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Intranasal Immunization with a Vaccinia Virus Vaccine Vector Expressing Pre-Fusion Stabilized SARS-CoV-2 Spike Fully Protected Mice against Lethal Challenge with the Heavily Mutated Mouse-Adapted SARS2-N501Y$_{MA30}$ Strain of SARS-CoV-2

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Citation: Kibler, K.V.; Szczerba, M.; Lake, D.; Roeder, A.J.; Rahman, M.; Hogue, B.G.; Roy Wong, L.-Y.; Perlman, S.; Li, Y.; Jacobs, B.L. Intranasal Immunization with a Vaccinia Virus Vaccine Vector Expressing Pre-Fusion Stabilized SARS-CoV-2 Spike Fully Protected Mice against Lethal Challenge with the Heavily Mutated Mouse-Adapted SARS2-N501Y$_{MA30}$ Strain of SARS-CoV-2. *Vaccines* **2022**, *10*, 1172. https://doi.org/10.3390/vaccines10081172

Academic Editor: Luis Martinez-Sobrido

Received: 1 June 2022
Accepted: 18 July 2022
Published: 23 July 2022

Abstract: The Omicron SARS-CoV-2 variant has been designated as a variant of concern because its spike protein is heavily mutated. In particular, the Omicron spike is mutated at five positions (K417, N440, E484, Q493, and N501) that have been associated with escape from neutralizing antibodies induced by either infection with or immunization against the early Washington strain of SARS-CoV-2. The mouse-adapted strain of SARS-CoV-2, SARS2-N501Y$_{MA30}$, contains a spike that is also heavily mutated, with mutations at four of the five positions in the Omicron spike associated with neutralizing antibody escape (K417, E484, Q493, and N501). In this manuscript, we show that intranasal immunization with a pre-fusion stabilized Washington strain spike, expressed from a highly attenuated, replication-competent vaccinia virus construct, NYVAC-KC, fully protected mice against symptoms and death from SARS2-N501Y$_{MA30}$. Similarly, immunization by scarification on the skin fully protected against death, but not from mild disease. This data demonstrates that the Washington strain spike, when expressed from a highly attenuated, replication-competent poxvirus—administered without parenteral injection—can fully protect against the heavily mutated mouse-adapted SARS2-N501Y$_{MA30}$.

Keywords: vaccine; SARS-CoV-2; replication-competent

1. Introduction

The recently identified Omicron variant of SARS-CoV-2 has been designated as a variant of concern because of its highly mutated spike protein [1]. In particular, the Omicron spike is mutated at five positions (K417, N440, E484, Q493, and N501), which have been associated with escape from neutralizing antibodies induced by either infection with or immunization against the early Washington strain of SARS-CoV-2 (see Table 1) [2–4]. Thus, Omicron may be able to at least partially escape from immunization with the current vaccines, which are all based on early, unmutated spike proteins.

Table 1. RBD Mutations. The mutations shown are in comparison to the Washington strain of SARS-CoV-2.

Residue	Beta	Gamma	Delta	Omicron	SARS2-N501Y$_{MA30}$
G339				G339D	
S371				S371L	
S373				S373P	
S375				S375F	
K417 *	K417N	K417T		K417N	K417M
N440 *				N440K	
G446				G446S	
L452			L452R		
S477				S477N	
T478			T478K	T478K	
E484 *	E484K	E484K		E484A	E484K
Q493 *				Q493K	Q493R
G496				G496S	
Q498				Q498R	Q498R
N501 *	N501Y	N501Y		N501Y	N501Y
Y505				Y505H	

* Mutations associated with antibody escape.

While the vaccines currently licensed or authorized for emergency use in the United States provide excellent protection against early variants of SARS-CoV-2, including Delta, they have limitations that may hinder their widespread worldwide use. They require maintenance of a significant cold-chain, and are administered parenterally, both of which may make widespread use difficult. We have generated a highly attenuated, replication-competent vaccinia virus vector, NYVAC-KC [5], which does not require an extensive cold-chain and can be administered either by scarification on the skin or intranasally (this manuscript). NYVAC-KC is fully replication competent in human primary keratinocytes and primary human dermal fibroblasts [5]. Despite being replication competent, NYVAC-KC is highly attenuated in the very sensitive newborn intra-cranial mouse model, as well as in immune-deficient mice [5]. NYVAC-KC induced mild induration on the skin of rabbits, with no signs of systemic spread [5]. NYVAC-KC was highly immunogenic, inducing improved T cell and antibody responses to HIV inserts, compared to its replication deficient parental vector, NYVAC [5–10]. Thus, NYVAC-KC may have properties that will make it useful in the worldwide fight against SARS-CoV-2.

In this manuscript we describe protection against the challenge of a mouse-adapted variant of SARS-CoV-2—SARS2-N501Y$_{MA30}$ [11]. Early strains of SARS-CoV-2 are not pathogenic in mice. SARS2-N501Y$_{MA30}$ was generated by serially passaging through mice of the Washington strain SARS-CoV-2 that had an N501Y spike mutation. After 30 passages, the virus became pathogenic for mice, which was associated with increased affinity for mouse ACE2 protein [11]. During the passage through mice, four mutations accumulated in the spike (along with three mutations in orf1a and a non-coding mutation in TRS), K417, E484, Q493, and Q498 along with maintenance of the previous mutation at N501 (Figure 1). All five spike sites mutated in SARS2-N501Y$_{MA30}$ are also mutated in Omicron, and four of the five mutated sites are at residues, which when mutated allow escape from neutralizing antibodies induced by spike from early strains of SARS-CoV-2 [2–4]. Thus, SARS2-N501Y$_{MA30}$ expresses a highly mutated spike, which may also allow for escape from neutralizing antibodies induced by the current vaccines. However, we show that intranasal immunization with a pre-fusion stabilized Washington strain spike, expressed from the highly attenuated, replication-competent vaccinia virus vector NYVAC-KC, fully protected mice against both death and symptoms after infection with SARS2-N501Y$_{MA30}$. Immunization by scarification fully protected against death, but not from mild disease. Thus, Washington strain spike, when expressed from a highly attenuated, replication-competent heat-stable poxvirus vector, administered without parenteral injection, can fully protect against severe disease and death following a challenge with the heavily mutated, mouse-adapted SARS2-N501Y$_{MA30}$ variant of SARS-CoV-2.

Pre-fusion stabilizing mutations in Washington strain SARS-CoV-2 Spike

1 mfvflvllpl vssqcvnltt rtqlppaytn sftrgvyypd kvfrssvlhs tqdlflpffs
61 nvtwfhaihv sgtngtkrfd npvlpfndgv yfasteksni irgwifgttl dsktqslliv
121 nnatnvvikv cefqfcndpf lgvyyhknnk swmesefrvy ssannctfey vsqpflmdle
181 gkqgnfknlr efvfknidgy fkiyskhtpi nlvrdlpqgf saleplvdlp iginitrfqt
241 llalhrsylt pgdsssgwta gaaayyvgyl qprtfllkyn engtitdavd caldplsetk
301 ctlksftvek giyqtsnfrv qptesivrfp nitnlcpfge vfnatrfasv yawnrkrisn
361 cvadysvlyn sasfstfkcy gvsptklndl cftnvyadsf virgdevrqi apgqtgkiad
421 ynyklpddft gcviawnsnn ldskvggnyn ylyrlfrksn lkpferdist eiyqagstpc
481 ngvegfncyf plqsygfqpt ngvgyqpyrv vvlsfellha patvcgpkks tnlvknkcvn
541 fnfngltgtg vltesnkkfl pfqqfgrdia dttdavrdpq tleilditpc sfggvsvitp
601 gtntsnqvav lyqdvnctev pvaihadqlt ptwrvystgs nvfqtragcl igaehvnnsy
661 ecdipigagi casyqtqtns p**rrar**svasq siiaytmslg aensvaysnn siaiptnfti
721 svtteilpvs mtktsvdctm yicgdstecs nlllqygsfc tqlnraltgi aveqdkntqe
781 vfaqvkqiyk tppikdfggf nfsqilpdps kpskrs**f**ied llfnkvtlad agfikqygdc
841 lgdiaardli caqkfngltv lpplltdemi aqytsallag titsgwtfga g**a**alqipf**a**m
901 qmayrfngig vtqnvlyenq klianqfnsa igkiqdslss t**a**salgklqd vvnqnaqaln
961 tlvkqlssnf gaissvlndi lsrld**kv**eae vqidrlitgr lqslqtyvtq qliraaeira
1021 sanlaatkms ecvlgqskrv dfcgkgyhlm sfpqsaphgv vflhvtyvpa qeknfttapa
1081 ichdgkahfp regvfvsngt hwfvtqrnfy epqiittdnt fvsgncdvvi givnntvydp
1141 lqpeldsfke eldkyfknht spdvdlgdis ginasvvniq keidrlneva knlneslidl
1201 qelgkyeqyi kwpwyiwlgf iagliaivmv timlccmtsc csclkgccsc gscckfdedd
1261 sepvlkgvkl hyt

RRAR>GSAS, F817P, A892P, A899P, A942P, KV986/7>PP (12)

Figure 1. Pre-fusion stabilizing mutations in Washington strain Spike. The indicated mutations (in bold) were made to stabilize Washington strain Spike in the pre-fusion conformation, as previously described [12].

2. Materials and Methods

2.1. Viruses

Mouse-adapted SARS-CoV-2 SARS2-N501Y$_{MA30}$ was propagated in A549-huACE2 cells [11]. For insertion of foreign genes into the NYVAC-KC genome, we constructed a cassette (pGNR-cmr^S) that encodes an *E. coli* gyrase/PKR fusion protein that confers coumermycin (cmr) sensitivity [13], and a neo^R gene fused to the GFP gene [14]. The cassette has arms that are homologous to the sequence flanking the TK deletion in NYVAC-KC, to allow for in vivo recombination with the viral genome. The pGNR-cmr^S cassette was added to NYVAC-KC through an in vivo recombination (IVR) [15] done in BSC-40 cells; cells were transfected with a linear cassette DNA using Lipofectamine 2000 (Invitrogen) according to manufacturer instructions. Infection with NYVAC-KC was at an MOI of 0.05. After 48 h, the infected cells were scraped into the medium (1.2 mls Opti-Pro (Gibco) with glutamine and 1% FBS). Following two cycles of freeze/thaw, the cell supernatant was used to infect 100 mm dishes of BSC-40 cells, at 1:10, 1:100, and 1:1000 dilutions of the IVR stock. DMEM 2% FBS plus G418 at 1 mg/mL was added after the infection incubation. Green, G418^R plaques were picked at 48 h post infection, following the addition of an agarose overlay. Plaques were screened in 6-well plates for sensitivity to cmr, and the two showing the highest sensitivity were chosen for continuing to the next round of plaque purification in BSC-40 cells. The plaque from this round that demonstrated the highest sensitivity to cmr was amplified in a 60 mm dish. This virus (NYVAC-KC-pGNR-cmr^s) was used in an IVR to replace the pGNR-cmr^S cassette with the coding sequence for a vaccinia virus optimized and pre-fusion-stabilized SARS-CoV-2 Washington strain spike protein [12], under control of a vaccinia virus synthetic early/late promoter [16], yielding a cmr^R, non-fluorescent virus. For this selection, 100 ng/mL cmr was added at 24 hpi of the IVR, and subsequent infections were carried out in the presence of cmr until the final plaque was chosen. Correct insertion was confirmed by PCR and Western blotting. Plaques were amplified twice to obtain P2 stocks [5] that were used for immunization of mice.

2.2. Cell Lines

BSC40 cells obtained from ATCC (CRL-2761) were cultured in (DMEM; Gibco catalog No. 11965), supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, 100 µg/mL streptomycin, 50 µg/mL gentamicin, and 1 mM sodium pyruvate. African green monkey kidney Vero cells (E6) or (CCL81) (obtained from ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco catalog No. 11965), supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, 100 µg/mL streptomycin, 50 µg/mL gentamicin, 1 mM sodium pyruvate, and 10 mM HEPES. Human A549 cells (Verified by ATCC) were cultured in RPMI 1640 (Gibco catalog No. 11875) supplemented with 10% FBS, 100 U/mL of penicillin, and 100 µg/mL streptomycin. The generation of A549-ACE2 cells was described previously [17].

2.3. Plaque Assay

Briefly, virus supernatant was serially diluted 10-fold and inoculum was absorbed on Vero cells for 1 h at 37 °C. Inoculum was overlaid with DMEM plus 0.7% agarose and incubated for 3 days at 37 °C. Cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet for counting plaques. All infections and virus manipulations were conducted in a biosafety level 3 (BSL-3) laboratory using appropriate and IBC-approved personal protective equipment and protocols.

2.4. Immunization

BALB/c mice at age 7 weeks were immunized with 10^6 pfu of NYVAC-KC-pfsSpike. Immunization was performed either intranasally (in 10 µL), or by tail scarification (20 µL) and under anesthesia with a cocktail containing 37.5 mg/kg ketamine, 7.5 mg/kg xylazine, and 2.5 mg/kg acepromazine. Following vaccination, mice were allowed to recover on heating pads and were monitored until ambulatory, at which point they were placed in their cages. Mice were boosted 1 month and 4 months after initial vaccination. Throughout the duration of the study before challenge, mice were weighed weekly and blood draws were taken on a bi-weekly basis.

2.5. Inhibition of RBD/huACE2 Interaction

Neutralizing antibodies were assessed using a lateral flow assay that semi-quantitatively measures levels of antibodies that prevent binding of Washington strain RBD to ACE2, as previously described [18]. Briefly, 3 µL of serum was diluted to 6 µL in PBS and loaded onto lateral flow strips that had soluble gold-labeled Washington strain RBD, and bound huACE2. Serum and gold-labeled RBD were chased through the strip with chase buffer [18]. After 20 min, blue color at the site of the bound ACE2 was quantified by densitometry. Percent inhibition was calculated as previously described [19], using the following formula: $1 - (\text{Test sample line density/Limit of Detection, LoD}) \times 100$, where the LoD for the non-neutralizing sera for the rapid test was 570,229.

2.6. SARS2-N501Y$_{MA30}$ Challenge

Mice either immunized or not immunized with NYVAC-KC-pfsSpike were moved to the ABSL3 for SARS-CoV-2 challenge. SARS2-N501Y$_{MA30}$ was administered intranasally at a dose of 2×10^3 pfu per animal in a volume of 50 µL. Mice were anesthetized by intraperitoneal route with a cocktail of 50 mg/kg ketamine and 7.5 mg/kg xylazine for the inoculation. Following the inoculation, mice were allowed to recover in their cages, which were placed on heating pads, and mice were monitored until ambulatory. Mice were weighed daily unless their weight fell below 85% of their original weight, at which time they were monitored twice daily. Symptoms were scored in a blinded manner for ruffled fur, hunching, and activity, and scored from 0 to 3 (0 normal, 3 severe) for 10 days and mice were euthanized when their aggregate clinical score reached 8 (including a score of 0–3 for weight loss), as detailed in the approved IACUC protocol. Mice that recovered or were asymptomatic were monitored for 10 days. All animal procedures were approved by the ASU IACUC.

3. Results

3.1. Generation of NYVAC-KC-pfsSpike

A vaccinia virus-optimized Washington strain spike was stabilized in the pre-fusion state by mutation of the furin cleavage site, and insertion of 6 proline residues, preventing the conformational change to the post-fusion conformation (pfsSpike) [12] (Figure 1). PfsSpike, flanked by TK locus homologous flanking arms, was inserted into the TK locus of NYVAC-KC by homologous recombination (Figure 2). The TK locus of NYVAC-KC was modified by insertion of a pGNR-cmr^S cassette [14] prior to homologous recombination with TK flanked pfsSpike. pGNR-cmr^S encodes a neo^r gene and a GFP gene, to allow for selection and identification of virus that has taken up pGNR-cmr^S, as well as a cmr^S gene that acts as a negative selectable marker [13]. Cells were infected with NYVAC-KC-neo^R-GFP-cmr^S and transfected with TK-flanked pfsSpike. Recombinant virus that had replaced the pGNR-cmr^S cassette with pfsSpike was selected for as cmr^R, non-fluorescent plaques. Insertion of pfsSpike was confirmed by PCR and Western blot of individual plaques. This technology allows for rapid insertion (approximately 1 month from obtaining DNA to having a P2 stock) of new genes into NYVAC-KC.

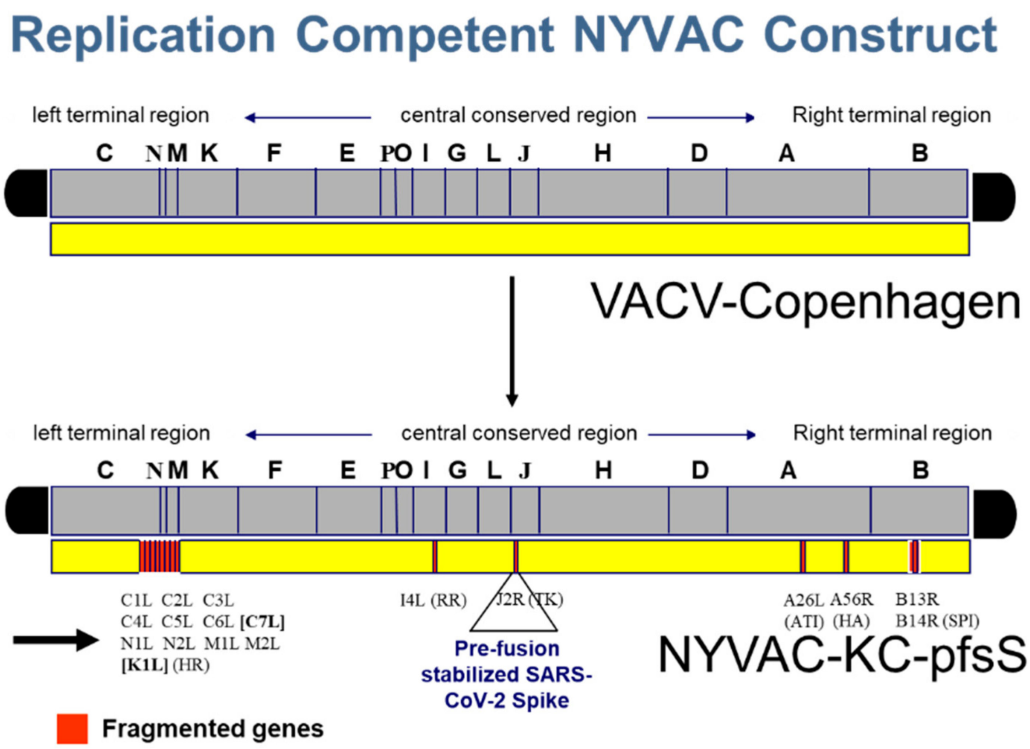

Figure 2. NYVAC-KC-pfsSpike. NYVAC-KC is a highly attenuated, replication-competent derivative of the Copenhagen strain of vaccinia virus, that has been deleted of 16 open reading frames. A pre-fusion stabilized spike, under control of a synthetic early/late promoter was inserted into the TK locus of NYVAC-KC to generate NYVAC-KC-pfsSpike. NYVAC-KC was constructed by inserting the K1L and C7L genes back into the replication-deficient NYVAC strain [5]; location of these two genes is indicated by a bold arrow and bold brackets.

3.2. Immunization with NYVAC-KC

Mice were immunized with 10^6 pfu of NYVAC-KC-pfsSpike, either by scarification or intranasally (Figure 3). Mice were boosted at one month post immunization, rested for 3 months, and boosted a second time. Blood was obtained one month after the primary immunization, one and three months after the first boost and two weeks after the second boost. Serum was assayed for the ability to block binding of Washington strain Spike protein RBD to human ACE2 [18]. Immunization by scarification with NYVAC-KC-pfsSpike gave a modest serum response inhibiting RDB binding to huACE2 (Figure 4A). The response was boosted to high levels, which waned after three months. The second boost increased the serum response, inhibiting binding of RBD to huACE to moderate levels. A single intranasal immunization with NYVAC-KC-pfsSpike induced a potent serum response that inhibited RDB binding to huACE2 (Figure 4B). This response remained high after the first boost and did not appreciably wane three months after the first boost, and remained high after the second boost. Thus, intranasal immunization was able to induce a potent durable serum RBD binding response.

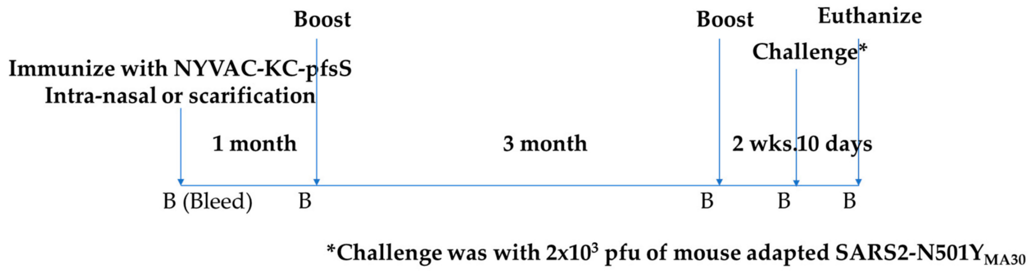

Figure 3. Immunization/challenge schedule. Animals were immunized on day 0 and boosted 1 month and 4 months after the first immunization. Animals were challenged 2 weeks after the second boost and monitored for signs of morbidity for up to 10 days. Animals were bled (indicated by "B") one day prior to each immunization, one day prior to challenge and for all surviving animals, at the termination of the experiment.

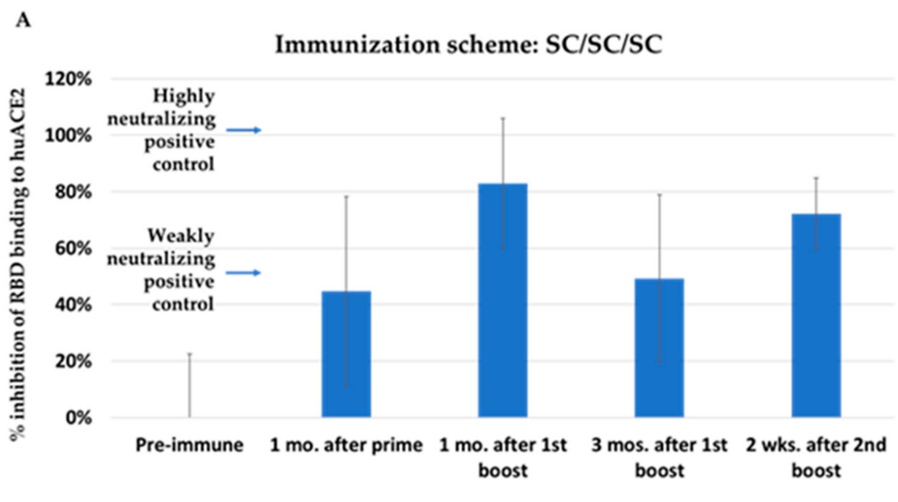

Figure 4. *Cont.*

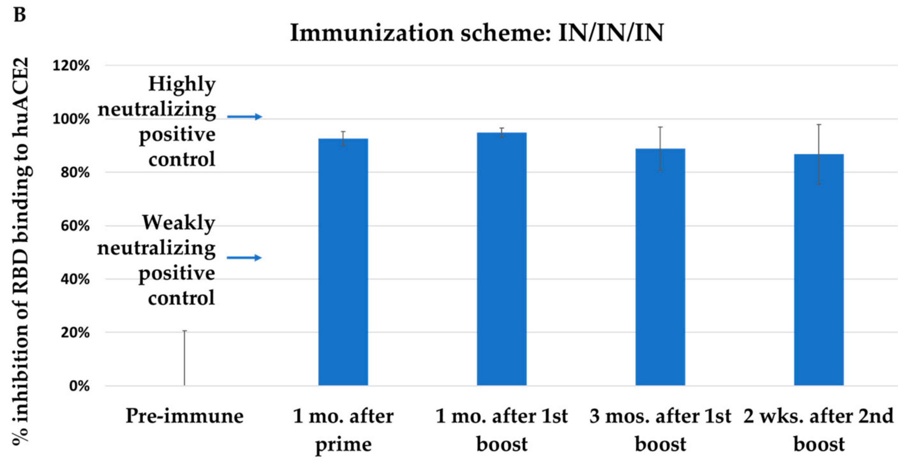

Figure 4. RBD binding antibodies. Serum from animals at the indicated times were assayed for the ability to inhibit binding of gold-labeled Washington strain RBD to huACE2. Controls indicated inhibition of binding by a strongly neutralizing positive control, and a weakly neutralizing positive control. (**A**) Immunization scheme: SC/SC/SC. (**B**) Immunization scheme: IN/IN/IN.

3.3. Challenge with SARS2-N501Y$_{MA30}$

Animals immunized with NYVAC-KC-pfsSpike were challenged intranasally two weeks after the second boost with approximately 2 × 10^3 pfu of SARS2-N501Y$_{MA30}$ [11]. Animals were monitored and scored from 0 to 3 according to the severity for each criterion: weight loss, ruffled fur, hunching, and loss of activity. All animals were scored in a blinded fashion. An aggregate clinical score of 8 was an endpoint for humane euthanasia. Fifteen of 17 animals not immunized with NYVAC-KC-pfsSpike reached a clinical score of 8 by 4 days post-infection and were humanely euthanized (Figure 5, red line). None of the animals immunized with NYVAC-KC-pfs-Spike needed to be euthanized (Figure 5, blue line). Figure 6 shows the clinical score for each animal in aggregate groups from 0 to 9 days post challenge. Mock challenged animals had scores of 0–1 throughout the course of the experiment (Figure 6A). Animals not immunized with NYVAC-KC-pfsSpike, and challenged with SARS2-N501Y$_{MA30}$, all showed signs of illness by days 2–3 post-challenge, and for 15 of the 17 animals, symptoms were serious enough to warrant humane euthanasia (Figure 6B). Intranasally immunized animals were asymptomatic after the SARS2-N501Y$_{MA30}$ challenge, with clinical scores of 0–1 (Figure 6C), indistinguishable from mock challenged animals (Figure 6A). Two of the animals immunized by scarification had mild disease, with maximal clinical scores of 2 and 4 (Figure 6D).

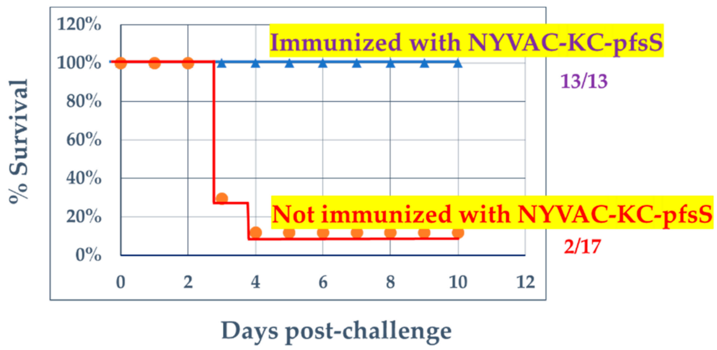

Figure 5. Survival after challenge with mouse-adapted SARS-CoV-2, SARS2-N501Y$_{MA30}$. Animals either not immunized with NYVAC-KC-pfsSpike or immunized with NYVAC-KC-pfsSpike were challenged with 2 × 10^3 pfu of mouse adapted SARS2-N501Y$_{MA30}$. Animals were monitored for morbidity daily in a blinded manner for up to 10 days (see Figure 6). Animals with a clinical score of 8 or higher were humanely euthanized.

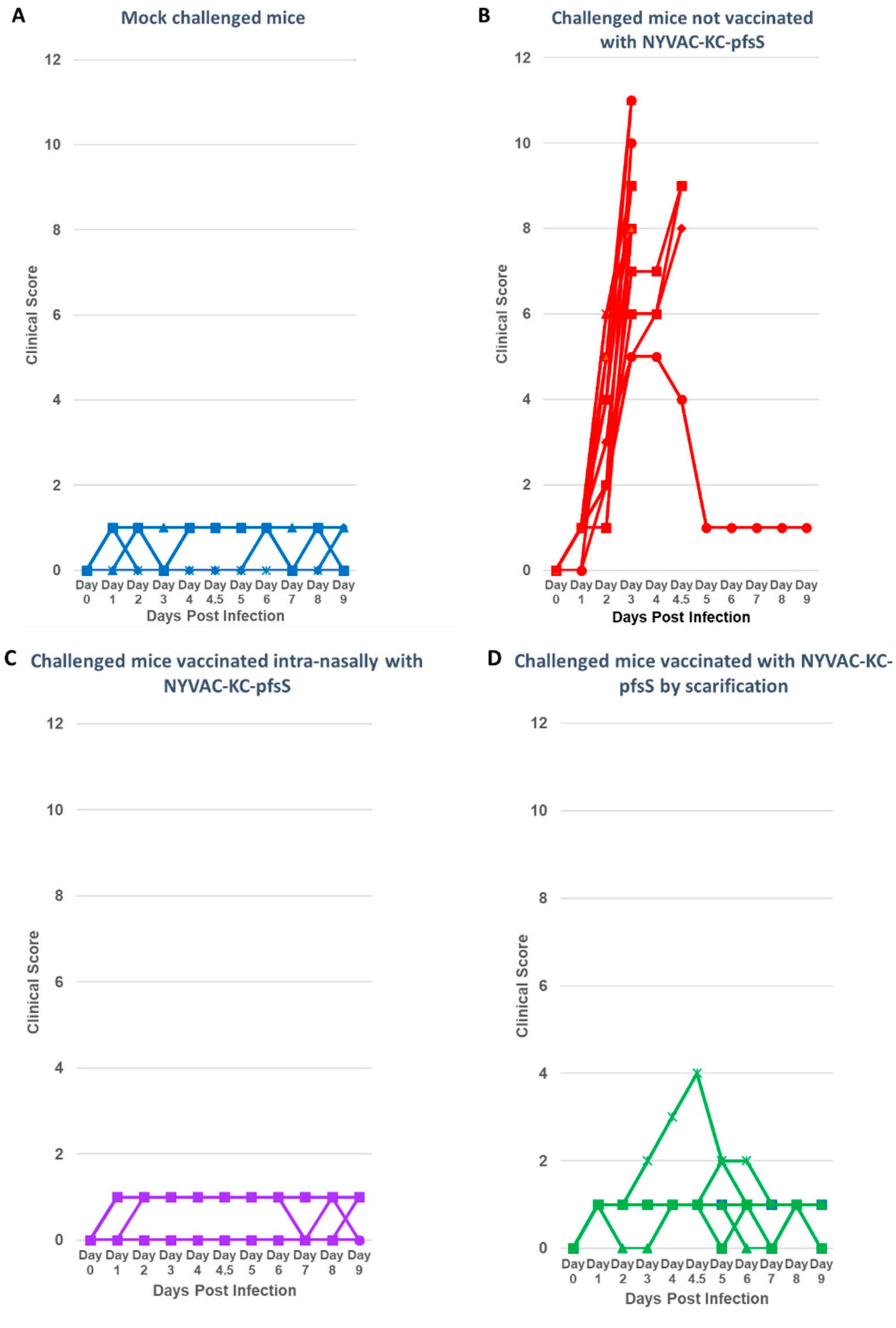

Figure 6. Clinical scores of challenged animals. Animals were monitored for morbidity (weight loss, ruffled fur, hunching, diminished activity, with a range or 0-3 for each parameter, with 0 being no symptoms, 1, mild symptoms, 2, moderate symptoms, and 3, severe symptoms) for up to 10 days after challenge. Animals with an aggregate score of 8 or greater were humanely euthanized. (**A**) Animals not immunized with NYVAC-KC-pfsSpike and not challenged. (**B**) Animals not immunized with NYVAC-KC-pfsSpike and challenged with mouse adapted SARS2-N501Y$_{MA30}$. (**C**) Animals immunized intra-nasally with NYVAC-KC-pfsSpike and challenged with mouse adapted SARS2-N501Y$_{MA30}$. (**D**) Animals immunized by scarification with NYVAC-KC-pfsSpike and challenged with mouse adapted SARS2-N501Y$_{MA30}$.

4. Discussion

In this manuscript we demonstrate that the highly attenuated, replication-competent vaccinia virus vector NYVAC-KC expressing Washington strain spike fully protected mice from severe disease after challenge with a heavily mutated, mouse adapted strain of SARS-CoV-2, SARS2-N501Y$_{MA30}$. NYVAC-KC was originally developed as an improved poxvirus-based vector for immunization against HIV [5]. NYVAC-KC is highly attenuated, yet induces a potent T cell and antibody response against HIV *gag*, *pol*, *nef*, and *env* inserts [5–10]. Since poxvirus vectors are heat stable and generally do not require an extensive cold-chain [20], NYVAC-KC based vectors will likely be easy to distribute worldwide. In this manuscript we demonstrate that NYVAC-KC expressing a pre-fusion stabilized spike can induce protective immune responses when administered either by scarification or intranasally. Thus, these vectors may be easy to administer after widespread distribution. Furthermore, multiple antigens can be expressed from NYVAC-KC. There are six deletion sites in NYVAC-KC (Figure 2), each of which can be used to express foreign antigens. It is also possible to express multiple antigens from each insertion site. We have successfully generated a stable construct expressing HIV *gag*, *pol*, *nef*, and *env* from the TK locus [5]. Thus, it may be possible to express spikes from multiple SARS-CoV-2 variants in a single vector, and to express antigens encoding stable T cell epitopes, in addition to the highly variant Spike proteins.

Early strains of SARS-CoV-2 do not cause disease in wild type mice. On the contrary, mouse adapted SARS2-N501Y$_{MA30}$ is highly virulent in wild type mice [11]. Intranasal administration of a dose of 2×10^3 pfu uniformly induced serious disease in infected animals, with the majority of animals being euthanized by four days post-infection. During the course of adaptation in mice, SARS2-N501Y$_{MA30}$ fixed five mutations in the Spike RBD [11]. These mutations were associated with increased binding to mouse ACE2. Interestingly, while SARS2-N501Y$_{MA30}$ was selected for in immunologically naïve mice, four of the five RBD mutations are at loci associated with resistance to neutralizing antibodies induced by early strain Spike [2–4]. Thus, these mutations may have multiple effects, enhancing binding to murine ACE2, while possibly providing at least partial resistance to neutralizing antibodies. All five of the loci in RBD with fixed mutations in SARS2-N501Y$_{MA30}$ are also mutated in the heavily mutated Omicron variant of SARS-CoV-2 (Table 1).

Immunization with NYVAC-KC-pfsSpike fully protected mice from lethal challenge with the heavily mutated SARS2-N501Y$_{MA30}$, despite the immunogen in NYVAC-KC-pfsSpike having a wild type—unmutated RBD. Thus, either NYVAC-KC-pfsSpike induces neutralizing antibodies to regions not mutated in SARS2-N501Y$_{MA30}$ spike or induces a high enough neutralizing antibody titer to cross-neutralize the divergent SARS2-N501Y$_{MA30}$. While we are in the process of measuring neutralizing antibody levels induced by NYVAC-KC-pfsSpike, we have shown that NYVAC-KC-pfsSpike can potently induce antibodies that inhibit binding of Washington strain RBD to human ACE2 (Figure 4A,B). It is not clear if these antibodies can inhibit binding of SARS2-N501Y$_{MA30}$ RBD to mouse ACE2, or if NYVAC-KC-pfsSpike induces antibodies to other regions of Spike.

While either intranasal or scarification immunization with NYVAC-KC-pfsSpike protected mice from serious disease induced by SARS2-N501Y$_{MA30}$, intranasal immunization appeared to give superior protection, with animals being fully asymptomatic after challenge. Intranasal immunization in animals also induced more potent serum antibody responses that inhibited binding of Washington strain SARS-CoV-2 Spike RBD to human ACE2, than immunization by scarification. Others have noted strong serum antibody responses after intra-nasal immunization [21]. Immunization by scarification yielded weaker serum neutralizing antibody responses, perhaps due to limited replication of this vector, compared to a more wild type VACV vector [5]. It is unclear if these higher titers of serum RBD binding antibodies led to enhanced protection after intranasal immunization, or if intranasal immunization led to an enhanced mucosal immune response that fully protected against disease. It is also unclear if induction of adaptive immunity was fully responsible for the extensive protection seen in these experiments. Although NYVAC-KC is replication competent, it is a relatively poor inducer of innate immunity [5,6,9]. Even though NYVAC-KC is cleared from mice within two days of infection [5], it is still possible that lingering innate immunity to the vector could play a role in the extensive protection seen in these experiments.

In conclusion, in this manuscript we demonstrate that the highly attenuated, replication-competent vaccinia virus vector NYVAC-KC, expressing Washington strain spike, administered without parenteral injection, fully protected mice from severe disease after challenge with a heavily mutated, mouse adapted strain of SARS-CoV-2—SARS2-N501Y$_{MA30}$.

Author Contributions: Conceptualization, B.L.J., K.V.K., M.R., B.G.H. and Y.L.; methodology, B.L.J., K.V.K., A.J.R., D.L., M.R. and M.S.; resources, B.L.J., D.L., L.-Y.R.W., S.P., B.G.H., Y.L. and M.R.; writing—original draft preparation, B.L.J.; writing—review and editing, K.V.K., M.S., D.L., A.J.R., M.R., B.G.H., L.-Y.R.W., S.P. and Y.L.; visualization, B.L.J. and K.V.K.; supervision, B.L.J., D.L. and K.V.K.; project administration, B.L.J. and K.V.K.; funding acquisition, B.L.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by grants from the National Institutes of Health USA (P01 AI060699 and RO1 AI129269) to S.P., by grants from National Institutes of Health USA (R01 AI095394), The ASU Catalyst Fund (PG13155) and ASU Presidents Club (GF2391) to B.L.J., and a grant from Mercatus Center Fast Grant (G09202-300) to B.G.H. and M.R.

Institutional Review Board Statement: The study was conducted according to the guidelines and approved by the Institutional Animal Care and Use Committee (IACUC), protocol 20-1746R.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available upon reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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