

A Novel, Killed-virus Nasal Vaccinia Vaccine.

(Killed-virus smallpox vaccine, nasal adjuvant, nanoemulsion)

Anna U. Bielinska^a, Alexander A. Chepur^a, Jeffrey J. Landers^a, Katarzyna W. Janczak^a, Tatiana S. Chepur^a, Gary D. Luker^b, and James R. Baker, Jr.^{a,*}

^aMichigan Nanotechnology Institute for Medicine and Biological Sciences (MNIMBS),
University of Michigan, Ann Arbor, MI 48109

^bDepartment of Radiology, University of Michigan, Ann Arbor, MI 48109

*Corresponding author: James R. Baker, Jr.

Michigan Nanotechnology Institute for Medicine and Biological Sciences (MNIMBS)

University of Michigan

9220 MSRB III / 1150 West Medical Center Drive

Ann Arbor, MI 48109-5648

Phone: (734) 647-2777 Fax: (734) 936-2990

E-mail: jbakerjr@umich.edu.

1 **ABSTRACT**

2 Live virus vaccines for smallpox are effective but have risks that are no longer
3 acceptable for routine use in populations at minimal risk of infection. We have
4 developed a mucosal, killed-vaccinia virus (VV) vaccine based on antimicrobial
5 nanoemulsion (NE) of soybean oil and detergent. Incubation of VV with 10% NE for at
6 least 60 minutes causes complete disruption and inactivation of VV. Simple mixtures of
7 NE and VV (Western Reserve serotype) applied to the nares of mice resulted in both
8 systemic and mucosal anti-VV immunity, virus neutralizing antibodies and Th1-biased
9 cellular responses. Nasal vaccination with VV/NE vaccine produced protection against
10 lethal infection equal to vaccination by scarification, with 100% survival after challenge
11 with $77 \times LD_{50}$ of live vaccinia virus. However, animals protected with VV/NE
12 immunization did have more extensive clinical symptoms after virus challenge than
13 animals vaccinated by scarification. VV/nanoemulsion based vaccines are highly
14 immunogenic and induce protective mucosal and systemic immunity without the need for
15 an inflammatory adjuvant or infection with live virus.

16 **INTRODUCTION**

17 Smallpox is a dreaded human disease that was eradicated due to the efforts of the
18 World Health Organization's vaccination program in 1980 (38). Ending compulsory
19 smallpox vaccination was welcomed because the live, attenuated virus (vaccinia) vaccine
20 (Dryvax, Wyeth Laboratories) was associated with serious adverse effects, including
21 inadvertent transfer of replicating vaccinia and mortality (24). Unfortunately, concerns
22 about the use of smallpox as a biological weapon has led to consideration of the
23 reintroduction of live vaccinia vaccination (33) despite the current risk of the disease and
24 the known risks of the vaccine. The vaccination program undertaken in the US before the
25 Iraq war demonstrated significant safety issues with the live vaccinia vaccine (2, 33).

26 Given the aforementioned discussion, there is substantial interest in new vaccines
27 for orthopoxviruses that represent potential pathogens for humans including monkeypox,
28 cowpox and variola viruses. An optimal smallpox vaccine would be safer by employing
29 inactivated virus or recombinant viral antigens while retaining the efficacy of the live
30 vaccine. Also, some form of rapid mucosal administration would be advantageous.
31 Unfortunately, current formulations of killed virus vaccines for mucosal application are
32 poorly immunogenic or use bacterial toxins as adjuvants, which have resulted in
33 inflammation and autoimmunity (4, 6, 11). We used a nanoscale (< 400 nm), oil-in-water
34 emulsion as a formulation for a killed-virus mucosal smallpox vaccine. The
35 antimicrobial spectrum of nanoemulsions is broad and includes enveloped viruses,
36 bacteria, fungi and spores (7, 17, 18, 30). Similar formulations to the one used for
37 vaccine have been demonstrated safe and effective in animal tests and in human trials for

38 herpetic cold sores(23). This lack of toxicity is partly due to the emulsion particles eing
39 too large to effectively penetrate the tissue matrix and disrupt organized tissues.

40 Previously, we reported that an antimicrobial nanoemulsion mixed with an
41 enveloped virus (influenza) disrupted the virus and produced efficient immunization
42 when applied topically to the nares (29). This present study evaluates the
43 immunogenicity and effectiveness of a potential smallpox vaccine, based on a
44 nanoemulsion adjuvant mixed with vaccinia virus purified from tissue culture. Our
45 findings indicate that the nanoemulsion inactivates vaccinia and that this mixture results
46 in protective mucosal and systemic immunity when applied to the nares of mice.

47 **MATERIAL AND METHODS**

48 **Animals.** Pathogen-free, 5 to 6-week-old, female BALB/c mice were purchased from
49 Charles River Laboratories. Vaccination groups were housed separately, five animals to
50 a cage, in accordance with the American Association for Accreditation of Laboratory
51 Animal Care standards. All procedures involving mice were performed according to the
52 University Committee on Use and Care of Animals (UCUCA) at the University of
53 Michigan.

54 **Viruses.** Two vaccinia viruses were used in these studies: VV_{WR} and VV_{WR-Luc}. VV_{WR}
55 (NIH TC-adapted) was obtained from the American Type Culture Collection (ATCC).
56 The recombinant vaccinia virus (VV_{WR-Luc}) is the same virus but expresses firefly
57 luciferase from the pH 7.5 early/late promoter and has been described previously (27).
58 VV_{WR-Luc} is not attenuated *in vitro* or *in vivo* because the virus was constructed using a

59 method that does not require deletion or disruption of any viral genes (1), as confirmed in
60 prior studies (27).

61 Stocks of all viruses were generated using the method of Lorenzo et al (25) with
62 some modifications. Virus was propagated on Vero cells infected at a 0.5 multiplicity of
63 infection. Cells were harvested at 48 to 72 hours and virus was isolated from culture
64 supernatants and cells lysates. Cell lysates were obtained by rapidly freeze-thawing the
65 cell pellet followed by homogenization in Dounce homogenizer in the 1 mM Tris pH 9.
66 The virus preparations contained both IMV and EMV virions. Cell debris was removed
67 by centrifugation at 2000 rpm. The purified virus stocks were obtained from the clarified
68 supernatants by layering on the 4% to 40% sucrose gradients which were centrifuged for
69 1 h at $25000 \times g$. Turbid bands, containing viral particles, were collected, diluted in 1
70 mM Tris pH 9 and then concentrated by 1 hour centrifugation at $25000 \times g$. Viral pellets
71 were resuspended in 1 mM Tris pH 9 and stored frozen at -80°C . The virus stocks were
72 sonicated and titered on Vero cells before use (29).

73 $\text{VV}_{\text{WR-Luc}}$ has identical surface proteins as the native strain, but expresses
74 luciferase protein during infection (27). This allows for a sensitive cytotoxicity and
75 morbidity assessment, and the monitoring of the viral infection in animals with imaging
76 techniques. Comparison of the serological response in VV_{WR} immunized animals either
77 by ELISA, Western blot or virus neutralization assays showed no difference in titer to
78 either VV_{WR} or $\text{VV}_{\text{WR-Luc}}$.

79 **Nanoemulsion.** Nanoemulsion (NE, $\text{W}_{205\text{EC}}$ formulation) was supplied by NanoBio
80 Corporation, Ann Arbor, MI. This nanoemulsion is manufactured by emulsification of

81 cetyl pyridum chloride (1%), Tween 20 (5%) and ethanol (8%) in water with soybean oil
82 (64%) using a high speed emulsifier. Resultant droplets have a mean particle size of 300
83 \pm 25 nm in diameter. W₂₀5EC has been formulated with surfactants and food substances
84 considered 'Generally Recognized as Safe' (GRAS) by the FDA. W₂₀5EC can be
85 economically manufactured under Good Manufacturing Practices (GMP) and is stable for
86 at least 18 months at 40°C.

87 **Virus inactivation assays.** Virucidal activity of NE was determined using both a
88 standard plaque reduction assay (PRA) (31) for VV_{WR}, and the inhibition of luciferase
89 activity, in addition to PRA, for the recombinant VV_{WR-Luc}. Generally, 10 μ l samples of
90 VV_{WR} or VV_{WR-Luc} were mixed with 1% to 10% concentration of NE and incubated 1, 2
91 and 3 hours at 37°C. Subsequently, undiluted and serially diluted samples were used for
92 infection of Vero cell monolayers. No cellular toxicity was observed even with samples
93 containing 10%, NE since the emulsion was diluted extensively before addition to the
94 cells (the final alcohol and detergent concentrations in the cell culture medium were
95 0.01% and 0.016%, respectively). For PRA, cells were fixed and stained with 1% crystal
96 violet to visualize plaques. Luciferase expression allowed for more sensitive assessment
97 of replicating virus in cells than plaque-based assays. Luciferase activity was determined
98 by measuring the light emission in cell Vero lysates incubated with a luciferin substrate
99 (Promega, Madison WI). Light emission was measured as a relative light units (RLU)
100 using LB96P chemiluminometer (EG&G, Berthold), and adjusted to the protein
101 concentration of the sample. The protein concentration in the cell lysates was measured
102 in a standard protein assay (BCA Pierce, Rockford, IL).

103 **PCR detection of viral DNA.** Primers for conserved regions of the HA gene of
104 orthopox viruses (the 5' region from start codon to residue 19 and 3' region segment
105 proximal to stop codon) were synthesized by Integrated DNA Technologies (IDT,
106 Coralville, IA). The sequence of the forward primer (5'-ATG ACA CGA TTG CCA
107 ATA C 3') and reverse primer (5'-CTA GAC TTT GTT TTC TG 3') were obtained from a
108 prior report (37). DNA was isolated from Vero cells or from lung tissue homogenates
109 with TriReagent according to the manufacturer's protocol (MRC, Cincinnati, OH). To
110 optimize virus detection and increase sensitivity, cells and lung tissue were collected
111 from 2 to 4 days after infection when viral replication was highest in control animals.
112 PCR amplification was performed with 10 µg of total cell or lung DNA using 0.5 µM of
113 each primer, 0.2 mM of each dNTP, 2.5 mM of MgCl₂, and 0.1 U/µl of *Taq* DNA
114 polymerase (Roche Molecular Biochemicals, Indianapolis, IN). The reaction was carried
115 out in a volume of 20 µl, incubated at 94°C for 1 minute, followed by 25 cycles with
116 annealing at 55°C, extension at 72°C and denaturation at 94°C. The PCR products were
117 analyzed by electrophoresis on 1% agarose gel in Tris-borate buffer for electrophoresis
118 and ethidium bromide for DNA staining. The purified VV DNA (1 ng) mixed with lung
119 DNA served as a positive control. Gel analysis was performed using a photoimaging
120 camera and software from BioRad (Hercules, CA).

121 **Bioluminescence imaging.** Bioluminescence imaging was performed with a
122 cryogenically-cooled CCD camera (IVIS) as described previously (26). Data for photon
123 flux were quantified by region-of-interest (ROI) analysis of the head, chest and abdomen
124 of infected mice. Background photon flux from an uninfected mouse injected with
125 luciferin was subtracted from all measurements.

126 **Preparation of the nanoemulsion-based vaccine.** Vaccinia virus inactivation studies
127 indicated that a minimum of 1 hour incubation with either 10% NE or 0.1% formalin was
128 needed to completely inactivate the virus (greater than a six log vaccinia viral titer
129 reduction in a plaque and luciferase assays). Based on these results, for NE-killed
130 vaccine, the aliquots of 1×10^6 pfu of VV_{WR} were incubated for 3 hours at 37°C in 10%
131 NE. Nasal instillation killed virus was diluted to obtain either 1×10^3 pfu or 1×10^5 pfu
132 per dose in 1% NE. Vaccine formulations containing formalin-killed virus were prepared
133 by incubation of vaccinia virus ($> 10^8$ pfu/ml) with 0.1% formalin (Sigma) at room
134 temperature for 3 hours. This mixture was then diluted in either saline or 1% NE to $1 \times$
135 10^3 and 1×10^5 pfu per dose, to reduce the formalin to nontoxic concentrations for
136 intranasal immunization. For every formulation in every experiment, virus inactivation
137 by either NE or formalin was confirmed *in vitro* by infecting Vero cells, followed with
138 two subsequent passages of culture supernatants after 3-4 days of incubation. None of
139 the control infections showed a presence of viral plaques. Additionally, a PCR assay for
140 viral DNA in Vero cells and in the lungs of animals harvested 2 to 4 days after
141 vaccination showed an absence of viral DNA (PCR limit of detection <0.001 ng viral
142 DNA).

143 **Immunizations.** Samples of pre-immune serum were collected from the mice prior to
144 initial immunization. For intranasal immunization mice were anesthetized with
145 isoflurane and vaccinated with 10-15 μ l of vaccine formulation per nare using a pipette
146 tip. Emulsion was applied slowly to minimize bronchial distribution and swallowing of
147 the material. After immunization, animals were carefully observed for adverse reactions.
148 Specific anti-vaccinia virus antibody response was measured in blood samples three

149 weeks after the initial immunization and at two to three week intervals after the second
150 and third immunizations (in those cases where multiple immunizations were performed).

151 Immunization by scarification was performed in the anesthetized mice by
152 superficial scarification at the base of the tail. Before procedure hair was removed by a
153 clipper to expose approximately 0.5-0.7 square centimeters and the naked skin was
154 disinfected with 70% ethanol. The alcohol was allowed to completely dry (time > 10 to
155 15 min) before scarification. A sterile bifurcate needle was used to superficially abrade
156 the epidermis and apply 1×10^5 pfu dose of live VV_{WR} in 10 μ l PBS. Animals were held
157 still for up to 10 minutes to ensure virus absorption into the skin.

158 **Collection of blood, bronchial alveolar lavage fluid (BAL) and splenocytes.** Blood
159 samples were obtained from the saphenous vein at various time points during the course
160 of the trials. The final sample was obtained by cardiac puncture from euthanized,
161 premorbid mice. Serum was obtained from the blood by centrifugation at $1500 \times g$ for 5
162 minutes after it coagulated for 30-60 minutes at room temperature. Serum samples were
163 stored at -20°C until needed.

164 BAL fluids were obtained at 16 weeks from mice euthanized by isoflurane
165 inhalation. After the trachea was dissected, a 22GA catheter (Angiocath, B-D) attached
166 to a 1 ml syringe was inserted into the trachea. The lungs were infused twice with 0.5 ml
167 of PBS containing 10 μ M DTT and 0.5 mg/ml aprotinin. Approximately 1 ml of aspirate
168 was recovered with a syringe. BAL samples were stored at -20°C until analyzed.

169 Murine splenocytes were mechanically isolated to obtain single-cell suspension in
170 PBS. Red blood cells (RBC) were removed by lysis with ACK buffer (150 mM NH₄Cl,
171 10 mM KHCO₃, 0.1 mM Na₂EDTA), and the remaining cells were washed twice in PBS.
172 For the antigen-specific proliferation or cytokine expression assays, splenocytes were
173 resuspended in RPMI 1640 medium supplemented with 5% FBS, 200 nM L-glutamine,
174 and penicillin/streptomycin (100 U/ml and 100 µg/ml).

175 **Specific anti-virus IgG and IgA determination.** Mouse anti-vaccinia antibodies were
176 determined by ELISA. Microtiter 96-well flat bottom NUNC-PolySorp polystyrene
177 plates were coated with a dilution of infected Vero cells lysate containing at least 5×10^4
178 pfu/well of vaccinia virus in PBS. Plates were incubated overnight at 4°C and fixed with
179 50% mixture of ethanol/acetone (EtOH/acetone) for 1 hour at -20°C. After the fixing
180 solution was removed, plates were washed twice with PBS containing 0.001% Tween 20
181 and then blocked for 1 hour at 37°C with 1% non-fat dry milk in PBS containing 0.2%
182 Tween 20. Mouse sera or BAL fluid were serially diluted in PBS with 0.1% BSA, 100 µl
183 aliquots were added to wells, and the plates were incubated for 2 hours at 37°C. Plates
184 were washed three times with PBS-0.05% Tween 20, followed by 1 hour incubation with
185 either anti-mouse IgG or anti-mouse IgA alkaline phosphatase (AP)-conjugated
186 antibodies (Rockland Immunochemicals) and then washed three times. The colorimetric
187 reaction was performed with AP substrate SigmaFast™ (Sigma, St. Louis, MO)
188 according to the manufacturer's protocol. Spectrophotometric readouts were done using
189 the Spectra Max 340 ELISA reader (Molecular Devices, Sunnyvale, CA) at 405 nm and
190 reference wavelength of 690 nm. The endpoint titers and antibody concentrations were
191 calculated as the serum dilution resulting in an absorbance greater than two standard

192 deviations above the absorbance in control wells. To aid comparisons in some
193 experiments, the IgG antibody concentration was calculated using the logarithmic
194 transformation of the linear portion of the standard curve generated with dilutions of a
195 mouse monoclonal antibody (Biodesign, clone B808M) multiplied by the serum dilution
196 factor. The serum antibody concentrations are presented as a mean value +/- standard
197 error (sem). Serum from the naive mice was used as a control for non-specific
198 absorbance. Since commercial mouse vaccinia-specific IgA antibodies were not
199 available, the concentration of anti-vaccinia IgA in BAL was inferred from the
200 logarithmic transformation of the linear portion of the standard curve generated with IgA
201 and detected by anti-IgA AP-conjugate. BAL fluid from the naive mice was used as a
202 control for non-specific absorbance.

203 Anti-VV IgG antibody activity targeted toward alcohol denaturated versus
204 formalin-alkylated viral epitopes was measured using ELISA, as described above with a
205 few modifications. The 96 well plates were coated with 1×10^5 pfu/well of purified
206 vaccinia virus and incubated overnight at 4°C. After virus was removed, wells were
207 treated for 1 hour with either 50% EtOH/acetone at -20°C or with 1% formalin solution in
208 PBS at 4°C. Plates were washed and blocked as described above. Pooled sera from mice
209 immunized with various formulations of vaccine (VV/NE, VV/Fk/NE, VV/Fk) and sera
210 from mice which survived sub-lethal infection with live vaccinia virus (VV live) were
211 serially diluted in 0.1% BSA, and 100 µL aliquots were added to EtOH/acetone and to
212 the formalin-fixed wells. The assay was performed as describe above for the anti-
213 vaccinia IgG determination. The optical density (OD) values at 405 nm were compared
214 between EtOH/acetone and formalin-fixed viral antigens. The differences in the activity

215 of anti-vaccinia antibodies were evaluated by the ratio of IgG titers on EtOH/acetone
216 versus formalin at the same OD 405 nm value.

217 **Virus neutralizing antibodies.** Neutralizing antibodies were determined using both a
218 standard plaque reduction assay (PRA) (31) and the inhibition of luciferase activity using
219 the recombinant VV_{WR-Luc}. The PRA was performed in duplicates by mixing 10 µl of
220 heat-inactivated mouse serum in serial, two-fold dilutions with 10 µl of serum-free RPMI
221 medium containing 200-300 pfu of vaccinia virus. Sera were incubated 6 hours at 37°C
222 and subsequently placed in 0.5 ml of serum-free medium and overlaid on Vero cell
223 monolayer. After 1 hour incubation, the virus/serum inocula were removed and fresh
224 medium was placed on the cell monolayers. After 48 to 72 hours, the cells were fixed
225 and stained with 0.1% crystal violet. Plaques were counted and the neutralization titer
226 was calculated using non-immune serum as a control. For the assessment of
227 neutralization titer with VV_{WR-Luc}, 10 µl of heat-inactivated mouse serum in serial, two-
228 fold dilutions were mixed with 10 µl of serum-free RPMI medium containing 2×10^3 pfu
229 of virus. As in the PRA-based neutralization assay, samples were incubated for 6 hours
230 at 37°C, then added to 100 µl of serum-free RPMI and incubated for 1 hour with Vero
231 cells in 24 well plates. After 24 to 36 hours, infected cells were lysed and virus-
232 dependent luciferase activity was assessed by the luciferase assay as described above.
233 Neutralization titers (NT₅₀) were calculated from the luciferase inhibition curves using
234 non-immune sera and virus in PBS as controls. Correlations between PRA and luciferase
235 inhibition activity was made for each sample.

236 **Vaccinia-specific cytokine expression in splenocytes.** Spleens from vaccinated mice
237 were harvested 12 weeks after initial vaccination. Splenocytes were obtained from
238 mechanically disrupted spleens and suspended at 3×10^6 cells/ml in RPMI 1640
239 supplemented with 5% FBS, L-glutamine and penicillin/streptomycin. Cells were
240 incubated with either 1×10^3 or 1×10^4 pfu per well of vaccinia virus for 72 hours at
241 37°C . Cell culture supernatants were harvested and analyzed for cytokine production.
242 PHA-P ($1\mu\text{g}$ per well) was incubated with the cells as a positive control. The IFN- γ
243 concentrations in splenocyte supernatants were determined using Quantikine[®] M ELISA
244 kits (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's directions.

245 **Induction of Vaccinia virus-specific T cell responses.** Detection of intracellular
246 cytokine expression in splenocytes was performed using a modification of the technique
247 described by Harrington et al. (19). Splenic lymphocytes were obtained 10 days after
248 BALB/c mice were given either second intranasal immunization of 1×10^5 pfu NE-
249 killed virus or scarification with 1×10^5 pfu live vaccinia virus. Splenocytes were
250 stimulated with the syngeneic H-2^d haplotype A-20 B-cell lymphoma cell line expressing
251 both major histocompatibility complex I and II antigens (MHC class I and MHC class II)
252 or the cl7 fibroblast cell line expressing only MHC class I antigens. The A-20 cells were
253 maintained in complete RPMI 1640 medium containing 10% fetal calf serum (FCS), 2
254 mM L-glutamine, and 1% penicillin-streptomycin while the cl7 cells were maintained in
255 Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and
256 1% penicillin-streptomycin. Both cell lines were infected with VV_{WR} at MOI
257 approximately 0.7, harvested after 12 hr incubation at 37°C , washed and re-suspended in
258 complete RPMI 1640. Single-cell splenocyte suspensions from immunized mice (3×10^6

259 cells/ml) were incubated with approximately 6×10^5 of A-20 or c17, with non-infected A-
260 20 cells serving as controls. All stimulations were performed for 5 hour at 37°C in
261 complete RPMI 1640 medium in presence of monensin (GolgiStop™, BD Bioscience
262 Pharmingen) according to manufacturer specification.

263 **Cell-surface and intracellular staining for the detection of CD antigens and**
264 **cytokines.** Cell surface staining and intracellular cytokine detection was performed with
265 antibodies specific for mouse CD4, CD8a and for interferon- γ (IFN- γ) (BD Bioscience
266 Pharmingen) according to the manufacturers' manual (BD Cytotfix/Cytoperm™ Plus
267 Fixation/Permealization Kit, using BD GolgiStop™ monensin protein transport
268 inhibitor). First, the cells were stained with anti-CD8a or anti-CD4 Cy5-conjugated
269 antibodies, then fixed and permeablized with ethanol. Subsequently, intracellular
270 staining was performed with anti-IFN- γ FITC-conjugated antibody. Samples were then
271 washed, fixed with 2% paraformaldehyde in PBS and re-suspended in PBS with 0.1%
272 bovine serum albumin for analysis. Flow cytometry was performed using a Coulter
273 EPICS-XL MCL Beckman-Coulter flow cytometer and data were analyzed with Expo32
274 software (Beckman-Coulter, Miami, FL).

275 **Vaccinia virus challenge.** Immunized mice were challenged with live vaccinia virus to
276 evaluate the effectiveness of the vaccine. Serum samples were collected two days before
277 the vaccinia challenge and animals were weighed on the day of the challenge. Aliquots
278 of purified recombinant VV_{WR-Luc} or VV_{WR} (sonicated and titered before use) were
279 thawed and diluted in saline the day of the challenge. Mice were anesthetized by
280 inhalation of isoflurane and challenged intranasally with a 20 μ l suspension of 2×10^6 pfu

281 live VV_{WR-Luc} corresponding to $10 \times LD_{50}$, or with live VV_{WR} doses ranging from $1 \times$
282 10^7 to 3.2×10^3 in 5 fold dilutions. Weight and body temperature was measured daily for
283 3 weeks following challenge. Mice that demonstrated a 30% loss in initial body weight
284 were euthanized. Lethal dose (LD_{50}) and the infectious dose (ID_{50}) calculations were
285 based on the animals death rates, and on the core body temperature and body weight loss,
286 respectively (36). Index of protection against lethal challenge (IP_{LD}) was calculated as
287 follows: $IP_{LD} = \log_{10} \text{Maximum dose} - \log_{10} LD_{50} \text{ controls}$. Similarly, index of protection
288 against infection (IP_{ID}) was calculated as: $IP_{ID} = \log_{10} ID_{50} \text{ vaccinated} - \log_{10} LD_{50}$
289 controls.

290 **Statistical analysis.** Statistical analysis of the results was performed using ANOVA, and
291 Student's T-test for the determination of the *p value*, using a Bonferroni's correction for
292 multiple comparisons, where appropriate.

293 RESULTS

294 **Nanoemulsion inactivation of vaccinia virus (VV).** To evaluate virucidal activity of
295 the NE *in vitro*, a range of NE concentrations was mixed with either VV_{WR} or VV_{WR-Luc}
296 and incubated for 1 to 3 hours at 37°C. Results of both plaque reduction (PRA) and
297 luciferase bioluminescence assays indicated NE concentration dependent inactivation of
298 both viruses. The 10% NE completely inactivates greater than 10^6 pfu of vaccinia within
299 1 hour of incubation (Fig 1A and B). Three subsequent passages of the culture
300 supernatants from cells infected with VV inactivated with 10% NE showed no evidence
301 of surviving virus (data not shown).

302 Complete inactivation of virus in the NE preparations was further demonstrated *in*
303 *vivo* after intranasal administration of inactivated VV_{WR} using PCR amplification of
304 DNA isolated from mice lungs after administration. No viral DNA was detected in any
305 of the treated mice (Figure 1C) while a control PCR (lung DNA spiked with VV_{WR} DNA)
306 resulted in the product of the expected size > 950 bp. In addition, *in vivo*
307 bioluminescence imaging of mice also indicated an absence of viral infection and no
308 evidence of virus amplification after administration of 10⁵ pfu of NE-inactivated VV_{WR}-
309 Luc, as compared to a strong signal from mice nasally infected either by 1 × 10⁵ or 1 ×
310 10⁶ pfu of live VV_{WR}-Luc (Figure 1D). Taken together, the *in vitro* and *in vivo* results
311 indicated that incubation with 10% NE for at least 60 minutes causes complete
312 inactivation of VV. Thus, all virus inactivations were performed with 10% NE for 3
313 hours and subsequently diluted to 1% NE for immunization.

314 **VV/NE vaccine induces potent immune responses.** NE efficacy evaluation as an
315 mucosal adjuvant for vaccinia immunization was based on a previous study which
316 demonstrated that nanoemulsion mixed with influenza virus and applied to the nares
317 induce anti-influenza serum IgG, mucosal IgA antibodies, serum virus neutralization, and
318 specific splenocyte responses (29). Mice (*n*=5) were intranasally (i.n) immunized with
319 six formulations containing either 10⁵ or 10³ pfu doses of VV_{WR} killed with NE (10⁵/NE
320 and 10³/NE, respectively), formalin-killed virus mixed with 1% NE (10⁵/Fk/NE and
321 10³/Fk/NE, respectively), and formalin-killed virus in saline (10⁵/Fk and 10³/Fk).
322 Control mice were treated with 1% NE alone. The weak anti-VV antibody responses
323 were detected at three weeks after single administration, and immunity was boosted with
324 subsequent administrations, at 5 and 9 weeks (Figure 2A). Increased anti-VV IgG levels

325 were detected after booster immunization in serum from mice vaccinated with either
326 10^5 /NE or 10^5 /Fk/NE with a mean anti-VV IgG concentrations of 1.5 μ g/ml and 1 μ g/ml,
327 respectively. After a second boost, anti-VV antibody concentrations increased in all
328 groups, and at the conclusion of the experiment (at 16 weeks), immunization with 10^3 /NE
329 and 10^5 /NE, produced highest responses with mean concentrations of 44 μ g/ml and 70
330 μ g/ml of anti-VV IgG, respectively, followed by 10^5 /Fk/NE (17 μ g/ml). Immunizations
331 with 10^3 /Fk/NE, and either 10^5 /Fk or 10^3 /Fk formulations of vaccine consistently
332 produced low levels of anti-VV antibodies, which did not increase significantly after
333 booster administrations (Figure 2A). A comparison of a single-dose with a three-dose
334 schedule of immunization with 10^5 /NE shows that a single dose of vaccine produced
335 significant (\sim 4 μ g/ml), albeit lower than three-dose, concentration of serum anti-VV IgG
336 at 12 weeks after immunization. This suggests that a single dose of VV/NE vaccine may
337 be sufficient to initiate mucosal responses, which are enhanced by subsequent
338 immunization (Figure 1A). No specific anti-VV antibodies were detected in any of the
339 control mice.

340 Analysis of cross-reactive anti-VV antibodies indicated that serum IgG from mice
341 immunized with NE-killed virus reacted with both formalin-crosslinked (alkylated) and
342 with alcohol-fixed (denatured but not alkylated) viral proteins. Vaccination with VV/NE
343 vaccine produced anti-VV IgG with 25 fold higher reactivity to the native, non-alkylated
344 epitopes (similar to serum from mice exposed to a live virus), and those antibodies were
345 also effective in recognizing formalin fixed viral proteins. In contrast, sera from animals
346 vaccinated with formalin-killed virus, either alone or mixed with NE, did not have
347 increased reactivity with native VV epitopes (data not shown).

348 Mucosal immunity was assayed by VV-specific secretory IgA antibody in
349 bronchialalveolar fluids (BAL). Anti-VV IgA was detected in BAL from animals
350 immunized with either 10^3 /NE or 10^5 /NE. Animals vaccinated with formulations
351 containing formalin-killed virus, whether diluted in saline or NE, did not produce
352 measurable mucosal response despite the presence of serum anti-VV IgG (Figure 2B).

353 The biological relevance of the anti-VV antibody response was assessed in the
354 virus neutralization assays. Neutralizing activity was detected in the serum of some mice
355 after the single vaccination (Figure 3A). However, consistent titers of serum neutralizing
356 antibodies were present after two immunizations with 10^3 /NE, 10^5 /NE or 10^5 /Fk/NE.
357 The mean 50% neutralization titer (NT_{50}) for each of these groups was ≥ 20 . In contrast,
358 animals vaccinated with 10^3 /Fk/NE, 10^3 /Fk or 10^5 /Fk, virus neutralization was observed
359 only in the lowest serum dilution. Subsequent immunization produced greater than a ten
360 fold increase in NT_{50} titers only in the mice vaccinated with NE-killed virus (10^3 /NE and
361 10^5 /NE, $NT_{50} = 180$ and $NT_{50} = 500$, respectively). Third vaccination with any of
362 formulations containing formalin-killed virus did not significantly increase VV
363 neutralization. Significant neutralizing activity was also detected in BAL fluids from
364 mice vaccinated with either 10^3 /NE or 10^5 /NE, and was lower in BAL from mice
365 immunized with either 10^3 /Fk/NE or 10^5 /Fk/NE (Figure 3 insert). Neutralizing activity
366 was absent in BAL of mice immunized with formalin-killed virus diluted in saline and in
367 the control, not vaccinated animals.

368 **Vaccinia-specific cellular responses.** The effect of NE based vaccine on cellular
369 response was explored using an *in vitro* cytokine expression assay in splenocytes.
370 Individual cultures of mouse splenocytes were stimulated with 10^3 and 10^4 pfu of live

371 vaccinia. VV-specific cellular immune responses were demonstrated by IFN- γ
372 expression *in vitro* in splenocytes from animals immunized with either 10^3 /NE or 10^5 /NE.
373 In contrast, no increase VV-specific IFN- γ production was observed in splenocytes from
374 animals immunized with formalin-killed virus, even when it was mixed with
375 nanoemulsion. Production of IFN- γ in cells from mice treated with VV/NE vaccine
376 indicates Th1 polarization of cellular response. No antigen specific cytokine expression
377 was detected in control splenocyte cultures (Figure 4).

378 To assess the VV-specific IFN- γ expression in effector CD4 and CD8 T cells, the
379 splenocytes from VV/NE and VV/scar immunized mice were stimulated *in vitro* with A-
380 20 cells infected with VV_{WR}. As shown in Table 1, nasal immunization with NE-killed
381 virus elicited a potent VV-specific CD8 T cell response in the animal's splenocytes. Up
382 to 40% of CD8 cells produced IFN- γ after stimulation with VV-infected A-20 cells as
383 compared to 7.9% of CD8 with non-infected A-20 controls. Analysis of virus-specific
384 CD4 T cells indicated approximately 18% of CD4 cells positive for IFN- γ production
385 following stimulation with VV-infected A-20 cells (Table 1). Analysis of either VV/NE
386 or scarification vaccinated mice showed that both techniques elicited strong virus-specific
387 CD8 and CD4 T cell responses (Table 1 and Figure 5)

388 **VV/NE immunization protects against live virus challenge.** Protective immunity
389 produced by mucosal immunization was evaluated in the challenge studies. Three groups
390 of mice ($n=5$) were nasally immunized with three doses of either 10^5 /NE, 10^5 /Fk/NE or
391 10^5 /Fk vaccine. Control animals ($n=5$) were treated with saline. At 12 weeks mice were
392 challenged with $10 \times LD_{50}$ (2×10^6 pfu) of live VV_{WR-Luc}. Body weight and temperature

393 was measured two times a day and animals were imaged for VV_{WR-Luc} luminescence once
394 a day. All 10⁵/NE vaccinated mice survived viral challenge (Figure 6A). Mice
395 vaccinated with 10⁵/Fk/NE and 10⁵/Fk had 40% and 20% survival rates, respectively.
396 Although not fully protective, vaccination with 10⁵/Fk/NE also extended mean time till
397 death (TTD) from 5 to 7 days. None of the control mice survived challenge.
398 Bioluminescence imaging used for assessment of viral infection demonstrated that two of
399 the five 10⁵/NE immunized mice had minimally detectable virus replication which did
400 not affect their weight and body temperature while the other three had more progressive
401 replication that resolved within 6 days after challenge (Figure 6B). However, none of
402 these animals had clinical evidence of infection. In contrast, all non-vaccinated controls
403 became ill and died or were humanely euthanized within 4 to 7 days of virus challenge.
404 These animals had massive virus replication and spreading of the infection throughout the
405 nasopharyngeal passage, lung and abdomen as presented in photon flux data. In 10⁵/NE
406 vaccinated mice, a low grade infection after i.n. challenge was limited to the head (nose)
407 of vaccinated animals, without spreading to the chest and abdomen (Table 2). Taken
408 together, the imaging studies suggested an inverse correlation between the dissemination
409 of infection and survival. The presence of self-limiting infection in some immunized
410 mice correlated with the levels of neutralizing antibodies in the individual animals.

411 To further investigate effectiveness of mucosal NE-based vaccine, the i.n.
412 immunization with three doses of 10⁵/NE was compared with vaccination by scarification
413 with live VV_{WR} (10⁵/scar). Serum anti-VV antibody response measured at 3 weeks
414 indicated that a higher titer of antibody was achieved with scarification (Figure 7A).
415 However, a repeated study examining multiple time points showed no difference in titer

416 at either 3 weeks or after the first booster (5 weeks), and no difference subsequent titers
417 out to 11 weeks after scarification. (Figure 7B). End titers were equivalent to those seen
418 in animals that survived infection with an LD₅₀ of virus (Figure 7B).

419 We i.n. challenged mice immunized three times with VV/NE with escalating
420 doses of live VV_{WR} at 12 weeks after initial immunization. Survival data indicate that
421 mucosal vaccination produced protective immunity equal to vaccination by scarification
422 (Table 3), with all mice surviving intranasal challenges with the maximal dose of 1×10^7
423 pfu of VV_{WR} ($77 \times \text{LD}_{50}$). The index of protection against lethal challenge (IP_{LD}) was
424 1.9 for both the NE-based vaccine and scarification. All control non-vaccinated animals
425 died after challenge with only $15 \times \text{LD}_{50}$ VV_{WR}. The high level of protection attained
426 with i.n immunization was also seen in weight loss analysis of surviving mice. Although
427 mucosal vaccination did not completely protect mice against respiratory infection with
428 high doses of VV_{WR} (Table 4), animals immunized with NE vaccine did not have clinical
429 evidence of illness and had average weight loss of 10 % or less, whereas surviving mice
430 in control groups lost more than 25% of weigh at much lower doses of VV_{WR}. Statistical
431 analysis indicated differences with *p value* <0.01 between body weight of immunized
432 and control mice. Index of protection against infection (IP_{ID}) was 1 for VV/NE vaccine
433 and 2.2 for scarification.

434 **DISCUSSION**

435 The current, licensed smallpox vaccines use live vaccinia virus obtained from
436 infected calf's skin and lymph nodes or tissue culture (43). These vaccines produce
437 infectious skin pustules (pox) and infrequent but severe side reactions that limit their use
438 in individuals (and their close contacts) with immunodeficiency, eczema, atopic

439 dermatitis, allergy or pregnancy (10). Although these vaccines confer the long-lasting
440 immunity against several different orthopox viruses, the adverse reactions argue for the
441 development of new, safer human vaccines (33). In contrast to the eradication of natural
442 infection, for which the current vaccines were developed, the most likely future use of a
443 smallpox vaccine would be for protection against either a bioterrorist attack or to a
444 natural outbreak of another rare orthopox infection, such as monkeypox (3, 9). These
445 scenarios suggest that the risk/benefit ration of vaccination might require new smallpox
446 vaccines with greater safety. Furthermore, a safe mucosal vaccine could aid emergent
447 public health situations. However, safe and effective mucosal vaccines for most
448 infectious agents based either on killed viruses or purified antigens have not been
449 achieved (15).

450 These studies document the development of antiviral immunity after mucosal
451 immunization with a unique inactivated vaccinia virus preparation. Nanoemulsions are
452 surface-active antimicrobials that when simply mixed with highly purified, cell culture-
453 derived vaccinia virus produces a stable adjuvant formulation for mucosal immunization.
454 This formulation has adjuvant activity without the addition of any specific pro-
455 inflammatory materials, toxins or cytokines. This activity appeared to result from the
456 ability of the nanoemulsion to penetrate the nasal mucosa and be readily endocytosed by
457 dendritic cells and macrophages (22, 40). Vaccinia/nanoemulsion immunization resulted
458 in high-titer mucosal and systemic antibody responses and specific Th1 cellular
459 immunity. Further, after 3 administrations vaccinated mice were fully protected against
460 an instillation challenge with 10 to $77 \times LD_{50}$ of vaccinia virus. In a direct comparison,
461 three-dose immunization with NE-killed vaccine attained a similar degree of protection

462 against vaccinia mortality as the scarification technique typically used for human
463 smallpox vaccine. However, differences in the IP_{ID} between these approaches indicated
464 that vaccination with live virus was more protective against challenge with higher viral
465 loads than the nasal NE-killed vaccine. Overall viral infection, measured both by virus
466 replication and changes in body weight and temperature in vaccinated animals, was
467 markedly attenuated with both approaches as compared to control mice. Comparison of
468 antibody response after nasal vaccination with NE-killed virus and scarification with live
469 virus indicated that although the NE-killed vaccine required repeated doses, it was
470 capable of producing similar immune responses. In addition, we found that after a single
471 immunization with the virus/nanoemulsion formulation serum concentrations of anti-VV
472 IgG developed in 10 to 12 weeks were comparable to a serological response in mice
473 immunized by intramuscular injection with live VV (Dryvax, Wyeth) (5). This suggests
474 that multiple immunizations with the NE/VV vaccine significantly enhance the immune
475 response and are necessary for full immunity. This is in accord with recent reports on the
476 development of safer parenteral recombinant or attenuated smallpox vaccines, which also
477 indicate that multiple administration might be necessary to achieve optimal immune
478 responses and protective immunity (12, 32).

479 In contrast to these results, protective immunity has been reported to not be
480 achieved with several other formulations of live or inactivated vaccinia. Intranasal
481 application of formalin-killed vaccinia virus, either with or without nanoemulsion,
482 produced inconsistent, low serum antibody responses that did not significantly augment
483 even after a third immunization. A similar absence of virus neutralizing activity was
484 detected in serum and BAL of mice vaccinated intramuscularly with formalin-killed

485 virus. In addition, neutralizing activity was not detected in BAL of animals vaccinated
486 with either IP or SQ injections of a live virus (data not shown). The reason for the
487 difference in activity is not entirely clear, but may involve several mechanisms. Our
488 studies examining anti-VV IgG activity suggests that in contrast to formalin, NE
489 inactivation may preserve the critical viral epitopes important in neutralizing the virus. In
490 addition, parenteral injections of VV/NE formulations failed to induce neutralizing
491 antibodies in bronchial secretions, suggesting benefits from mucosal immunity. The
492 ability to produce mucosal immunity without the addition of pro-inflammatory materials
493 is unique to nanoemulsion formulations since other vaccines use inflammatory toxins or
494 other immune stimuli to induce mucosal responses (20). It is possible that this material
495 may act as “physical adjuvant” that transports the antigens across the mucosa and
496 enhances vaccinia protein presentation to the immune system via dendritic cells. This has
497 been reported to occur with nanoemulsions used for cosmetic or drug delivery
498 applications (41, 42).

499 Work on a new generation of smallpox vaccine is hindered by the fact that the
500 exact mechanism of immune protection against smallpox is not completely understood.
501 This is due, in part, to the absence of human smallpox infections in the “modern” era of
502 immunology. However, it is well thought that both cellular and humoral immunity play a
503 role in protection against orthopox viruses and both were induced with the NE
504 formulations. Vaccinia-specific antibody titers are considered the most direct estimate of
505 protective immunity in humans and animal models of vaccination (16). Several studies
506 have identified proteins important for the elicitation of neutralizing antibodies (13, 21).
507 A recent trial with dilutions of the licensed smallpox vaccine (Dryvax) in human

508 volunteers confirmed that pustule formation strongly correlated with development of both
509 the specific antibodies and the induction of cytotoxic T lymphocytes (CTL) and elevated
510 INF- γ cell responses (14). Neither these studies nor our own work have fully
511 characterized the CTL responses, but assessment of virus-specific T cells expressing INF-
512 γ in mice vaccinated with VV/NE clearly indicated that nanoemulsion nasal vaccination
513 induces potent activation of vaccinia-specific MHC class I-restricted CD8+ T cells.
514 These cells have been implicated in the recognition and clearance of vaccinia infected
515 cells, and for maintenance of immunity after vaccination (8, 9, 16). The VV-specific
516 CD8 and CD4 T cell responses were comparable between nasal vaccination with NE-
517 killed virus and scarification with live virus, and were similar to the CTL results reported
518 previously in the vaccinia infected animals (19, 34, 35, 39). Live virus stimulation of
519 splenocytes from mice vaccinated with formalin-killed virus failed to significantly induce
520 the INF- γ production, again suggesting that the nanoemulsion formulations are unique in
521 inducing CTL responses. Finally, while it is not proven, a key benefit to this candidate
522 vaccine may be mucosal application, providing barrier immunity at sites where many
523 vaccinia infections initiate (28). Thus, a combination of systemic serological and cellular
524 immunity, and the mucosal immunity achieved with virus/nanoemulsion immunization
525 suggests the potential for protection against lethal mucosal exposure to vaccinia virus and
526 are probably important to yield an effective smallpox vaccine.

527 In summary, we have documented that a new formulation of nanoemulsion
528 inactivated vaccinia virus serves as an effective mucosal vaccine that induces both
529 mucosal and systemic antibody and TH1 cellular immunity. This prototype vaccine is

530 easily produced by simply mixing of the nanoemulsion with purified virus and may
531 provide an addition margin of safety over live virus vaccination.

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684 Figure Legends

685 **Figure 1. Complete virus inactivation with nanoemulsion.** (A) Plaque reduction
686 assay (PRA) of VV_{WR}. (B) Luciferase assay of VV_{WR-Luc}. Luciferase activity is
687 presented in relative light units (RLU). In standardized assays the limit of virus detection
688 was <10 pfu for PRA and <4 pfu for luciferase assay. (C) PCR analysis of lung DNA.
689 Lane 1: DNA size marker; lane 2: primers, no DNA; lane 3: no Taq; lane 4: 10⁵/Fk lung
690 DNA; lanes 5-7: 10⁵/Fk/NE lung DNA; lanes 8-10: 10⁵/NE lung DNA; lane 11: control -
691 VV DNA mixed with lung DNA collected from tissue harvested 4 days after vaccination.
692 Arrows indicate amplified viral template and primers. The limit of detection of this assay
693 was <0.001 ng viral DNA. (D) *In vivo* bioluminescence imaging of mice after intranasal
694 infection with live VV_{WR-Luc} and with 10⁵ pfu of NE-killed virus. Circles visible in some
695 images indicate the regions-of-interest (ROI) used for the photon flux analysis (Table 2).

696 **Figure 2. Immunogenicity of mucosal nanoemulsion vaccine in mice.** (A)
697 Development of serum anti-VV IgG antibody response in mice vaccinated with various
698 formulations of killed virus vaccine: 10⁵/NE (●), 10³/NE (○), 10⁵/Fk/NE (▲),
699 10³/Fk/NE (△), 10⁵/Fk (◆) and 10³/Fk (◇). Arrows indicate i.n administrations of the
700 vaccine. Insert: Comparison of serum anti-VV IgG after one or three vaccinations with
701 10⁵/NE vaccine. Data presented as mean of the individual anti-VV IgG concentrations ±
702 sem. (B) Secretory anti-VV IgA antibody in BAL. Results are presented as mean
703 concentrations (+/-sem) of IgA obtained in assays performed with individual and pooled
704 BAL fluids collected at 16 weeks.

705 **Figure 3. Virus neutralizing antibodies.** Assays for virus neutralization were
706 performed with both individual and with pooled sera obtained after one, two and three
707 vaccinations. Insert: Detection of virus neutralizing activity in BAL. Assays were
708 performed with individual and pooled BAL fluids collected at the conclusion of the
709 experiment at 16 weeks. Results were normalized and presented as NT₅₀ of the viral
710 PRA.

711 **Figure 4. Vaccinia-specific cellular immune responses.** The INF- γ expression *in vitro*
712 in splenocytes stimulated with 10³ and 10⁴ pfu of live VV_{WR}. The data show a specific
713 INF- γ response to the virus in splenocytes from animals immunized with vaccinia virus
714 inactivated by NE.

715 **Figure 5. Vaccinia virus-specific CD8 and CD4 response.** Splenocytes from mice
716 immunized i.n. with NE-killed vaccine were analyzed for VV-specific responses 10 days
717 after the 2nd booster administration of vaccine. The frequency of IFN- γ ⁺ VV-specific
718 CD8⁺ and CD4⁺ cells. Data presented as mean values obtained in splenocytes from
719 different groups of mice ($n=3$) immunized with either nasal NE-killed (VV-NE) vaccine
720 or scarification with live virus (VV-scar).

721 **Figure 6. Intranasal challenge with live vaccinia virus.** (A) Survival curves for mice
722 vaccinated with 10⁵ pfu of killed VV_{WR} in various vaccine formulations: VV/NE,
723 VV/Fk/NE and VV/Fk, after i.n. challenge with 10 \times LD₅₀ VV_{WR-Luc}. Significant
724 protection was observed in the vaccinated animals as compared to control mice -
725 vaccinated with either NE alone or PBS. (B) Bioluminescence images of representative

726 vaccinated (upper panel) and control mice (lower panel) challenged with a luciferase-
727 producing Vaccinia virus. Images were recorded 2 to 5 days after challenge.

728 **Figure 7. Comparison of IgG serum anti-VV titers with nasal vaccination using NE-**
729 **killed virus and scarification with live vaccinia.** Mice were vaccinated with either
730 three doses of VV/NE vaccine at 0, 3 and 8 weeks (VV/NE), or one live virus
731 scarification at week 0 (VV-scar). Serum samples VV/NE were analyzed at 3, 5 and 11
732 weeks and at 3 weeks VV-scar. Titers were assessed by ELISA at a 1:1000 serum
733 dilution to assure analysis in the linear range of the assay. Titers in the first assay showed
734 higher initial antibody concentrations in the mice immunized with scarification (Panel A,
735 $p < 0.5$). Subsequent serial studies again showed a higher titer for scarification at 3 weeks,
736 but no significant difference at later time points (up to 11 weeks) after booster
737 immunization with the NE/VV vaccine (Panel B). Overall titers after week 5 were
738 similar to those seen in naive animals who survived an LD₅₀ challenge with live virus
739 (Panel B, black bar).

740 **Table 1. T Cell subset response to vaccinia virus in immunized animals.**

741 Splenocytes were stimulated with A-20 cells infected with VV_{WR} with uninfected A-20
742 cells serving as a background control. VV-specific T cells expressing IFN- γ were
743 detected by intracellular staining and enumerated by flow cytometry, and percentages of
744 CD8⁺ and CD4⁺ cells that were IFN- γ ⁺ are indicated. A-20 – a syngeneic H-2^d
745 haplotype B-cell lymphoma cell line expressing both MHC class I and class II molecules
746 and c17 - a syngeneic H-2^d haplotype fibroblast cell line expressing only MHC class I
747 molecule were used as stimulator cells to show that different populations of cells could be

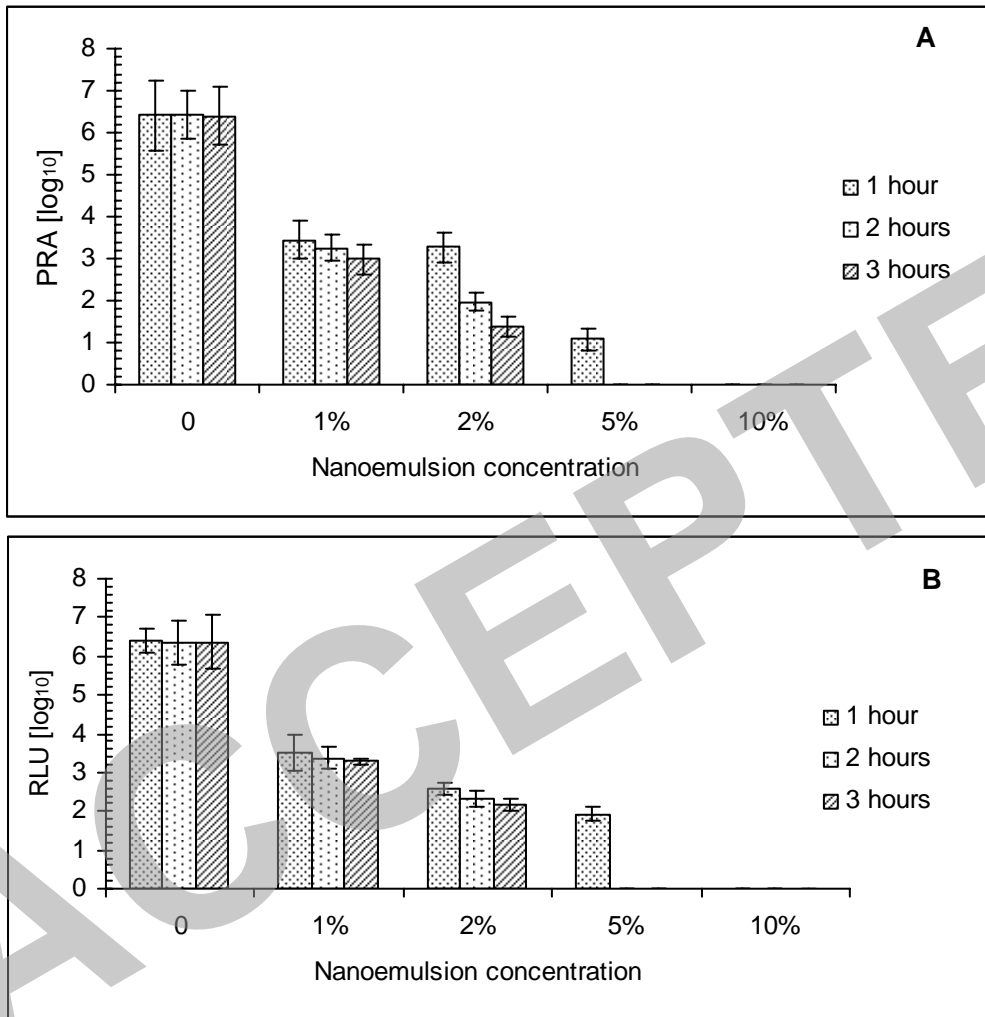
748 stimulated based on CD4/CD8 expression. INF- γ producing CD4 and CD8 cells were
749 specifically generated in response to vaccinia virus stimulation in animals vaccinated with
750 either scarification or intranasal NE.

751 **Table 2. Photon flux analysis of viral luciferase expression in vaccinated and**
752 **control mice.** Results are presented as mean fold of photon flux increase over
753 normalized 1×10^4 background values in the individual mice ($n=5$ per group). Photon
754 flux from an uninfected mouse injected with luciferin was subtracted from all
755 measurements.

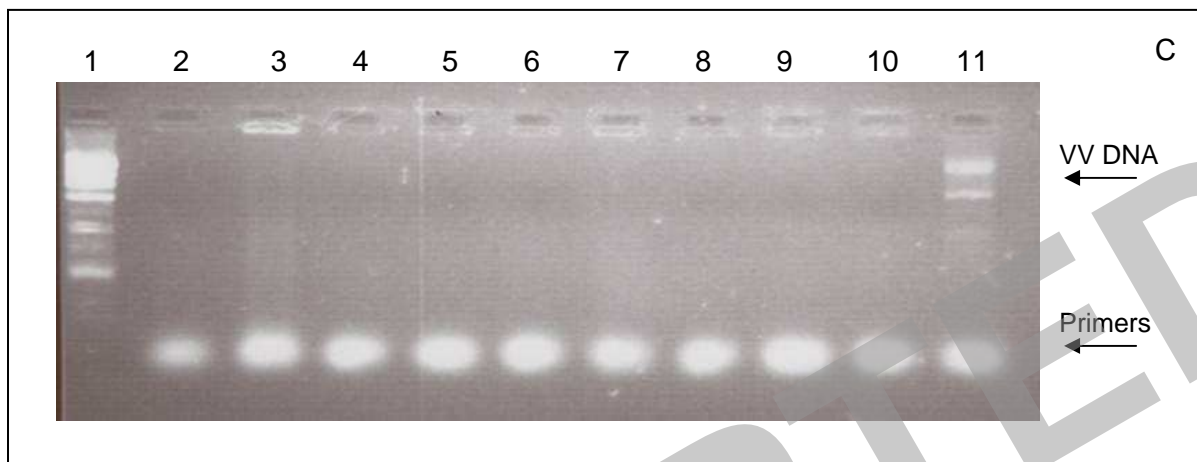
756 **Table 3. Comparison of protective immunity after intranasal vaccination with**
757 **10^5 /NE or scarification with live VV_{WR}.** Survival after challenge with escalating doses
758 of VV_{WR}. Three doses of NE-killed vaccine (10^5 /NE) were administered and compared
759 with a single vaccination by scarification with 10^5 pfu of live VV_{WR}. Viral challenge was
760 performed at 12 weeks after the initial vaccination. No difference in survival was
761 observed in animals based on their immunization protocol.

762 **Table 4. Protection against viral infection after intranasal vaccination with 10^5 /NE**
763 **or scarification with live VV_{WR}.** Protection against viral infection was assessed in mice
764 vaccinated with three doses of NE-killed vaccine (10^5 /NE) compared to a single
765 scarification with 10^5 pfu live VV_{WR}. Viral challenge was performed at 12 weeks after
766 initial vaccination. Mice were observed twice daily for changes in body weight and core
767 body temperature after challenge with live virus. Significantly more changes in body
768 weight and temperature were observed in the NE/VV immunized animals as compare to
769 those immunized with scarification, especially at higher virus challenge doses ($P>0.5$).

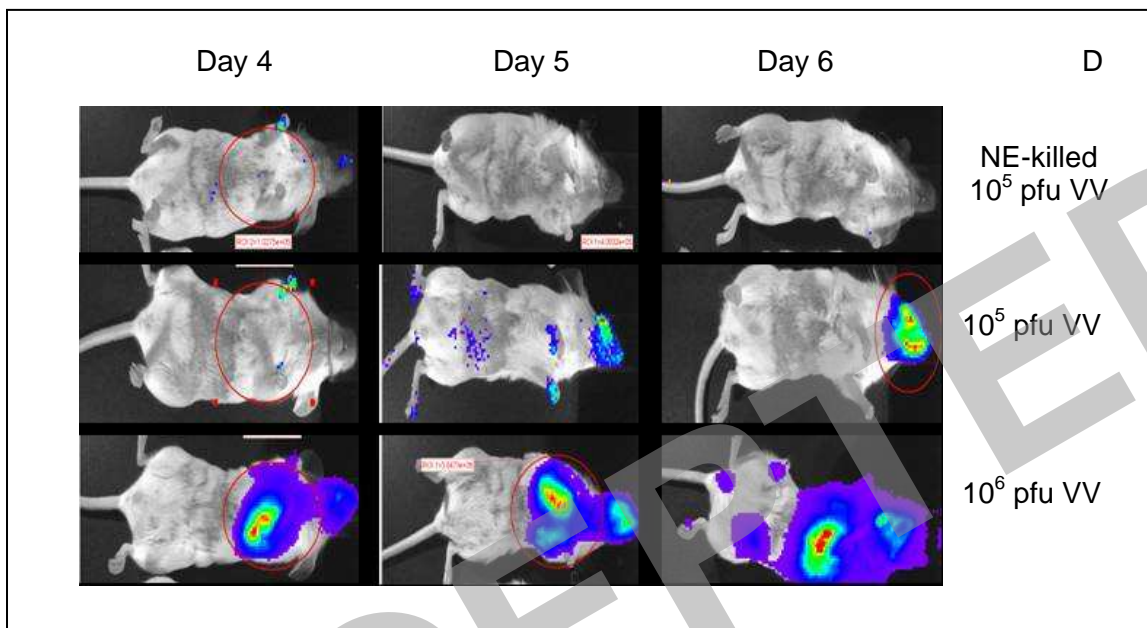
770 Figure 1 A and B



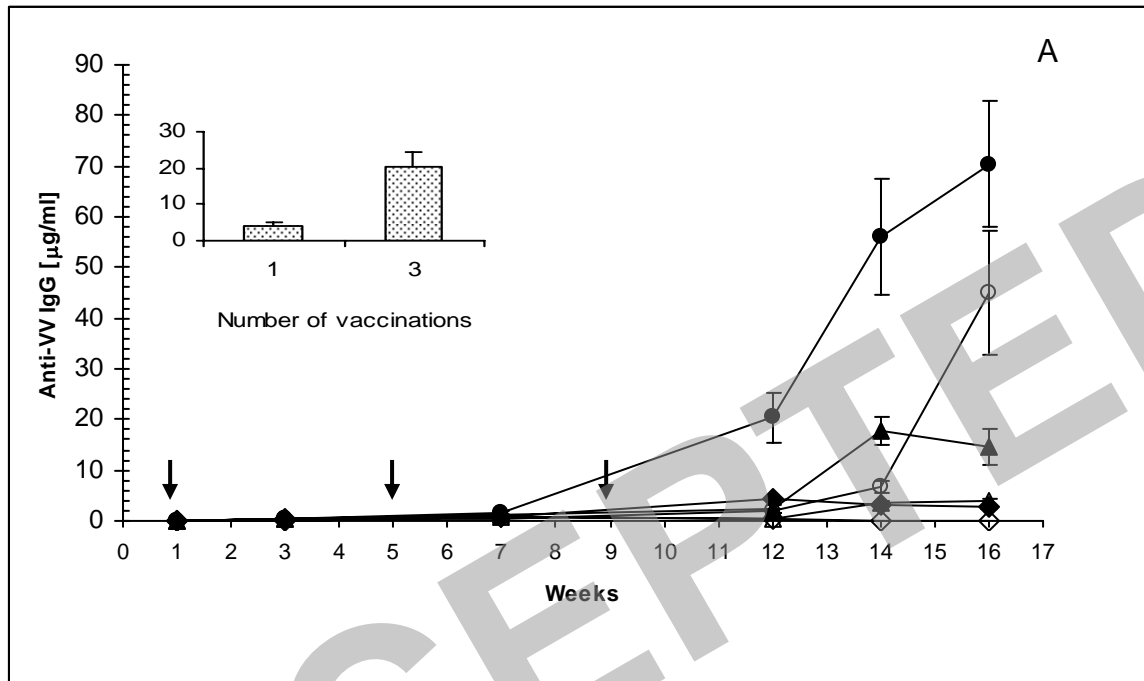
771 Figure 1C



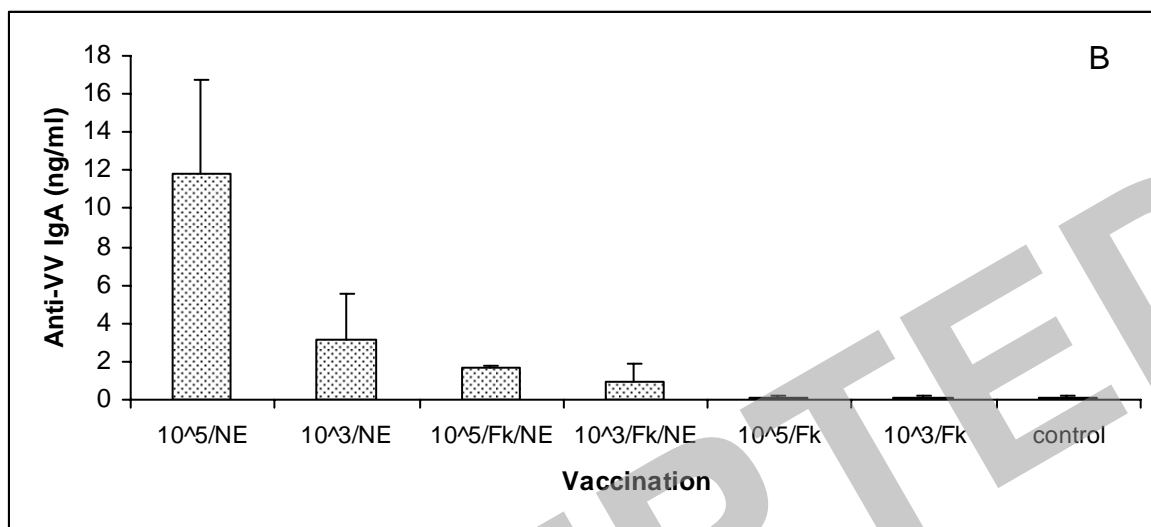
772 Figure 1D



773 Figure 2A

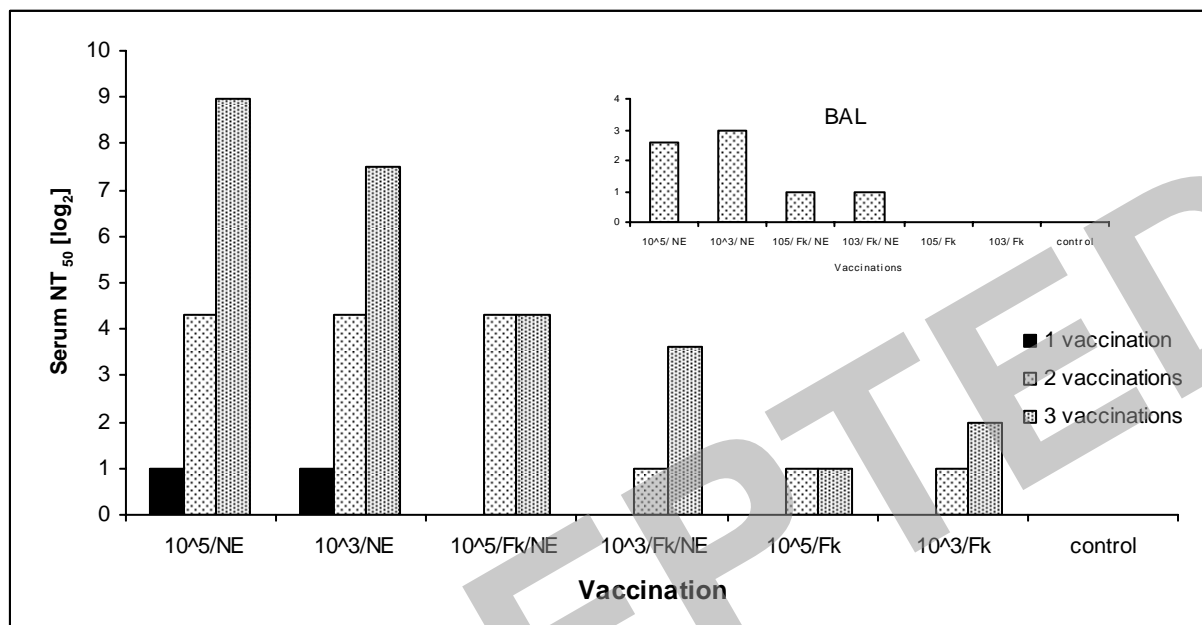


774 Figure 2B

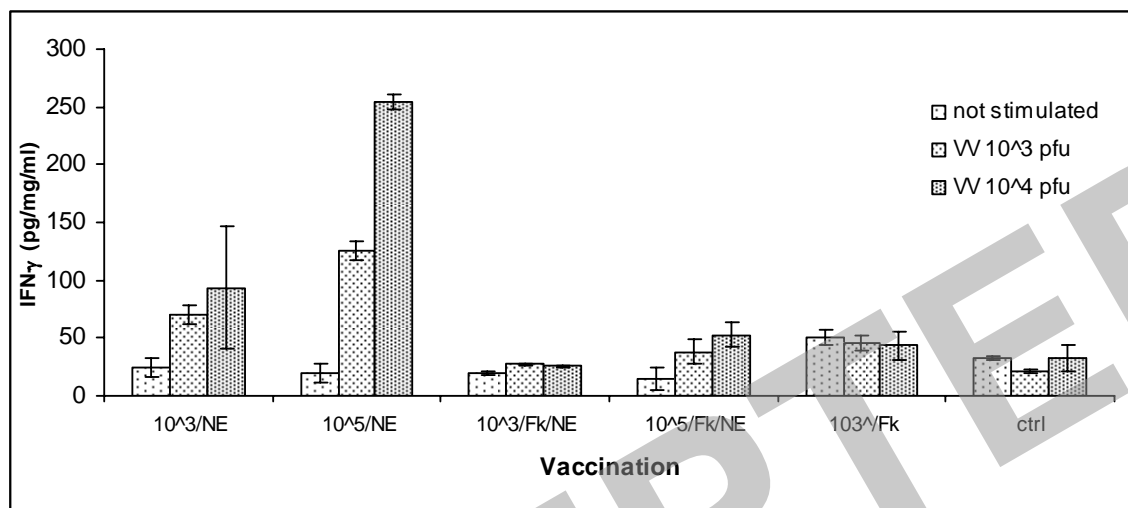


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775 Figure 3



776 Figure 4

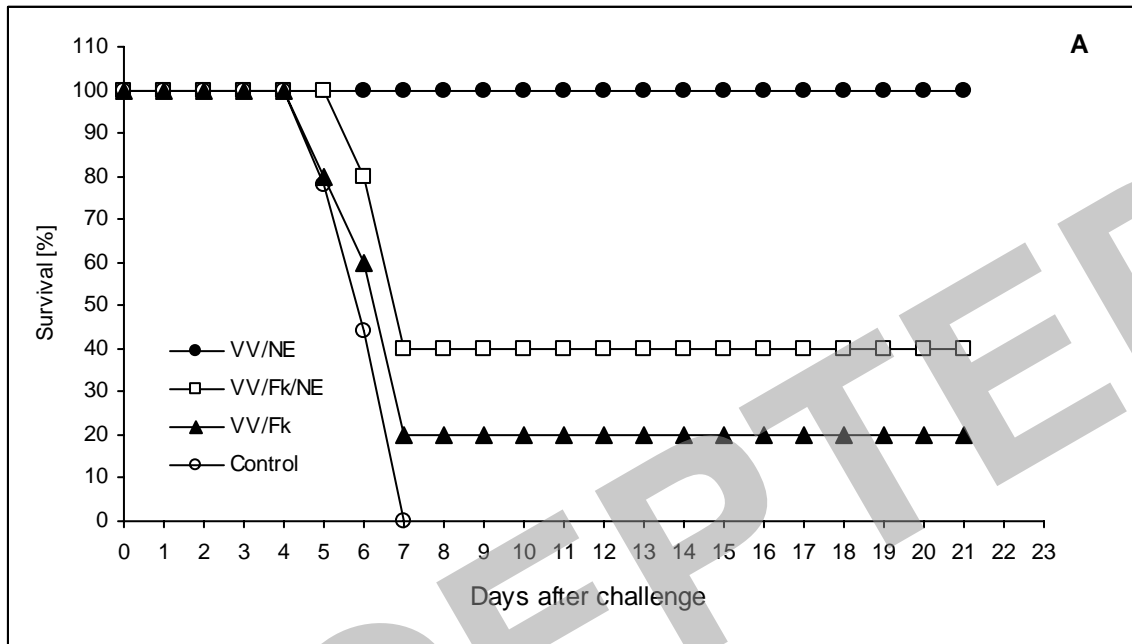


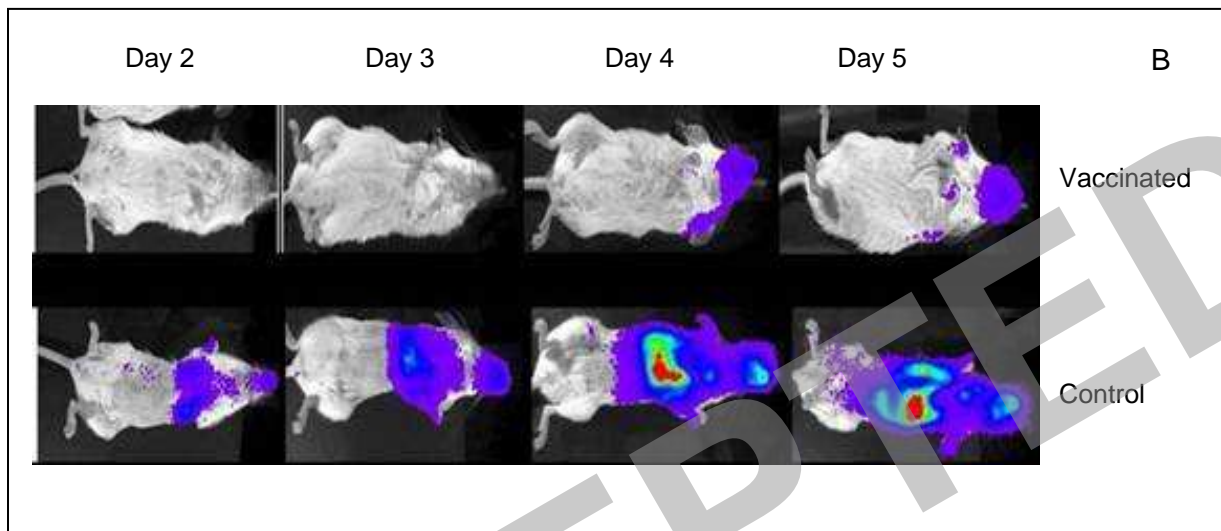
777 Figure 5



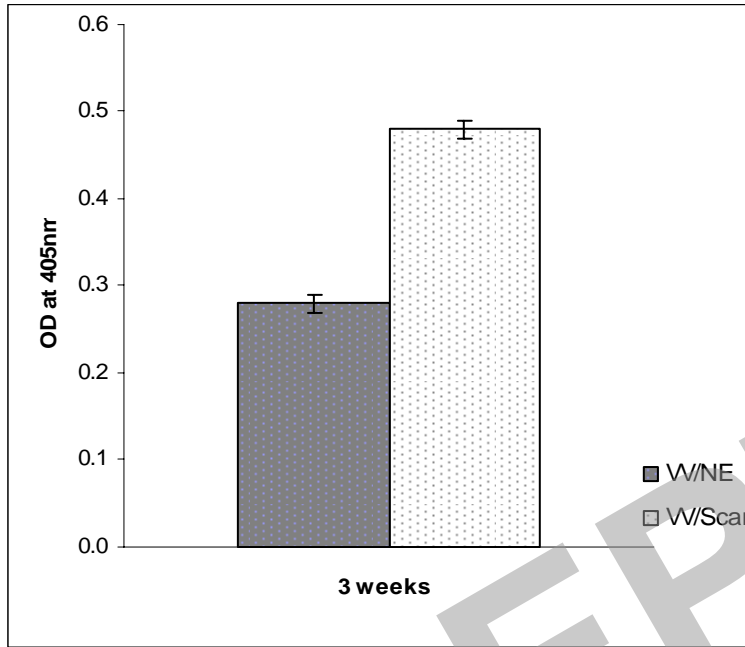
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778 Figure 6A



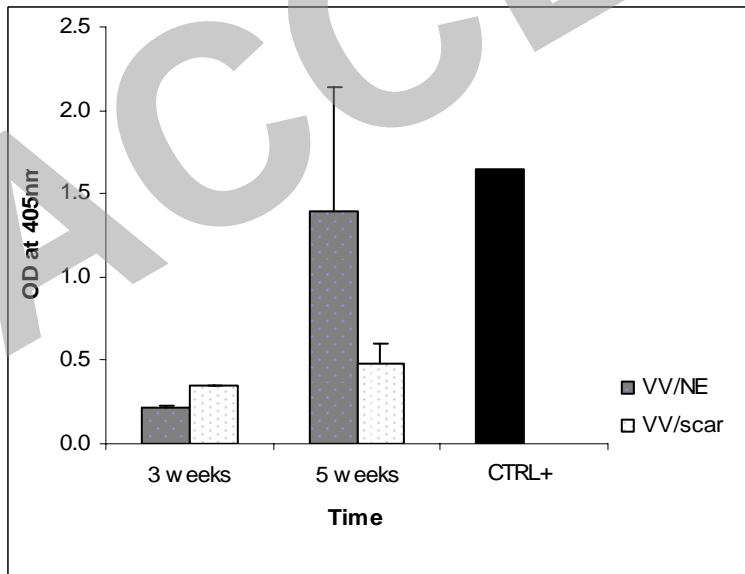


780 Figure 7A



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Figure 7B



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785 Table 1
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Vaccine	Stimulation	CD8+	CD8+/INF γ +	CD4+	CD4+/INF γ +
VV-NE	A20 (infVV)	836.0 (+/-87)	282.0 (+/-14)	1941.5 (+/- 47.5)	271.4 (+/- 25)
	A20	825.0 (+/-157)	40.5 (+/-17.5)	2073.0 (+/-170)	75.3 (+/-14)
	cl7(infVV)	815.4 (+/-91.2)	212.6 (+/-73)	N/A	N/A
	cl7	1086.3 (+/-113.5)	73.0 (+/-24)	N/A	N/A
VV-scarification	A20 (infVV)	475.1 (+/-43)	135.6 (+/-19.6)	1987.2 (+/-244)	303.5 (+/-65.5)
	A20	594.3 (+/-17)	52.0(+/-10.4)	2209.2 (+/-365)	81.3(+/-12.7)
	cl7(infVV)	589.5 (+/- 33.5)	173.0 (+/-22)	N/A	N/A
	cl7	679.0 (+/-32.6)	81.5 (+/-12.5)	N/A	N/A
Non-vaccinated controls	A20 (infVV)	644.0 (+/-48.6)	75.5 (+/-15.5)	1934.0 (+/-50)	82.5 (+/-9.6)
	A20	667.5 (+/-75.5)	42.5 (+/-14.8)	1767.0 (+/-56)	54.5 (+/-15.4)
	cl7(infVV)	518.5 (+/-10.6)	61.5 (+/-12.1)	N/A	N/A
	cl7	691.5 (+/-22.1)	44.0 (+/-25.7)	N/A	N/A

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789 Table 2

	Days	Vaccinated		Controls	
		avg	sem	avg	sem
Head	2	7.0	3.3	33.8	12.0
	3	26.1	13.1	135.9	86.1
	4	75.2	16.5	431.0	252.0
	5	56.6	17.1	924.7	355.6
Chest	2	8.2	1.8	24.0	3.8
	3	13.3	2.0	119.8	25.0
	4	22.1	6.3	216.3	62.0
	5	16.6	1.3	618.2	135.8
Abdomen	2	12.1	1.0	19.2	5.1
	3	13.7	1.3	23.8	8.2
	4	23.0	2.8	28.3	10.0
	5	14.8	1.8	32.1	6.9

790 Table 3
791

Challenge		Survival ^a		
Dose [pfu]	x LD50 ^b	NE vaccine	Scarification	Controls
1.0.E+07	77	5/5	5/5	0/5
2.0.E+06	15	5/5	5/5	0/5
4.0.E+05	3	5/5	5/5	1/5
8.0.E+04	0.62	5/5	5/5	3/5
1.6.E+04	0.12	5/5	5/5	5/5
3.2.E+03	0.02	5/5	5/5	5/5

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a - presented as a ratio of surviving to all mice
b - calculated 1 x LD₅₀ was 1.3 x 10⁵ pfu of VV_{WR}.

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Table 4

Challenge		Protected mice ^a		
Dose [pfu]	x LD ₅₀ ^b	NE vaccine	Scarification	Controls
1.0.E+07	77	0/5	3/5	0/5
2.0.E+06	15	0/5	5/5	0/5
4.0.E+05	3	1/5	5/5	0/5
8.0.E+04	0.62	2/5	5/5	0/5
1.6.E+04	0.12	4/5	5/5	0/5
3.2.E+03	0.02	5/5	5/5	5/5

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800 *a* - presented as a ratio of mice which did *not* have decrease in body weight or decreased
801 temperature at any time after challenge to all mice

802 *b* - calculated 1 x LD₅₀ was 1.3 x 10⁵ pfu of VV_{WR}.

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