L, Voigt M, Wahlers T, Richter J, Gundersen HJ.** 2004.
703 The number of alveoli in the human lung. *American journal of respiratory and critical care
704 medicine* **169**:120-124.
- 705 33. **Pollmacher J, Figge MT.** 2014. Agent-based model of human alveoli predicts chemotactic
706 signaling by epithelial cells during early *Aspergillus fumigatus* infection. *PloS one* **9**:e111630.

- 707 34. **Holt PG, Strickland DH, Wikstrom ME, Jahnsen FL.** 2008. Regulation of immunological
708 homeostasis in the respiratory tract. *Nature reviews. Immunology* **8**:142-152.
- 709 35. **Rivera R, Hutchens M, Luker KE, Sonstein J, Curtis JL, Luker GD.** 2007. Murine alveolar
710 macrophages limit replication of vaccinia virus. *Virology* **363**:48-58.
- 711 36. **Benoit A, Huang Y, Proctor J, Rowden G, Anderson R.** 2006. Effects of alveolar macrophage
712 depletion on liposomal vaccine protection against respiratory syncytial virus (RSV). *Clinical and*
713 *experimental immunology* **145**:147-154.
- 714 37. **Reed JL, Brewah YA, Delaney T, Welliver T, Burwell T, Benjamin E, Kuta E, Kozhich A, McKinney**
715 **L, Suzich J, Kiener PA, Avendano L, Velozo L, Humbles A, Welliver RC, Sr., Coyle AJ.** 2008.
716 Macrophage impairment underlies airway occlusion in primary respiratory syncytial virus
717 bronchiolitis. *The Journal of infectious diseases* **198**:1783-1793.
- 718 38. **Pribul PK, Harker J, Wang B, Wang H, Tregoning JS, Schwarze J, Openshaw PJ.** 2008. Alveolar
719 macrophages are a major determinant of early responses to viral lung infection but do not
720 influence subsequent disease development. *Journal of virology* **82**:4441-4448.
- 721 39. **Murphy EA, Davis JM, McClellan JL, Carmichael MD, Rooijen NV, Gangemi JD.** 2011.
722 Susceptibility to infection and inflammatory response following influenza virus (H1N1,
723 A/PR/8/34) challenge: role of macrophages. *J Interferon Cytokine Res* **31**:501-508.
- 724 40. **Schneider C, Nobs SP, Heer AK, Kurrer M, Klinke G, van Rooijen N, Vogel J, Kopf M.** 2014.
725 Alveolar macrophages are essential for protection from respiratory failure and associated
726 morbidity following influenza virus infection. *PLoS pathogens* **10**:e1004053.
- 727 41. **Kolli D, Gupta MR, Sbrana E, Velayutham TS, Chao H, Casola A, Garofalo RP.** 2014. Alveolar
728 macrophages contribute to the pathogenesis of human metapneumovirus infection while
729 protecting against respiratory syncytial virus infection. *American journal of respiratory cell and*
730 *molecular biology* **51**:502-515.
- 731 42. **Kooguchi K, Hashimoto S, Kobayashi A, Kitamura Y, Kudoh I, Wiener-Kronish J, Sawa T.** 1998.
732 Role of alveolar macrophages in initiation and regulation of inflammation in *Pseudomonas*
733 *aeruginosa* pneumonia. *Infection and immunity* **66**:3164-3169.
- 734 43. **Duan M, Li WC, Vlahos R, Maxwell MJ, Anderson GP, Hibbs ML.** 2012. Distinct macrophage
735 subpopulations characterize acute infection and chronic inflammatory lung disease. *J Immunol*
736 **189**:946-955.
- 737 44. **Naidu BV, Krishnadasan B, Farivar AS, Woolley SM, Thomas R, Van Rooijen N, Verrier ED,**
738 **Mulligan MS.** 2003. Early activation of the alveolar macrophage is critical to the development of
739 lung ischemia-reperfusion injury. *The Journal of thoracic and cardiovascular surgery* **126**:200-
740 207.
- 741 45. **Ferreira CS, Frenzke M, Leonard VH, Welstead GG, Richardson CD, Cattaneo R.** 2010. Measles
742 virus infection of alveolar macrophages and dendritic cells precedes spread to lymphatic organs
743 in transgenic mice expressing human signaling lymphocytic activation molecule (SLAM, CD150).
744 *Journal of virology* **84**:3033-3042.
- 745 46. **Copenhaver AM, Casson CN, Nguyen HT, Fung TC, Duda MM, Roy CR, Shin S.** 2014. Alveolar
746 macrophages and neutrophils are the primary reservoirs for *Legionella pneumophila* and
747 mediate cytosolic surveillance of type IV secretion. *Infection and immunity* **82**:4325-4336.
- 748 47. **Hartwig SM, Holman KM, Varga SM.** 2014. Depletion of alveolar macrophages ameliorates
749 virus-induced disease following a pulmonary coronavirus infection. *PLoS one* **9**:e90720.
- 750 48. **Raftery MJ, Lalwani P, Krautkrmer E, Peters T, Scharffetter-Kochanek K, Kruger R, Hofmann J,**
751 **Seeger K, Kruger DH, Schonrich G.** 2014. beta2 integrin mediates hantavirus-induced release of
752 neutrophil extracellular traps. *The Journal of experimental medicine* **211**:1485-1497.

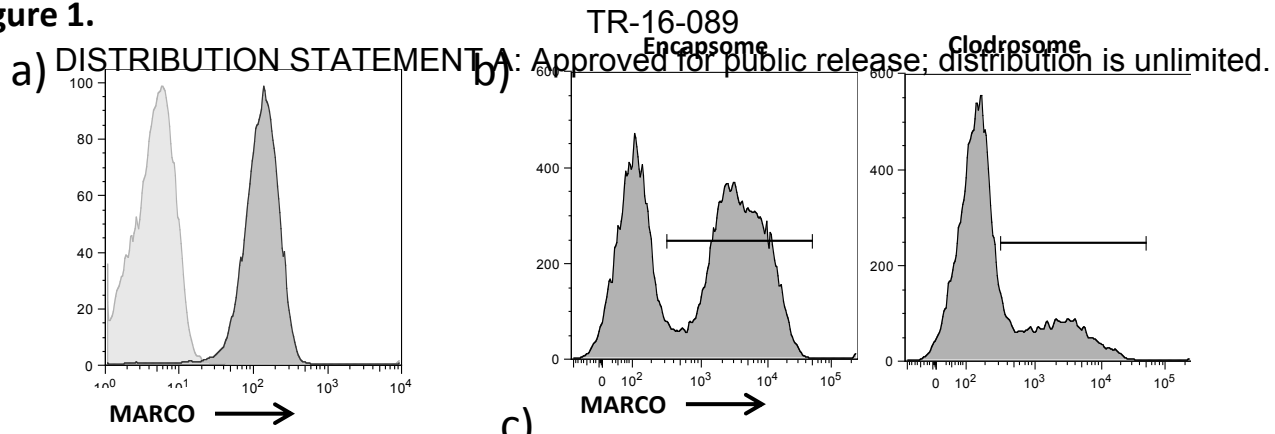
- 753 49. **Hodge S, Hodge G, Ahern J, Jersmann H, Holmes M, Reynolds PN.** 2007. Smoking alters alveolar
754 macrophage recognition and phagocytic ability: implications in chronic obstructive pulmonary
755 disease. *American journal of respiratory cell and molecular biology* **37**:748-755.
- 756 50. **Gil M, McCormack FX, Levine AM.** 2009. Surfactant protein A modulates cell surface expression
757 of CR3 on alveolar macrophages and enhances CR3-mediated phagocytosis. *The Journal of*
758 *biological chemistry* **284**:7495-7504.
- 759 51. **Simmons G, Reeves JD, Grogan CC, Vandenberghe LH, Baribaud F, Whitbeck JC, Burke E,**
760 **Buchmeier MJ, Soilleux EJ, Riley JL, Doms RW, Bates P, Pohlmann S.** 2003. DC-SIGN and DC-
761 SIGNR bind ebola glycoproteins and enhance infection of macrophages and endothelial cells.
762 *Virology* **305**:115-123.
- 763 52. **Gibb TR, Norwood DA, Jr., Woollen N, Henchal EA.** 2002. Viral replication and host gene
764 expression in alveolar macrophages infected with Ebola virus (Zaire strain). *Clinical and*
765 *diagnostic laboratory immunology* **9**:19-27.
- 766 53. **Barreto DF, Takiya CM, Schatzmayr HG, Nogueira RM, Farias-Filho Jda C, Barth OM.** 2007.
767 Histopathological and ultrastructural aspects of mice lungs experimentally infected with dengue
768 virus serotype 2. *Memorias do Instituto Oswaldo Cruz* **102**:175-182.
- 769 54. **Povoa TF, Alves AM, Oliveira CA, Nuovo GJ, Chagas VL, Paes MV.** 2014. The pathology of severe
770 dengue in multiple organs of human fatal cases: histopathology, ultrastructure and virus
771 replication. *PloS one* **9**:e83386.
- 772 55. **Hussell T, Bell TJ.** 2014. Alveolar macrophages: plasticity in a tissue-specific context. *Nature*
773 *reviews. Immunology* **14**:81-93.
- 774 56. **Thepen T, Van Rooijen N, Kraal G.** 1989. Alveolar macrophage elimination in vivo is associated
775 with an increase in pulmonary immune response in mice. *The Journal of experimental medicine*
776 **170**:499-509.
- 777 57. **Strickland DH, Thepen T, Kees UR, Kraal G, Holt PG.** 1993. Regulation of T-cell function in lung
778 tissue by pulmonary alveolar macrophages. *Immunology* **80**:266-272.
- 779 58. **Holt PG, Oliver J, Bilyk N, McMenamin C, McMenamin PG, Kraal G, Thepen T.** 1993.
780 Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by
781 resident alveolar macrophages. *The Journal of experimental medicine* **177**:397-407.
- 782 59. **Gwinn MR, Vallyathan V.** 2006. Respiratory burst: role in signal transduction in alveolar
783 macrophages. *Journal of toxicology and environmental health. Part B, Critical reviews* **9**:27-39.
- 784 60. **Underhill DM, Ozinsky A.** 2002. Phagocytosis of microbes: complexity in action. *Annual review*
785 *of immunology* **20**:825-852.
- 786 61. **Bilyk N, Holt PG.** 1995. Cytokine modulation of the immunosuppressive phenotype of
787 pulmonary alveolar macrophage populations. *Immunology* **86**:231-237.
- 788 62. **Maus UA, Koay MA, Delbeck T, Mack M, Ermert M, Ermert L, Blackwell TS, Christman JW,**
789 **Schlondorff D, Seeger W, Lohmeyer J.** 2002. Role of resident alveolar macrophages in leukocyte
790 traffic into the alveolar air space of intact mice. *American journal of physiology* **282**:L1245-1252.
- 791 63. **Cohen TS, Prince AS.** 2013. Activation of inflammasome signaling mediates pathology of acute P.
792 aeruginosa pneumonia. *The Journal of clinical investigation* **123**:1630-1637.
- 793 64. **Broug-Holub E, Toews GB, van Iwaarden JF, Strieter RM, Kunkel SL, Paine R, 3rd, Standiford TJ.**
794 1997. Alveolar macrophages are required for protective pulmonary defenses in murine
795 *Klebsiella pneumoniae*: elimination of alveolar macrophages increases neutrophil recruitment but
796 decreases bacterial clearance and survival. *Infection and immunity* **65**:1139-1146.
- 797 65. **Cheung DO, Halsey K, Speert DP.** 2000. Role of pulmonary alveolar macrophages in defense of
798 the lung against *Pseudomonas aeruginosa*. *Infection and immunity* **68**:4585-4592.

- 799 66. **Machado-Aranda D, M VS, Yu B, Dolgachev V, Hemmila MR, Raghavendran K.** 2014. Alveolar
800 macrophage depletion increases the severity of acute inflammation following nonlethal
801 unilateral lung contusion in mice. *The journal of trauma and acute care surgery* **76**:982-990.
- 802 67. **Nakamura T, Abu-Dahab R, Menger MD, Schafer U, Vollmar B, Wada H, Lehr CM, Schafers HJ.**
803 2005. Depletion of alveolar macrophages by clodronate-liposomes aggravates ischemia-
804 reperfusion injury of the lung. *The Journal of heart and lung transplantation : the official*
805 *publication of the International Society for Heart Transplantation* **24**:38-45.
- 806 68. **Elder A, Johnston C, Gelein R, Finkelstein J, Wang Z, Notter R, Oberdorster G.** 2005. Lung
807 inflammation induced by endotoxin is enhanced in rats depleted of alveolar macrophages with
808 aerosolized clodronate. *Experimental lung research* **31**:527-546.
- 809 69. **Sadik CD, Kim ND, Luster AD.** 2011. Neutrophils cascading their way to inflammation. *Trends*
810 *Immunol* **32**:452-460.
- 811 70. **Hashimoto S, Pittet JF, Hong K, Folkesson H, Bagby G, Kobzik L, Frevert C, Watanabe K,**
812 **Tsurufuji S, Wiener-Kronish J.** 1996. Depletion of alveolar macrophages decreases neutrophil
813 chemotaxis to *Pseudomonas* airspace infections. *The American journal of physiology* **270**:L819-
814 828.
- 815 71. **Fujimoto J, Wiener-Kronish JP, Hashimoto S, Sawa T.** 2002. Effects of Cl2MDP-encapsulating
816 liposomes in a murine model of *Pseudomonas aeruginosa*-induced sepsis. *Journal of liposome*
817 *research* **12**:239-257.
- 818 72. **Zhao M, Fernandez LG, Doctor A, Sharma AK, Zarbock A, Tribble CG, Kron IL, Laubach VE.** 2006.
819 Alveolar macrophage activation is a key initiation signal for acute lung ischemia-reperfusion
820 injury. *American journal of physiology* **291**:L1018-1026.
- 821 73. **Gavrilovskaya IN, Gorbunova EE, Mackow NA, Mackow ER.** 2008. Hantaviruses direct
822 endothelial cell permeability by sensitizing cells to the vascular permeability factor VEGF, while
823 angiopoietin 1 and sphingosine 1-phosphate inhibit hantavirus-directed permeability. *Journal of*
824 *virology* **82**:5797-5806.
- 825 74. **Gorbunova E, Gavrilovskaya IN, Mackow ER.** Pathogenic hantaviruses Andes virus and Hantaan
826 virus induce adherens junction disassembly by directing vascular endothelial cadherin
827 internalization in human endothelial cells. *Journal of virology* **84**:7405-7411.
- 828 75. **Shrivastava-Ranjan P, Rollin PE, Spiropoulou CF.** 2010. Andes virus disrupts the endothelial cell
829 barrier by induction of vascular endothelial growth factor and downregulation of VE-cadherin.
830 *Journal of virology* **84**:11227-11234.
- 831 76. **Wang W, Zhang Y, Li Y, Pan L, Bai L, Zhuang Y, Huang CX, Wang JP, Yu HT, Wei X, Jiang W, Nan**
832 **YY, Yang DQ, Su WJ, Wang PZ, Bai XF.** 2012. Dysregulation of the beta3 integrin-VEGFR2
833 complex in Hantaan virus-directed hyperpermeability upon treatment with VEGF. *Archives of*
834 *virology* **157**:1051-1061.
- 835 77. **Gavrilovskaya I, Gorbunova E, Koster F, Mackow E.** Elevated VEGF Levels in Pulmonary Edema
836 Fluid and PBMCs from Patients with Acute Hantavirus Pulmonary Syndrome. *Adv Virol*
837 **2012**:674360.
- 838 78. **Li Y, Wang W, Wang JP, Pan L, Zhang Y, Yu HT, Jiang W, Wang PZ, Bai XF.** 2012. Elevated
839 vascular endothelial growth factor levels induce hyperpermeability of endothelial cells in
840 hantavirus infection. *The Journal of international medical research* **40**:1812-1821.
- 841 79. **Medford AR, Douglas SK, Godinho SI, Uppington KM, Armstrong L, Gillespie KM, van Zyl B,**
842 **Tetley TD, Ibrahim NB, Millar AB.** 2009. Vascular Endothelial Growth Factor (VEGF) isoform
843 expression and activity in human and murine lung injury. *Respiratory research* **10**:27.
- 844 80. **Maretta M, Toth S, Jonecova Z, Kruzliak P, Kubatka P, Pingorova S, Vesela J.** 2014.
845 Immunohistochemical expression of MPO, CD163 and VEGF in inflammatory cells in acute

- 846 respiratory distress syndrome: a case report. *International journal of clinical and experimental*
847 *pathology* **7**:4539-4544.
- 848 81. **Song C, Ma H, Yao C, Tao X, Gan H.** 2012. Alveolar macrophage-derived vascular endothelial
849 growth factor contributes to allergic airway inflammation in a mouse asthma model.
850 *Scandinavian journal of immunology* **75**:599-605.
- 851 82. **Nishigaki Y, Fujiuchi S, Fujita Y, Yamazaki Y, Sato M, Yamamoto Y, Takeda A, Fujikane T,**
852 **Shimizu T, Kikuchi K.** 2006. Increased serum level of vascular endothelial growth factor in
853 *Mycobacterium avium* complex infection. *Respirology* **11**:407-413.
- 854 83. **Shariati F, Perez-Arellano JL, Lopez-Aban J, El Behairy AM, Muro A.** 2010. Role of angiogenic
855 factors in acute experimental *Strongyloides venezuelensis* infection. *Parasite immunology*
856 **32**:430-439.
- 857 84. **Shariati F, Perez-Arellano JL, Carranza C, Lopez-Aban J, Vicente B, Arefi M, Muro A.** 2011.
858 Evaluation of the role of angiogenic factors in the pathogenesis of schistosomiasis. *Experimental*
859 *parasitology* **128**:44-49.
- 860 85. **Koyama S, Sato E, Nomura H, Kubo K, Miura M, Yamashita T, Nagai S, Izumi T.** 1999. The
861 potential of various lipopolysaccharides to release monocyte chemotactic activity from lung
862 epithelial cells and fibroblasts. *Eur Respir J* **14**:545-552.
- 863 86. **Koyama S, Sato E, Tsukadaira A, Haniuda M, Numanami H, Kurai M, Nagai S, Izumi T.** 2002.
864 Vascular endothelial growth factor mRNA and protein expression in airway epithelial cell lines in
865 vitro. *Eur Respir J* **20**:1449-1456.
- 866 87. **Tan KW, Chong SZ, Wong FH, Evrard M, Tan SM, Keeble J, Kemeny DM, Ng LG, Abastado JP,**
867 **Angeli V.** 2013. Neutrophils contribute to inflammatory lymphangiogenesis by increasing VEGF-
868 A bioavailability and secreting VEGF-D. *Blood* **122**:3666-3677.
- 869 88. **Taichman NS, Young S, Cruchley AT, Taylor P, Paleolog E.** 1997. Human neutrophils secrete
870 vascular endothelial growth factor. *Journal of leukocyte biology* **62**:397-400.
- 871 89. **Pham I, Uchida T, Planes C, Ware LB, Kaner R, Matthay MA, Clerici C.** 2002. Hypoxia
872 upregulates VEGF expression in alveolar epithelial cells in vitro and in vivo. *American journal of*
873 *physiology* **283**:L1133-1142.
- 874 90. **Koma T, Yoshimatsu K, Nagata N, Sato Y, Shimizu K, Yasuda SP, Amada T, Nishio S, Hasegawa**
875 **H, Arikawa J.** 2014. Neutrophil depletion suppresses pulmonary vascular hyperpermeability and
876 occurrence of pulmonary edema caused by hantavirus infection in C.B-17 SCID mice. *Journal of*
877 *virology* **88**:7178-7188.
- 878 91. **Hammerbeck CD, Hooper JW.** 2011. T cells are not required for pathogenesis in the Syrian
879 hamster model of hantavirus pulmonary syndrome. *Journal of virology* **85**:9929-9944.
- 880 92. **Prescott J, Safronetz D, Haddock E, Robertson S, Scott D, Feldmann H.** 2013. The adaptive
881 immune response does not influence hantavirus disease or persistence in the Syrian hamster.
882 *Immunology* **140**:168-178.

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Figure 1.



□ Isotype control
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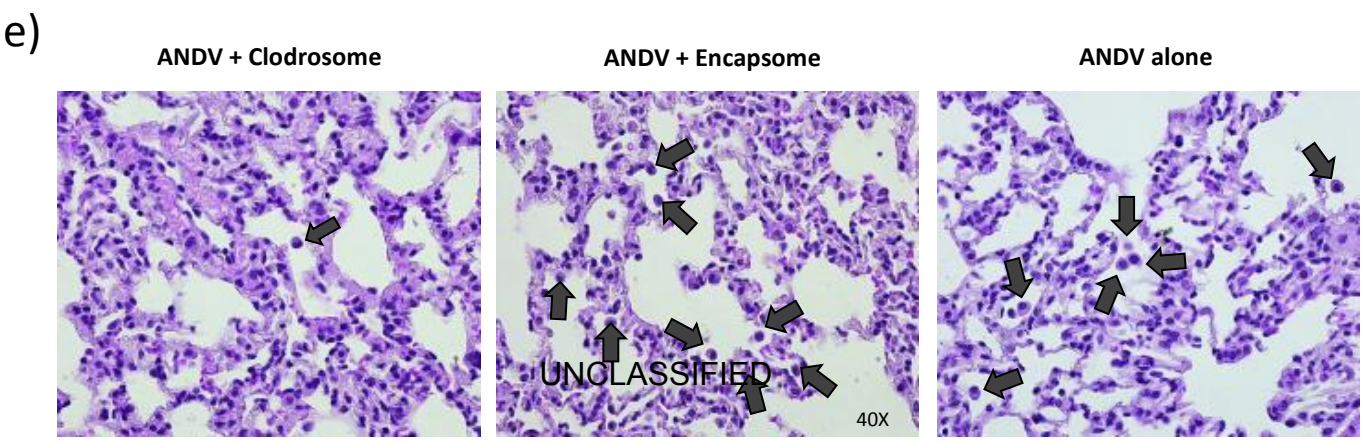
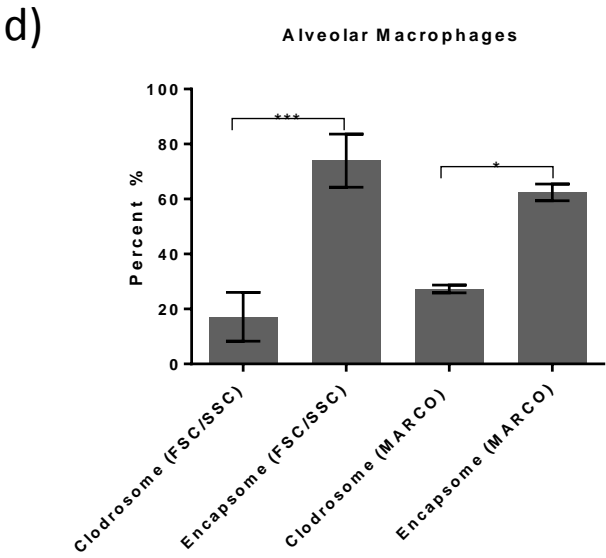
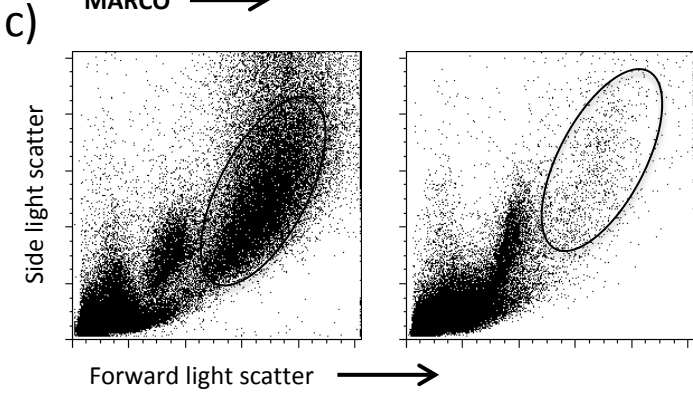
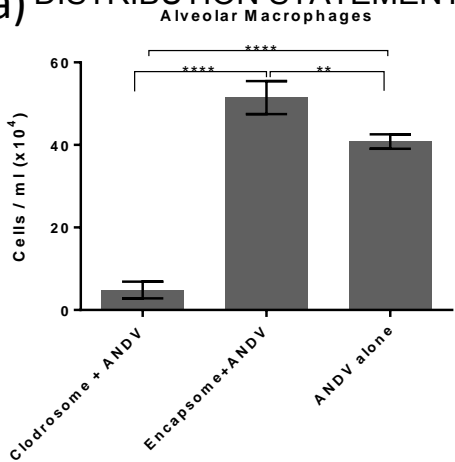


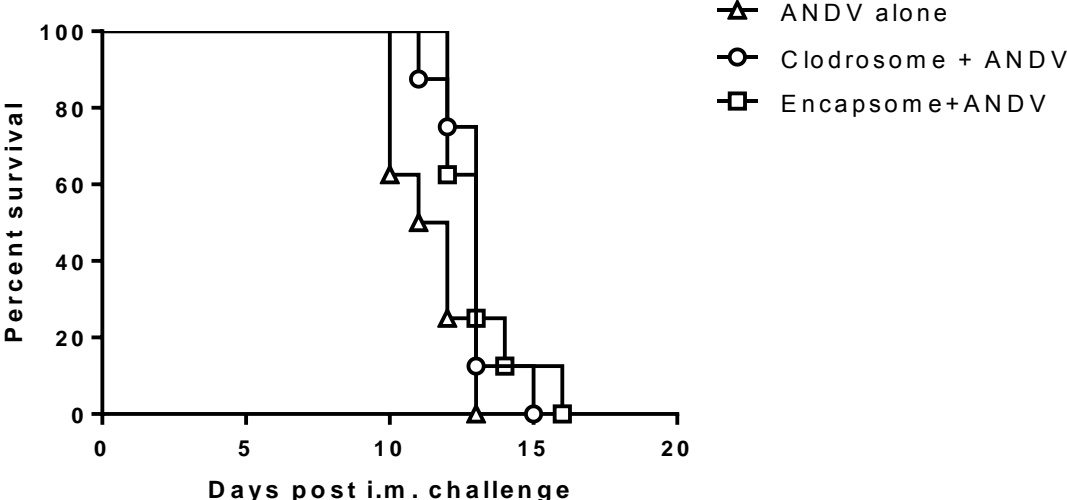
Figure 2.

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c)

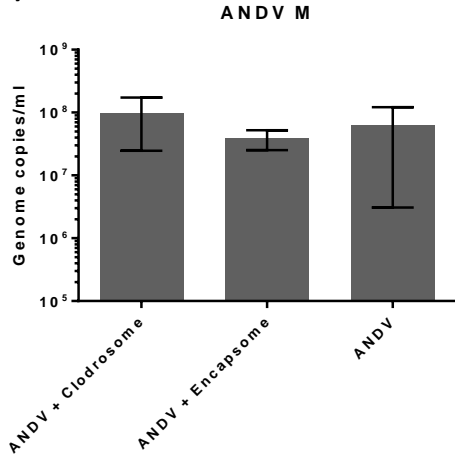


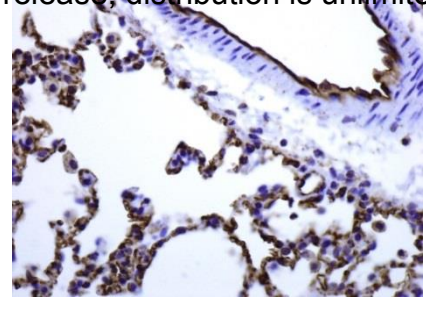
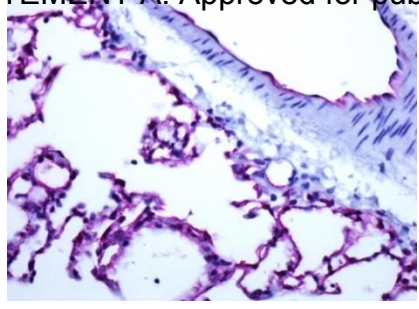
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ANDV NP 16-089

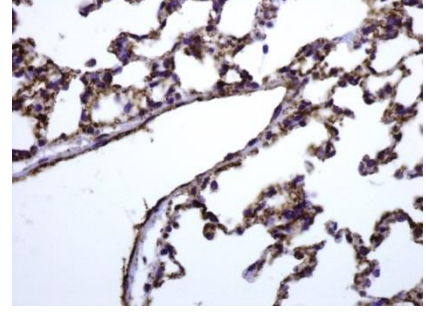
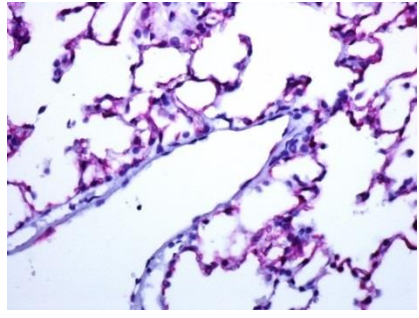
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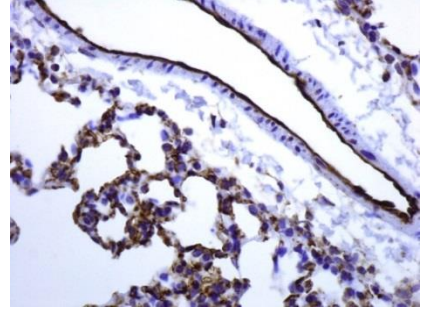
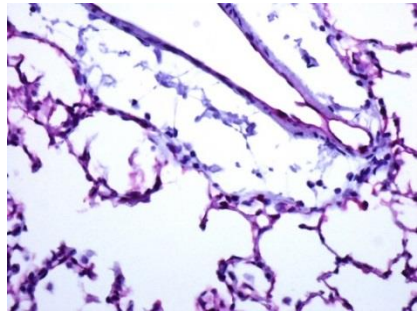
a) ANDV + Clodrosome



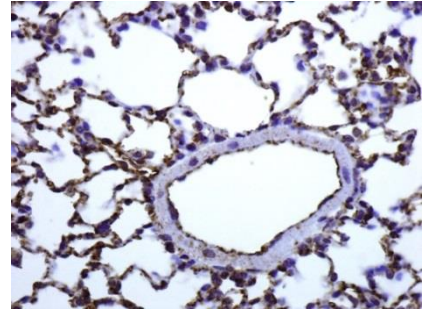
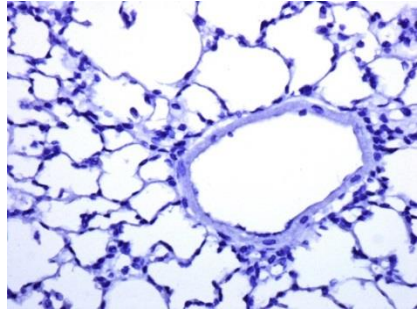
b) ANDV + Encapsome



c) ANDV alone



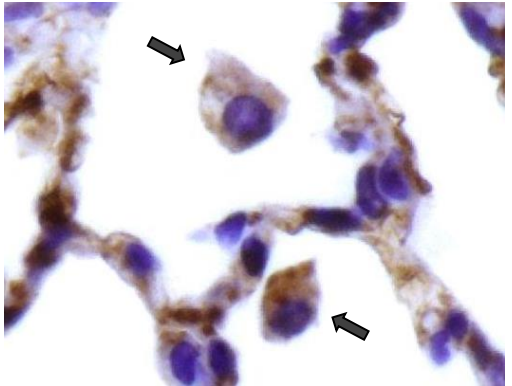
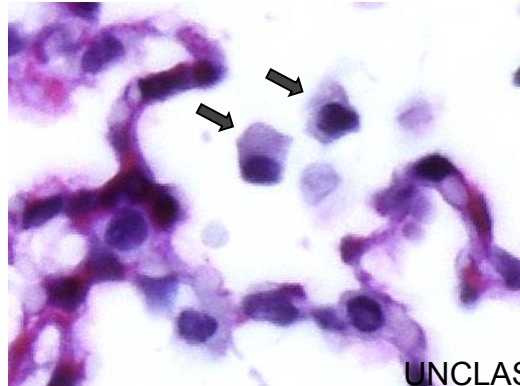
d) Normal hamster



e)

ANDV NP

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Figure 4.

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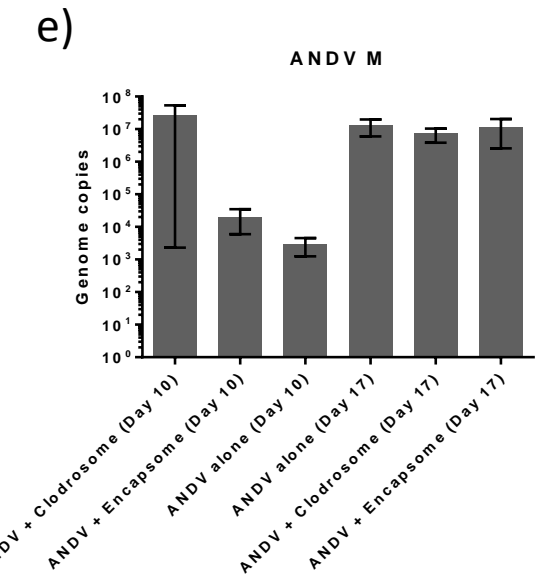
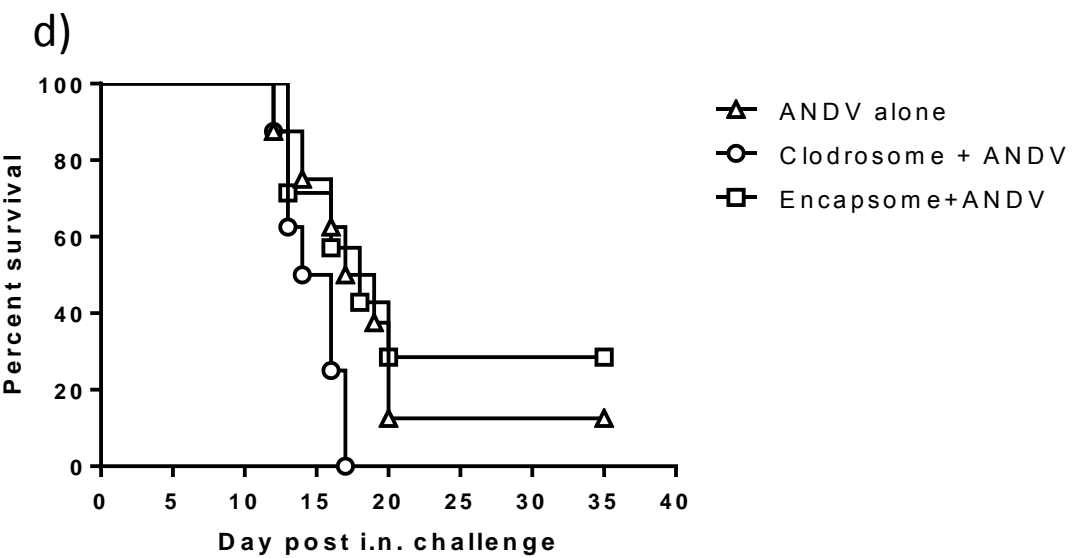
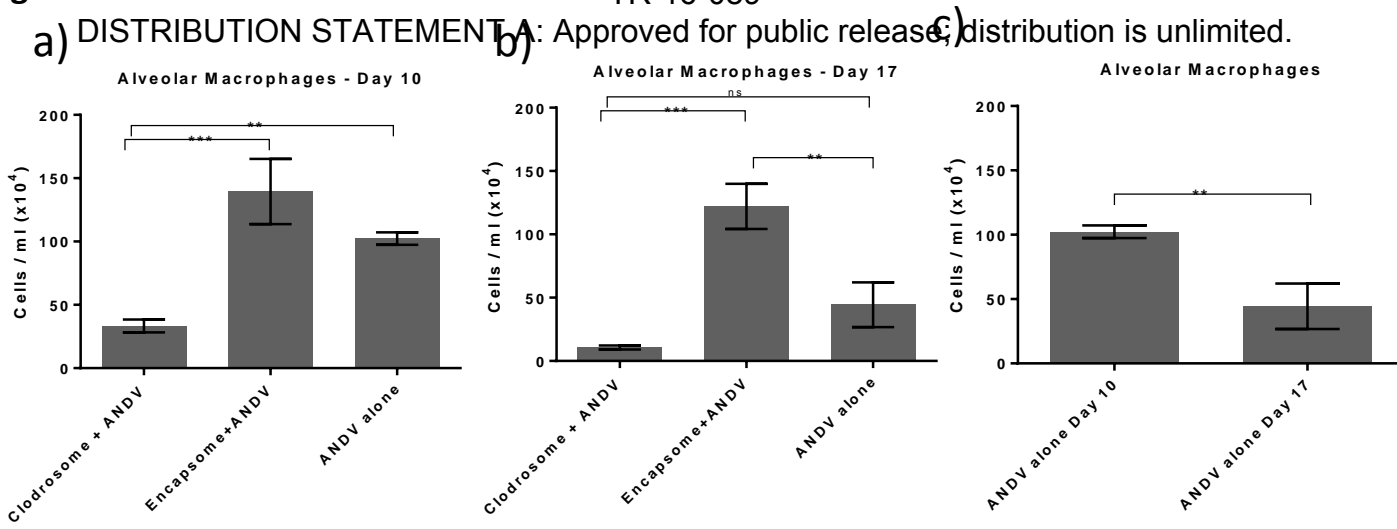
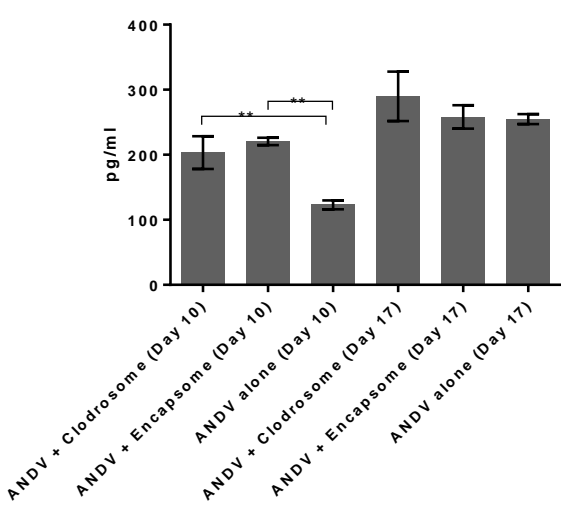


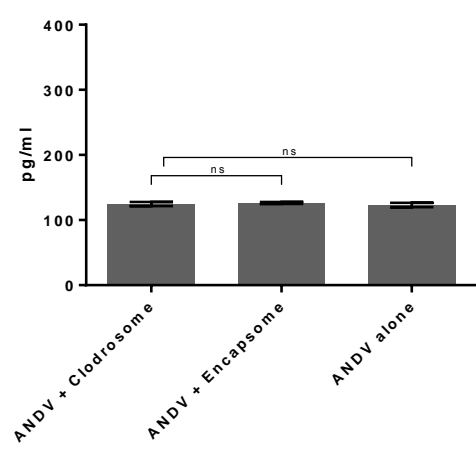
Figure 5.

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b) DISTRIBUTION STATEMENT A: Approved for public release; distribution is unlimited.



c) TNF-α

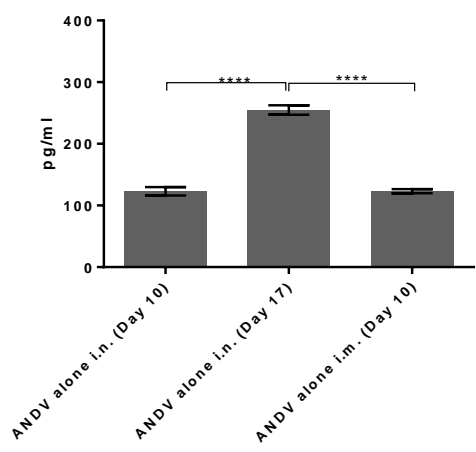


Figure 6.

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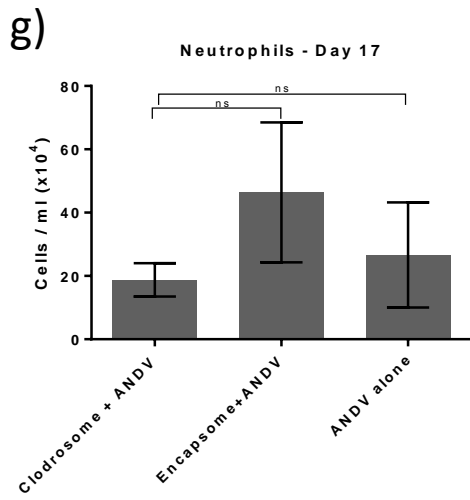
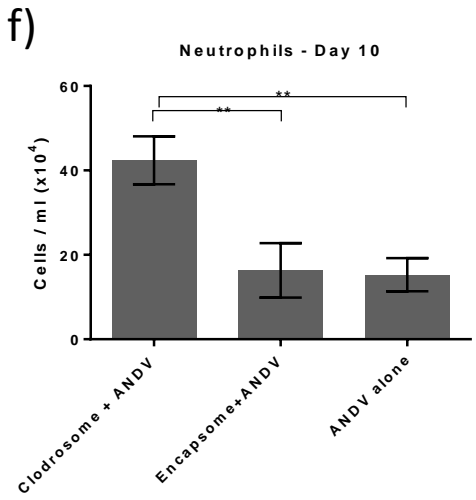
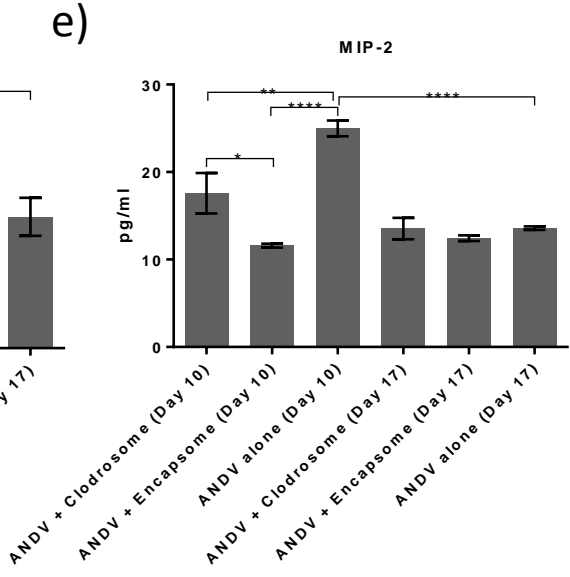
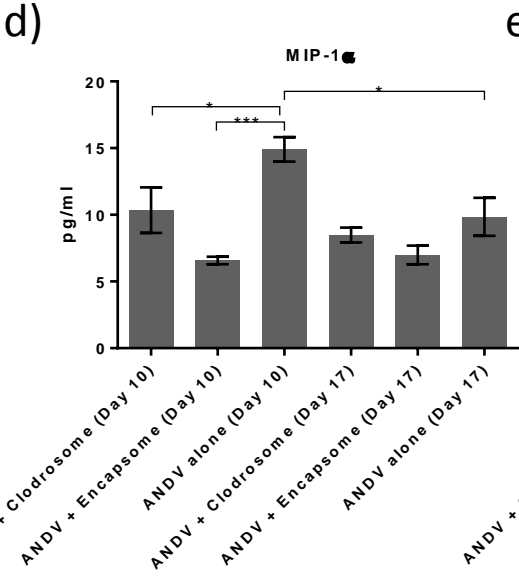
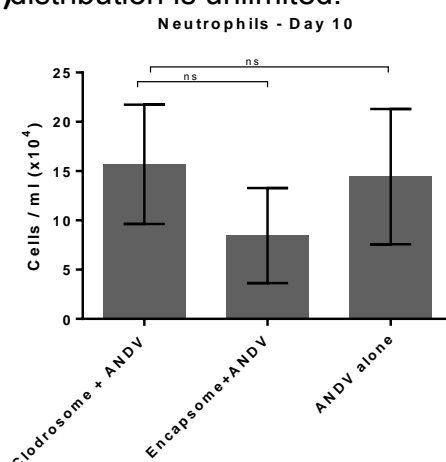
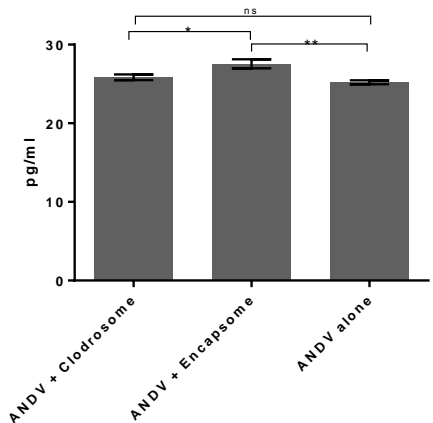
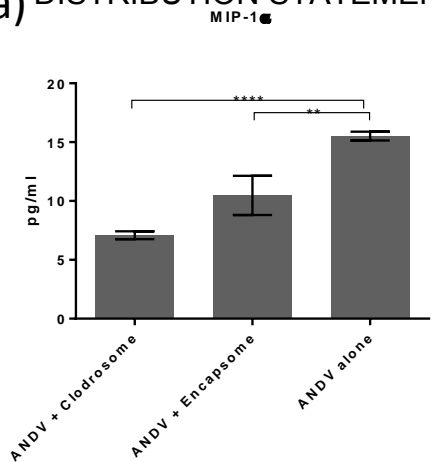


Figure 7.

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