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1 **Protective efficacy of recombinant Modified Vaccinia virus Ankara (MVA) delivering**
2 **Middle East Respiratory Syndrome coronavirus spike glycoprotein**

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22 **ABSTRACT**

23 **Middle East Respiratory Syndrome coronavirus (MERS-CoV) causes severe respiratory**
24 **disease in humans. We tested a recombinant MVA vaccine expressing full-length**
25 **MERS-CoV spike glycoprotein (S) by immunizing BALB/c mice using either**
26 **intramuscular or subcutaneous regimens. In all cases MVA-MERS-S induced MERS-**
27 **CoV-specific CD8+ T-cells and virus-neutralizing antibodies. Vaccinated mice were**
28 **protected against MERS-CoV challenge infection after transduction with the human**
29 **dipeptidyl peptidase 4 receptor. This MERS-CoV infection model demonstrates the**
30 **safety and efficacy of the candidate vaccine.**

31 In 2012 the MERS-CoV emerged as the causative agent of severe human respiratory disease
32 in Saudi Arabia. Since then the virus continues to circulate and cases of human infections are
33 regularly reported, mostly linked to Middle East countries. The highest incidence of MERS-
34 CoV infection occurs in the elderly or immunocompromised individuals. The virus is
35 suspected to persist in dromedary camels and cause sporadic zoonotic infections, followed by
36 intra-familial or health-care-related transmissions (1-3). MERS-CoV uses a cell surface amino
37 peptidase, dipeptidyl peptidase 4 (DPP4) or CD26, as a functional receptor (4). Expression of
38 human DPP4 in mice using adenovirus transduction or transgenesis permits productive
39 infection of MERS-CoV in mouse model systems (5, 6). Rapid development of MERS-CoV
40 specific vaccines is warranted (3, 7), and several initial candidate vaccines based on the spike
41 glycoprotein have been shown to elicit MERS-CoV neutralizing antibodies (8-13).

42 Modified Vaccinia virus Ankara (MVA), a safety-tested and replication-deficient vaccinia
43 virus, is an advanced viral vector platform for developing new vaccines against infectious
44 diseases and cancer (14-16). Recently, we constructed a recombinant MVA stably expressing
45 the full-length MERS-CoV spike (S) protein (MVA-MERS-S) (13). Here, we assessed safety,
46 immunogenicity and protective capacity of this MVA-MERS-S candidate vaccine in a

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47 BALB/c mouse/MERS-CoV infection model using dose escalation and two different
48 application routes.

49 The MVA-MERS-S vaccine was prepared and quality-controlled following standard
50 procedures (17). The recombinant virus MVA-MERS-S proved genetically stable after five
51 repetitive large-scale amplifications in primary chicken embryo fibroblasts (CEF) under
52 serum-free conditions with >95% of the resulting virus population producing the MERS-S
53 target antigen (data not shown).

54 **Antibody response induced after vaccination with recombinant MVA-MERS-S.** Single
55 subcutaneous (s.c.) immunization with doses of 10^7 or 10^8 pfu MVA-MERS-S elicited
56 detectable MERS-CoV neutralizing antibodies (Fig. 1A). S.c. booster immunizations resulted
57 in increased titers of MERS-CoV neutralizing antibodies, and even the low dose of 10^6 pfu
58 MVA-MERS-S induced measurable neutralizing antibodies. Vaccination doses of 10^7 and 10^8
59 pfu MVA-MERS-S resulted in similar antibody levels.

60 Intramuscular (i.m.) immunization resulted in MERS-CoV neutralizing antibodies with all
61 dosages of MVA-MERS-S after a single primary immunization (Fig. 1B). Repeated i.m.
62 immunization further increased the levels of MERS-CoV neutralizing antibodies to higher
63 titers than those obtained upon s.c. immunization. However, the peak antibody titers elicited
64 by s.c. or i.m. immunizations did not differ significantly.

65 **T-cell immune responses after immunization with MVA-MERS-S.** To evaluate T-cell
66 responses in BALB/c mice we measured MERS-CoV-specific CD8+T-cells by IFN-γ-
67 ELISPOT. We tested several S antigen-derived peptides for CD8+ T-cell specificities
68 recognizing the MERS-S antigen (6). Primary immunizations with MVA-MERS-S given s.c.
69 or i.m. elicited CD8+T-cells specific for both MERS-S antigen epitopes S291 (KYYSIIPHSI)
70 and S823 (EYGQFCSKI) (data not shown). We chose peptide S291 for *in vitro* stimulations

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71 since this peptide consistently activated high numbers of S antigen-specific T-cells. Single s.c.
72 immunizations with 10^6 and 10^7 pfu MVA-MERS-S induced nearly equivalent levels of
73 S291-specific CD8+T-cells; however immunization with 10^8 pfu MVA-MERS-S resulted in
74 about three-fold higher responses (Fig. 2A). Booster s.c. immunizations further increased the
75 magnitude of IFN-γ-secreting MERS-S291-specific CD8+T-cells, particularly with the lower
76 dosage of 10^6 or 10^7 pfu MVA-MERS-S. Notably, i.m. immunizations resulted in comparable
77 levels of CD8+T-cell responses for all doses of MVA-MERS-S vaccine after single and
78 prime-boost immunizations (Fig. 2B). The i.m. booster increased the level of MERS-S291-
79 specific T-cell responses about three–fold. Moreover, we detected MERS-S291-specifc IFN-
80 γ-producing T-cells in splenocytes 56 days following the primary or secondary immunization,
81 demonstrating an antigen-specific memory CD8+ T-cell response (Fig. 2C).

82 **Protective capacity of MVA-MERS-S upon MERS-CoV challenge.** To model a productive
83 infection of MERS-CoV, we intranasally transduced MVA-MERS-S-vaccinated BALB/C
84 mice with 2.5×10^8 pfu of an adenoviral vector encoding both the human DPP4 receptor and
85 mCherry (ViraQuest) at 45 days post prime-boost immunization. Five days later the animals
86 were infected with 7×10^4 $TCID_{50}$ MERS-CoV (strain EMC/2012), and 4 days post challenge
87 the animals were sacrificed and the lungs harvested to measure viral loads and for
88 histopathological analysis. High virus loads, on average >11,000 to >20,000 MERS-CoV
89 genome equivalents/ng of total RNA, were found in both mock-immunized and non-
90 recombinant MVA-immunized control groups. In sharp contrast, the lung tissue of MVA-
91 MERS-S-immunized subjects contained significantly lower levels of MERS-CoV RNA,
92 indicating efficient inhibition of MERS-CoV replication by vaccine-induced immune
93 responses (Fig. 3). Furthermore, adenoviral vector transduction levels were also monitored by
94 real-time RT-PCR analysis for mCherry RNA.

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95 Histopathological examination demonstrated that the total percentage of lung tissue affected
96 by MERS-CoV infection varied greatly between the groups (Fig. 4). Lung tissue of control
97 mice revealed large areas of densely packed inflammatory cells, mainly comprising
98 macrophages, lymphocytes, and to a lesser extent, neutrophils (Fig. 4A,C). Inflamed foci were
99 mainly seen around larger bronchi, and some bronchi were filled with cellular debris and
100 inflammatory cells, while other areas of the lungs remained unaffected. Lungs from control
101 mice showed extensive MERS-CoV-S-specific staining, primarily in areas severely affected
102 by inflammation (Fig.4E). Tissues from MVA-MERS-S immunized animals showed minimal
103 lesions, mostly mild hyperplasia of the bronchus associated lymphoid tissue and little positive
104 staining of virus infected cells in lung tissues (Fig. 4B,F). Occasionally, small areas of
105 inflammation resembling those prominently seen in tissues from control mice were also noted
106 (Fig.4D).

107 **Conclusions**. Here we report that the MVA-MERS-S vector vaccine is compatible with
108 clinical use and industrial scale production. The vector can be grown in CEF without the need
109 for additional animal-derived components in culture and MVA-MERS-S stably synthesizes S
110 glycoprotein antigen upon serial amplifications at low multiplicity-of-infection.

111 The immunogenicity data required before initiating clinical trials (18) include evaluating
112 immune responses according to dosage, route of administration and intervals of application,
113 as well as characterizing humoral and cell-mediated immunity. In this study, s.c. and i.m.
114 routes were associated with comparable immune responses, particularly when using the
115 standard dosage of 10^8 pfu MVA-MERS-S in prime-boost applications. The present results
116 are in good agreement with other data in support of the licensing of MVA as replacement
117 smallpox vaccine demonstrating nearly equivalent immunogenicity of s.c. or i.m.
118 immunization (19-23). Moreover the efficiency of i.m. MVA-MERS-S immunization here in
119 inducing humoral and cell-mediated immune responses is similar to the immunogenicity data

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from other recombinant MVA vaccine studies in clinical testing (15, 24). Interestingly, i.m. immunizations induced nearly equal amounts of MERS-S-specific CD8+T-cells across all doses used here, and also in prime and prime-boost vaccination schemes. These findings are also in agreement with the previously observed induction of fully protective levels of virus-specific CD8+ T-cells upon low dose MVA immunization (25). S.c. vaccination was somewhat less immunogenic when using lower dosages of virus; only higher dose of 10^8 pfu MVA-MERS-S immunization resulted in high levels of MERS-specific CD8+ T-cells and MERS-CoV neutralizing antibodies after the prime-boost regimen.

An examination of the efficacy of MVA-MERS-S vaccination in a mouse model of MERS-CoV lung infection revealed that all immunized mice exhibited little or no replication of MERS-CoV, irrespective of the route or dose of vaccination. This data confirms that the S glycoprotein of MERS-CoV, like that of SARS-CoV (26), is an important and safe vaccine antigen. Notably we found no evidence of an increased inflammatory response or the potential enhancement of MERS-CoV infection through S-antigen-specific antibody induction, as has been previously speculated for SARS-CoV infections (27-29). Thus, the MVA-MERS-S vector merits further development as candidate vaccine against MERS-CoV for potential human use.

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145 **Figure Legends**

146 **Figure 1.** Antibody response induced by MVA-MERS-S vaccination. Groups of BALB/c
147 mice (n=5) were immunized subcutaneously (A) or intramuscularly (B) with 10^6, 10^7, or 10^8
148 pfu MVA-MERS-S, 10^8 pfu non-recombinant MVA (WT) or PBS (Mock). To monitor
149 antibody responses we analyzed the MERS-CoV neutralizing capacity of mouse sera taken at
150 d21 and d40. Serum antibodies against MERS-CoV were measured by virus neutralization
151 assays (VNT) after primary vaccination (prime) and after prime-boost vaccination (prime-
152 boost). Shown is the mean of serum titers (log2) from individual animals. The statistical
153 evaluation was performed with GraphPad Prism for Windows (GraphPad Prism Software,
154 USA). Statistical significance of differences between groups is indicated by * for p-value
155 <0.05, ** for p-value <0.01 and *** for p-value <0.001.

156 **Figure 2.** Virus-specific CD8+ T-cell responses induced by MVA-MERS-S. BALB/c mice
157 were immunized by single shot and prime-boost vaccinations with 10^6, 10^7, or 10^8 pfu MVA-
158 MERS-S vaccine via the subcutaneous (A) or intramuscular (B) route. Animals inoculated
159 with non-recombinant MVA (WT) or PBS (Mock) were used as controls. Splenocytes were
160 prepared at 8 days after prime or prime-boost vaccination, and S291-specific IFN-g-producing
161 CD8+ T-cells (IFN-γ-spot forming cells) were measured by ELISPOT. (C) Virus-specific
162 memory CD8+ T-cell responses induced by MVA-MERS-S. Spleen cells were harvested at
163 56 days after prime or prime-boost vaccination. MERS S-specific CD8+ T-cells were
164 stimulated with peptide S291. Peptide SPYAAGYDL (F2L) served for comparative analysis
165 of MVA-specific CD8+ T-cells (30). MERS-CoV S-specific T cells were quantified by IFN-g
166 ELISPOT (A.EL.VIS, Hannover, Germany). The statistical evaluation by t-test was
167 performed with GraphPad Prism for Windows (GraphPad Prism Software, USA). For
168 statistical significant results the following convention was used: * - p-value < 0.05, ** - p-
169 value < 0.01 and *** - p-value < 0.001.

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170 **Figure 3.** Protective capacity of MVA-MERS-S immunization against challenge with MERS-
171 CoV in human DPP4 transduced BALB/c mice. BALB/c mice were infected with $7x10^4$ tissue
172 culture infectious doses 50 (TICD50) MERS-CoV 45 days after immunization with 10^6, 10^7,
173 10^8 pfu MVA-MERS. MERS-CoV RNA loads in lung tissues were determined by
174 quantitative real-time RT-PCR (31). Viral genome copies/ng RNA are shown for groups of
175 animals (n, number of animals per group) immunized by (A) subcutaneous route with 10^6
176 (n=5), 10^7 (n=2), 10^8 (n=2) pfu MVA-MERS-S (MVA-S), non-recombinant MVA (WT)
177 (n=1) and PBS (Mock) (n=4) or (B) intramuscular vaccination with10^6 (n=5), 10^7 (n=5), 10^8
178 (n=5) pfu MVA-MERS-S (MVA-S), non-recombinant MVA (WT) (n=3) and PBS (Mock)
179 (n=4). The statistical evaluation was performed with GraphPad Prism for Windows
180 (GraphPad Prism Software, USA). Statistical significance of differences between groups is
181 indicated by * for p-value <0.05, ** for p-value <0.01 and *** for p-value <0.001.

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186 **Figure 4.**

187 Histopathological and immunohistochemical examination of MVA-MERS-S immunized (B,
188 D, F, H), non-recombinant MVA vaccinated (A, C, E) and mock vaccinated (G) mice that had
189 been transduced with a non-replicating adenoviral vector encoding human DPP4 and
190 mCherry. Mice were infected with MERS-CoV (A-H) or mock infected to monitor for
191 inflammation caused by adenoviral vector transduction (I, J). Lungs were collected 4 days
192 post infection (A-H) or 5 days after transduction with control adenoviral vector (I, J); fixed
193 tissue was routinely embedded in paraffin and stained with hematoxylin and eosin (H&E). For
194 immunohistochemical detection of MERS-CoV a rabbit polyclonal antibody against the spike
195 protein S1 (Sino Biological Inc., cat. no. 100208-RP) was used. Since all tested antibodies
196 against the human DPP4 showed partial cross-reactivity with murine DPP4, a mouse
197 monoclonal antibody against mCherry (abcam®, cat no. ab125096) was used to monitor
198 adenoviral transduction. H&E staining (A-D, I), immunohistochemistry for MERS-CoV spike
199 protein (E, F, J) or mCherry (G, H); scale bar: 500 µm (A, B), 200 µm (I, J), 100 µm (C-H).

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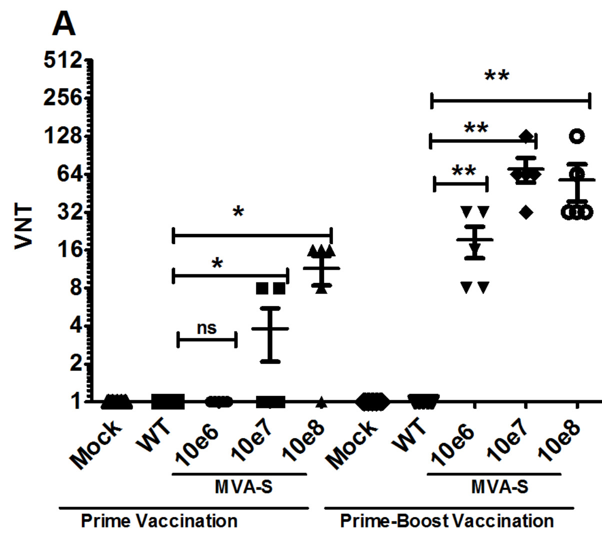

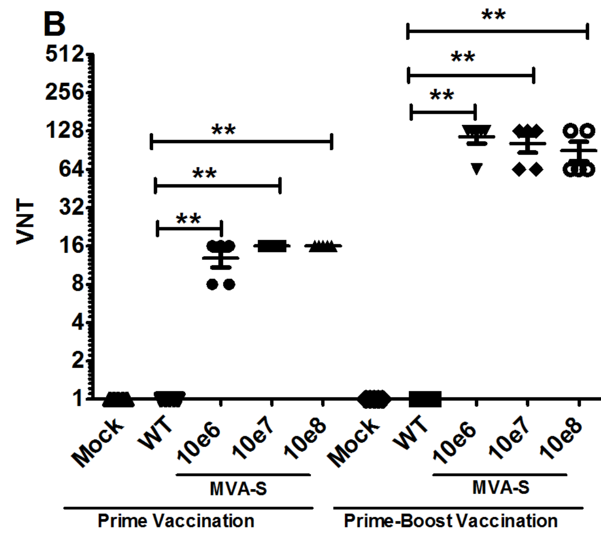

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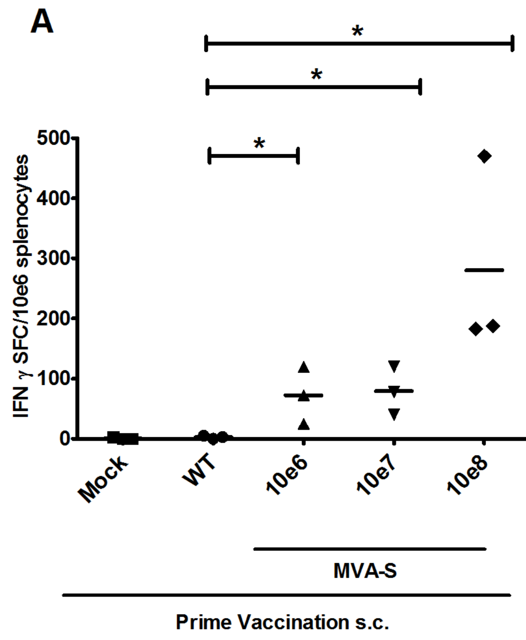

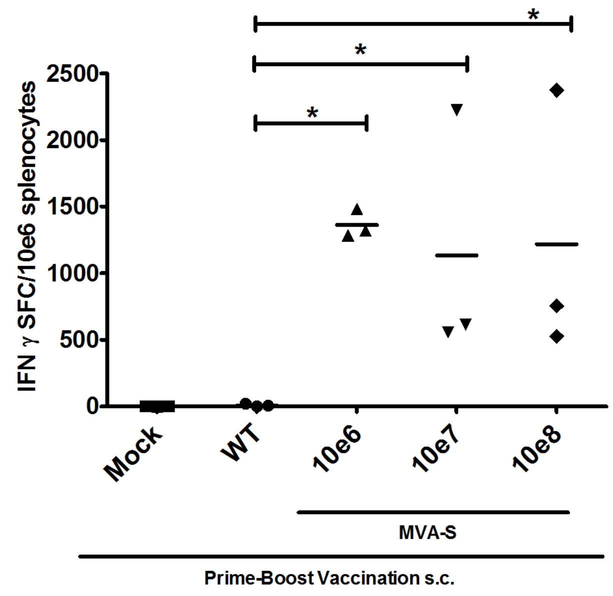

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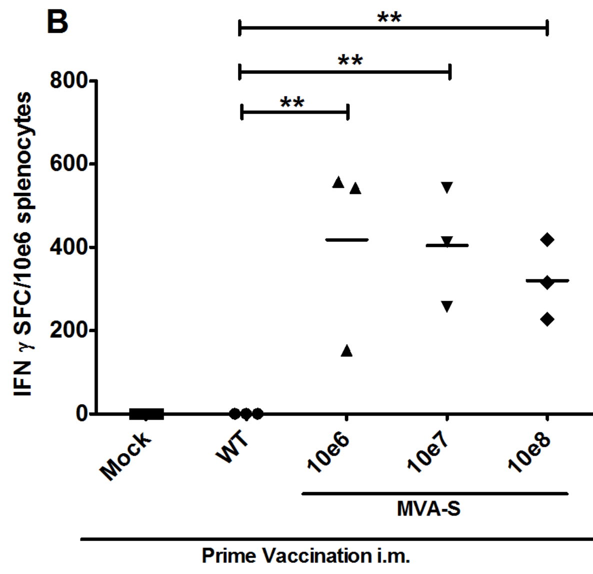

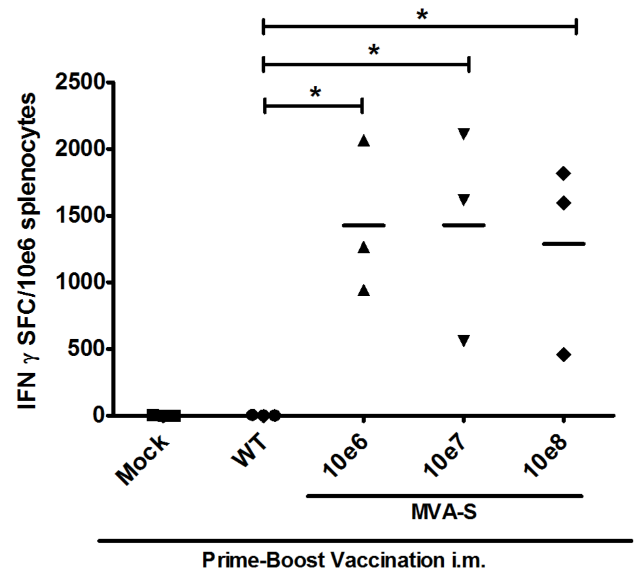

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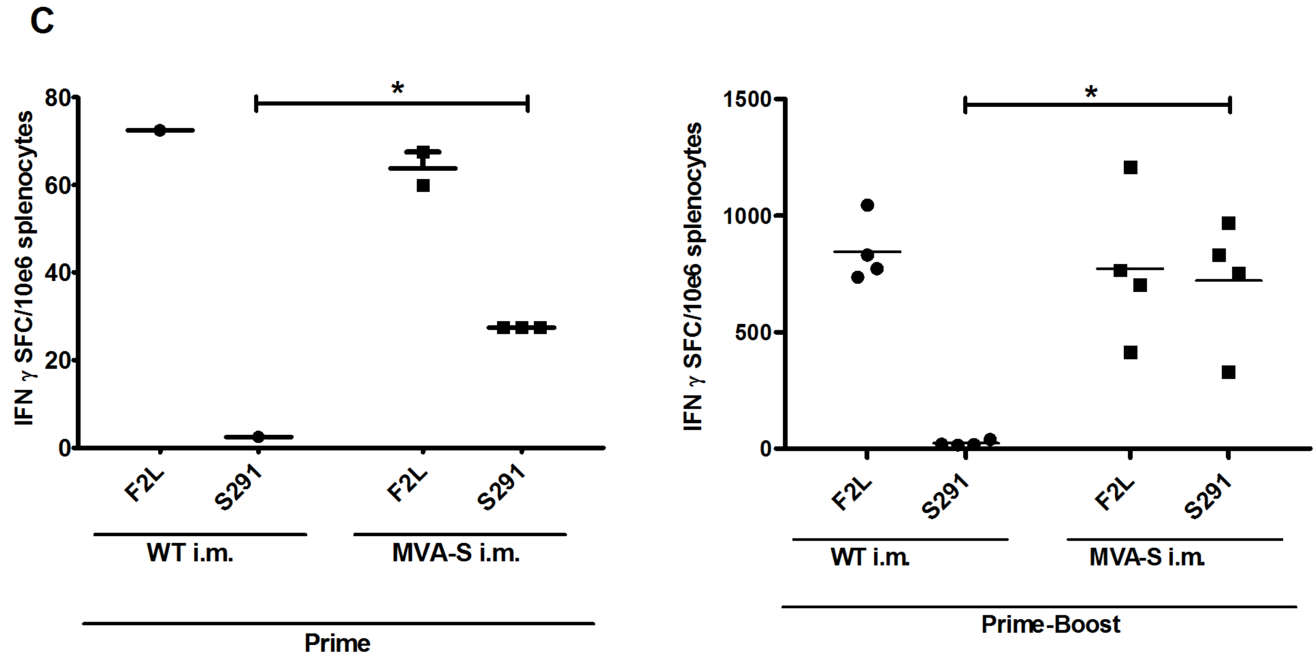

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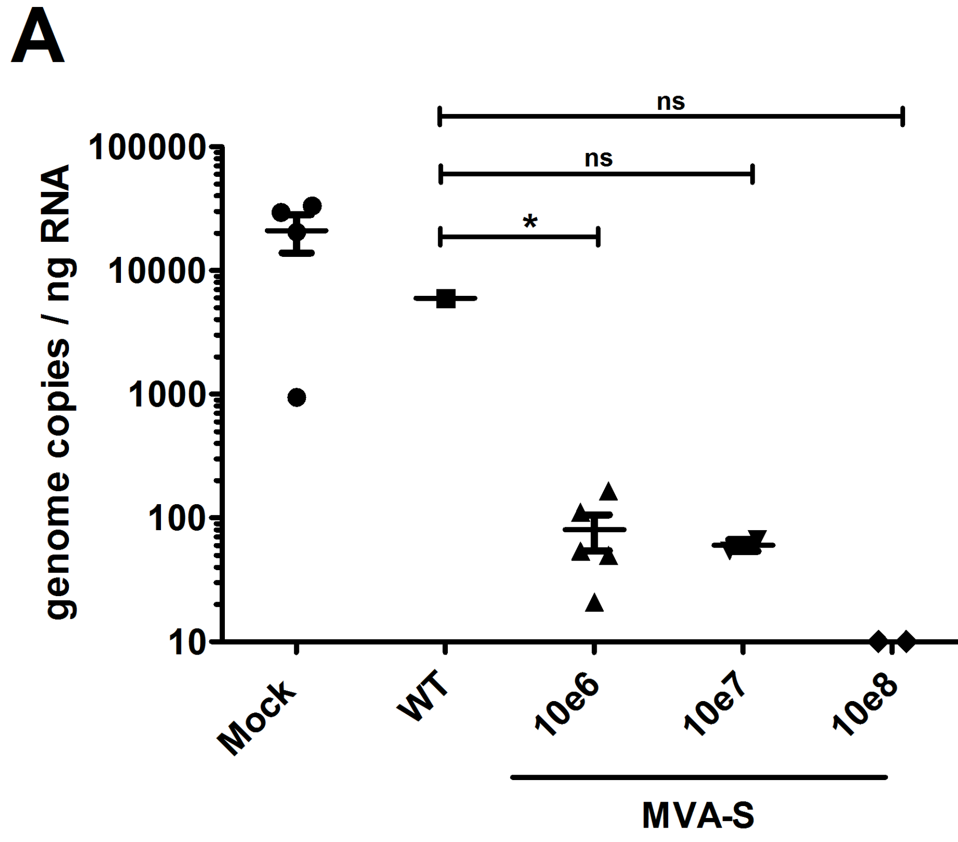

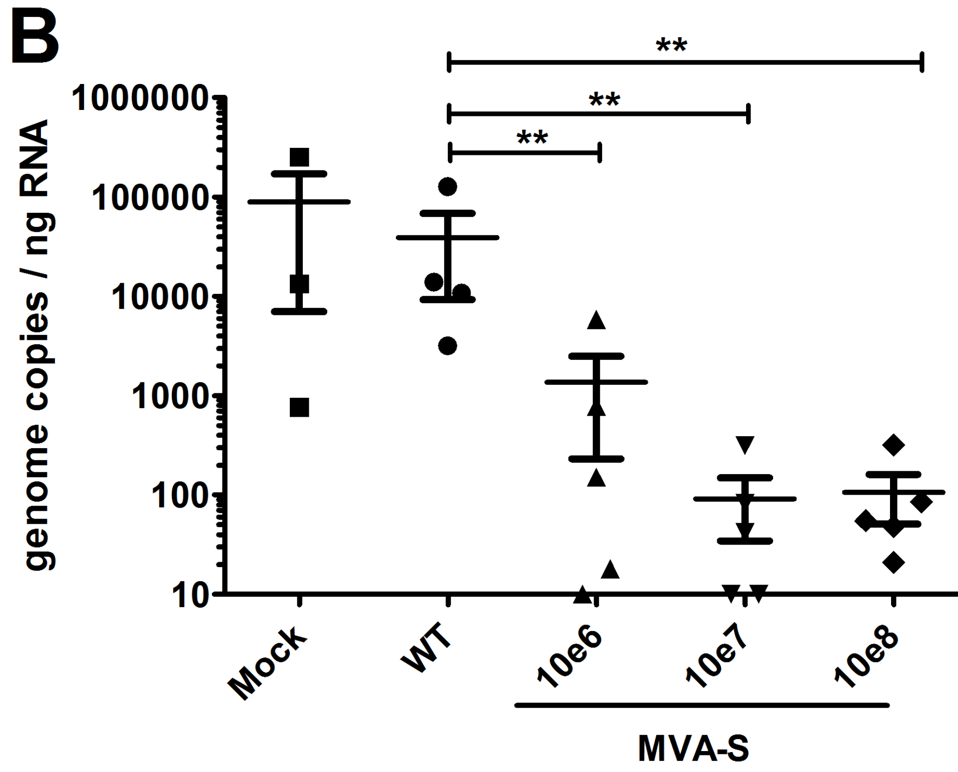

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