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Vaccination with Live Attenuated Simian Immunodeficiency Virus (SIV) Protects from Mucosal, but Not Necessarily Intravenous, Challenge with a Minimally Heterologous SIV

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ABSTRACT

Few studies have evaluated the impact of the viral challenge route on protection against a heterologous simian immunodeficiency virus (SIV) challenge. We vaccinated seven macaques with a live attenuated SIV that differed from SIVmac239Δnef by 24 amino acids, called m3KOΔnef. All animals were protected from an intrarectal SIVmac239 challenge, whereas only four animals were protected from subsequent intravenous SIVmac239 challenge. These data suggest that immune responses elicited by vaccination with live attenuated SIV in an individual animal can confer protection from intrarectal challenge while remaining insufficient for protection from intravenous challenge.

IMPORTANCE

Our study is important because we show that vaccinated animals can be protected from a mucosal challenge with a heterologous SIV, but the same animals are not necessarily protected from intravenous challenge with the same virus. This is unique because in most studies, either vaccinated animals are challenged multiple times by the same route or only a single challenge is performed. An individually vaccinated animal is rarely challenged multiple times by different routes, so protection from different challenge routes cannot be measured in the same animal. Our data imply that vaccine-elicited responses in an individual animal may be insufficient for protection from intravenous challenge but may be suitable for protection from a mucosal challenge that better approximates human immunodeficiency virus (HIV) exposure.

Two important variables that can influence the apparent efficacy of preclinical human immunodeficiency virus (HIV)/simian immunodeficiency virus (SIV) vaccines are the route of infection and the sequence of the challenge virus. For example, live attenuated SIV offers effective and consistent protection from intravenous challenge with a homologous virus but incomplete protection from intravenous challenge with a heterologous virus. This incomplete protection from heterologous challenge was observed after vaccination with several live attenuated SIV strains (e.g., SIVmac239Δnef, SIVmac239Δ3, SIVsmE543Δnef, and SIVmacC8), followed by challenge with different pathogenic SIV stocks (e.g., SIVsmE660, SHIV89.6p, SIV239/EnvE543, and SIVmac239) (1–6). Although those studies were each different, incomplete protection from an intravenous heterologous challenge was observed for both rhesus and cynomolgus macaques.

The failure to offer complete protection from an intravenous challenge with a heterologous virus may be because some immune responses elicited by live attenuated SIV are localized to the mucosa and are not mobilized systemically at the time of challenge, making an intravenous challenge too stringent for testing of the efficacy of live attenuated SIV vaccines (7, 8). Perhaps, immune responses elicited by a vaccine in an individual animal are sufficient to protect from mucosal challenge with a high dose of a heterologous virus, even if they cannot protect from intravenous challenge with the same virus.

In this study, we wanted to test the hypothesis that a live attenuated SIV vaccine can offer protection from a high-dose mucosal challenge with a heterologous SIV but not an intravenous challenge with the same virus in an individual animal. If this hypothesis is correct, then challenging vaccinated animals intravenously with SIV may be an inappropriately high bar for testing of preclinical HIV vaccines.

MATERIALS AND METHODS

Animals and virus infections. The seven experimental animals, animals cy0348, cy0379, cy0381, cy0382, cy0383, cy0384, and cy0385, were used in a previous study (9). Animals cy0379, cy0381, cy0384, and cy0385 were homozygous for the M3 major histocompatibility complex (MHC) haplotype and are referred to as "M3 positive." Animals cy0348, cy0382, and cy0383 expressed no MHC alleles from the M3 MHC haplotype and are referred to as "M3 negative." All seven animals were immunized intravenously with 10 ng p27 m3KOΔnef, a strain of live attenuated SIV that differs from SIVmac239Δnef by 24 amino acids.

Twenty weeks after immunization, animals cy0379, cy0381, cy0382, cy0383, cy0384, and cy0385 were challenged intrarectally with 7,000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239. Animal cy0348 was immunized with m3KOΔnef 98 days prior to the other six animals. Thus,

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TABLE 1 All seven animals vaccinated with m3KOΔnef are resistant to intrarectal challenge with SIVmac239[a]

Challenge virus and animal	Viral load (ceq/ml plasma)			
	Day 0	Day 7	Day 14	Day 21
ΔNef				
cy0348	<50	<50	<50	<50
cy0379	82,500	21,500	29,000	102,000
cy0381	681	178	358	273
cy0382	<50	<50	<50	<50
cy0384	<50	<50	<50	<50
cy0383	<50	<50	<50	54
cy0385	4,730	231	1,330	7,660
Full-length Nef				
cy0348	<50	<50	<50	<50
cy0379	<50	<50	<50	<50
cy0381	<50	<50	<50	<50
cy0382	<50	<50	<50	<50
cy0384	<50	<50	<50	<50
cy0383	<50	<50	<50	<50
cy0385	<50	<50	<50	<50

[a] The time points listed indicate the number of days after intrarectal challenge. *nef* qRT-PCR was performed on plasma samples after SIVmac239 challenge. Viral loads for the 182-bp *nef* deletion and full-length *nef* were measured. A viral load of <50 virus copy equivalents (ceq) per ml of blood plasma represents the limit of detection.

he was challenged intrarectally with 7,000 $TCID_{50}$ SIVmac239 20 weeks after m3KOΔnef vaccination and then rechallenged intrarectally with 7,000 $TCID_{50}$ SIVmac239 at day 98 after the first challenge, alongside the other six animals, so that the timeline for the intrarectal and intravenous challenge studies described in this study would align across all 7 animals. Fourteen weeks after this intrarectal challenge, all seven animals were rechallenged intravenously with 100 $TCID_{50}$ SIVmac239.

The four control animals, animals cy0684, cy0686, cy0687, and cy0689, were immunized originally with 10 ng p27 SIVmac239Δnef and then challenged intravenously 28 weeks later with 100 $TCID_{50}$ SIVmac239. All four of these animals were homozygous for the M3 MHC haplotype and are called M3-positive animals here.

All animals used in this study were purchased from Charles River Laboratories or the Bioculture Group and cared for by the Wisconsin National Primate Research Center (WNPRC) according to protocols approved by the University of Wisconsin Graduate School Animal Care and Use Committee.

Plasma viral load analysis. Viral loads were determined from plasma obtained from venous blood drawn into EDTA tubes. SIV *gag* loads were determined essentially as previously described (9, 10). Viral RNA (vRNA) was isolated from plasma, reverse transcribed, and amplified with the SuperScript III Platinum one-step quantitative reverse transcription-PCR (qRT-PCR) system (Invitrogen). Quantification of amplified templates was performed on a LightCycler 480 instrument (Roche). Serial dilutions of an SIV *gag in vitro* transcript were used as an internal standard curve for each run. LightCycler version 1.5 software was used to interpolate values for samples onto the standard curve to determine the copy number. The detection limit of the assay was either 50 or 100 vRNA copy equivalents per ml of plasma, depending on the vRNA isolation protocol used. The limit-of-detection value was reported when the viral load was at or below the limit of detection.

Full-length *nef* and Δ*nef* viral loads were determined as previously described (11). Briefly, highly specific, real-time RT-PCR assays were used with primers that accurately differentiate viruses containing full-length *nef* from those that contain *nef* with a 182-bp deletion, using the methods described above. Serial dilutions of *in vitro* transcripts for both full-length *nef* and *nef* with a 182-bp deletion were used as internal standards for each run. The same machines and software used for the *gag* viral load assay were used to detect and quantify the *nef* and Δ*nef* viral loads. The limit of detection was identical to that for the SIV *gag* viral load assay.

IFN-γ ELISPOT analysis. Fresh PBMCs (peripheral blood mononuclear cells) were subjected immediately to an gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay, as previously described (12). Briefly, a precoated monkey IFN-γ ELISPOTplus plate (Mabtech, Mariemont, OH) was blocked, and peptides corresponding to each of the wild-type epitope sequences were added to each well at a final concentration of 1 μM. Peptides were tested in duplicate. A total of 10^5 cells were added to each well, and plates were incubated overnight. Plates were developed according to the manufacturer's protocol. Spots were imaged with an ELISPOT reader (AID Autoimmun Diagnostika Gmbh). Numbers of SFCs (spot-forming cells) per 10^6 PBMCs were calculated by subtracting the average number of background spots (average of data from four wells not stimulated with any peptide, which served as negative controls) and then multiplying this value by 10. Values for duplicate wells were then averaged and graphed. A response was considered positive if it exceeded our threshold of the no-stimulation average plus 2 times the standard deviation for the nonstimulated wells or if it had 50 SFCs per 10^6 PBMCs, whichever was greater. Concanavalin A was used as a positive control at 10 μM.

Deep sequencing of SIV. Genome-wide deep sequencing of replicating virus populations from animals cy0381, cy0348, and cy0382 at necropsy was performed as previously described (12). Briefly, circulating plasma vRNA was isolated with the MinElute virus spin kit (Qiagen). The Superscript III One-Step RT-PCR system with Platinum *Taq* High Fidelity (Invitrogen) was used to generate viral cDNA and four overlapping amplicons spanning the entire SIV coding sequence. PCR products were purified with the MinElute gel extraction kit (Qiagen) and were then quantified with the Quant-IT dsDNA HS assay kit (Invitrogen). Libraries were generated from 1 ng of pooled amplicons. The libraries were tagged with barcodes by using the Nextera XT kit (Illumina) and then quantified with the Quant-IT dsDNA HS assay kit. An Agilent Bioanalyzer was used to assess the quality of the library preparation. Libraries were pooled and sequenced on an Illumina MiSeq instrument using 2-by-250 kits.

Sequences were analyzed with Geneious (Biomatters, Ltd.). Reads were initially quality trimmed. Paired reads were then mapped to the SIVmac239 sequence (GenBank accession number M33262). Variant nucleotides were called at a threshold of 5%. The variants detected in the analyzed virus populations were then compared to the mutant sites originally incorporated into m3KOΔnef that were 5′ of the 182-bp deletion in *nef*. The frequencies of variant nucleotides in the virus populations at necropsy that matched those of the original inoculum are reported in Table 2.

Nucleotide sequence accession number. Sequences have been deposited in the Sequence Read Archive (SRA) under accession number SRP073659.

RESULTS

To test our hypothesis, we used seven Mauritian cynomolgus macaques (MCMs) that had been vaccinated with m3KOΔnef, a live attenuated strain of SIV that differs from SIVmac239Δnef by 24 amino acids (9). Four of these amino acid mutations are located in known CD8 T cell epitopes presented by MHC class I molecules expressed in the four M3-positive animals (animals cy0379, cy0381, cy0384, and cy0385) (9). *gag* viral loads were measured in these seven animals during the first 20 weeks after vaccination with m3KOΔnef (9). Viral loads peaked at 10^5 virus copy equivalents per ml of blood plasma, or higher, in all 7 animals. We found that the four M3-positive animals had a mean viral load set point of 2,042 copies/ml, whereas the three M3-negative animals (animals cy0348, cy0382, and cy0383) had a mean viral load set point of 39 copies/ml (9).

At least 20 weeks after immunization with m3KOΔnef, all

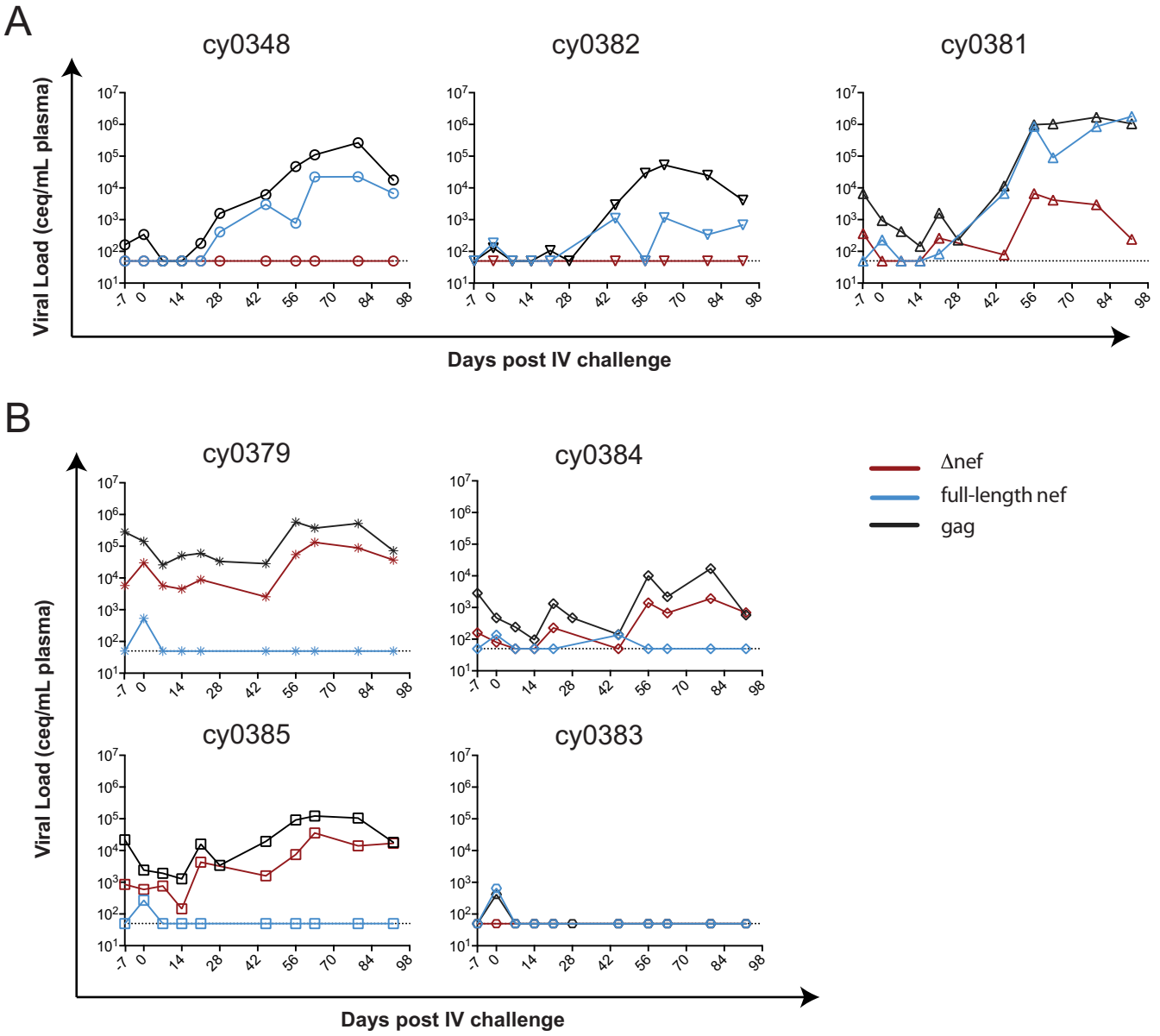

FIG 1 Animals vaccinated with m3KOΔnef are partially susceptible to intravenous (IV) challenge with SIVmac239. *gag* and *nef* qRT-PCRs were performed on plasma samples at the indicated times after intravenous SIVmac239 challenge. (A) Three animals with evidence of full-length *nef*. (B) Four animals without replicating full-length *nef*. Data for each animal are shown on independent plots. The horizontal dotted line at 50 copies/ml (copy equivalents [ceq] per milliliter) represents the limit of detection of the assay.

seven animals were challenged intrarectally with 7,000 $TCID_{50}$ SIVmac239, a dose and challenge route that have been used successfully to infect unvaccinated MCMs (13, 14). To distinguish between replication of SIVmac239 and m3KOΔnef, we used a qRT-PCR assay that uniquely quantifies the number of copies of full-length *nef* and Δ*nef* (a *nef* gene with a 182-bp deletion) (11). Even though three of the animals were unable to control the replication of m3KOΔnef (Table 1), we did not detect replication of the full-length *nef* sequence in the seven animals (Table 1).

We continued to monitor *gag* viral loads in these seven animals up to ~100 days after intrarectal challenge with SIVmac239. Of the four M3-positive animals, viral loads ranged from undetectable to $>10^5$ copies/ml. For the three M3-negative animals, viral loads remained at <500 copies/ml (data not shown).

We rechallenged all seven animals intravenously with 100 $TCID_{50}$ SIVmac239 at ~14 weeks (~100 days) after intrarectal challenge. Blood was sampled from these animals immediately after challenge (day 0) (Fig. 1) and then for several weeks. Determinations of *gag* viral loads were performed on plasma from all animals. In three of the animals (Fig. 1A), the *gag* viral loads became detectable ~2 to 3 weeks after infection and then continued to increase for several weeks thereafter (Fig. 1A, black lines). For these three animals, the increase in full-length *nef* viral loads paralleled the same increases in *gag* viral loads (Fig. 1A, blue lines). Notably, both M3-positive and M3-negative animals had detectable full-length *nef*, so we could not ascribe a link between MHC genetics and susceptibility to intravenous SIVmac239 challenge.

This delayed detection of *nef* is consistent with previous studies

TABLE 2 Frequencies of m3KOΔnef mutations detectable in virus populations at necropsy[a]

nt position of original mutation	Frequency (%) in animal:		
	cy0381	cy0348	cy0382
1316	99.8	95.7	96.6
1481	64.4	<5	47.6
1510	99.7	<5	48.3
2467	99.7	99.6	99.4
2723	99.6	84.1	99.8
2850	99.8	95.5	99.7
2860	99.9	96.6	99.5
3721	99.9	99.7	99.9
4260	99.8	92.6	99.8
4945	99.5	99.5	99.4
5815	100.2	99.9	99.8
6199	98.9	97.0	96.5
6639	99.4	65.6	99.7
6923	97.4	11.6	98.1
6925	97.4	11.6	98.1
7058	99.8	99.8	99.8
8390	98.0	11.3	97.7
8750	56.4	6.8	87.4
8850	92.1	52.3	25.5
9110	99.7	99.7	99.5
9176	85.0	5.2	5.3
9181	84.0	46.7	6.0

[a] The nucleotide (nt) positions in the m3KOΔnef inoculum virus that had the original mutations, relative to SIVmac239, are shown. The frequencies of the individual variants, relative to SIVmac239, are shown for each of the virus populations isolated from the three animals. This percentage reflects the frequency that the original mutation from the m3KOΔnef inoculum was still found in the virus population at necropsy.

suggesting that time is required for live attenuated SIV to recombine with the challenge virus prior to expansion (2). To assess recombination, we deep sequenced virus populations isolated at necropsy. We found that at least 50% of the variants upstream of the *nef* deletion, and present in m3KOΔnef, were present in the replicating virus population at a frequency of 90% or higher in all three animals (Table 2). This includes a variant present at nucleotide position 9110, which is ~500 bp upstream of the deletion in *nef*. Together, these data strongly suggest that the live attenuated SIV recombined with the SIVmac239 challenge virus.

Of the four animals that appeared to control intravenous challenge with SIVmac239 (Fig. 1B), all four of them tested positive for full-length *nef* in the blood sampled immediately after infection (day 0) (Fig. 1B, blue lines). This detection of full-length *nef* at day 0 was transient, indicating successful inoculation with SIVmac239 in these four animals that was subsequently controlled.

We wanted to determine if the specificity of the CD8 T cell response in these animals was different after intrarectal and intravenous challenges with SIVmac239. We performed IFN-γ ELISPOT assays 3 weeks after intrarectal and intravenous challenges. We used peptides for the epitopes that we predicted could be targeted in the animals with each MHC genotype. We found that there were no overt and consistent differences in the specificity of the T cell response after intrarectal or intravenous challenge (Fig. 2), independent of whether viruses with full-length *nef* ultimately replicated in the animals. Notably, 9 weeks after intravenous challenge with SIVmac239, we found that the M3-positive animal with replicating full-length *nef*, animal cy0381, had IFN-γ ELISPOT responses to two epitopes in Nef that are absent in m3KOΔnef but present in full-length Nef.

We wanted to confirm that SIVmac239Δnef vaccination protects MCMs from intravenous homologous SIVmac239 challenge, as observed previously in rhesus macaques (4). Four MCMs that were vaccinated with SIVmac239Δnef for 28 weeks were challenged intravenously with 100 $TCID_{50}$ SIVmac239. Again, blood was sampled from these animals immediately after challenge (day 0) and then for several weeks. *gag* qRT-PCR was performed to measure total SIV loads prior to and after SIVmac239 challenge (Fig. 3A). We observed minor transient increases in virus replication ~2 to 3 weeks after SIVmac239 challenge. We then quantified full-length *nef* and Δ*nef* for select time points, as described above (Table 1 and Fig. 1). All four animals had full-length *nef* detectable in their blood immediately after challenge (day 0), but this became undetectable at days 21, 42, and 63 (Fig. 3A). This stands in stark contrast to the detection of full-length *nef* observed in the three m3KOΔnef-immunized animals with replicating full-length *nef* (Fig. 1A). This control experiment demonstrates that SIVmac239Δnef vaccination protects from intravenous homologous challenge.

To confirm that there was no full-length Nef antigen in the four SIVmac239Δnef-vaccinated animals after SIVmac239 challenge, we performed IFN-γ ELISPOT assays at days 21 and 63 using two CD8 T cell epitopes that are present in full-length Nef but absent in SIVmac239Δnef: $Nef_{103-111}$RM9 and $Nef_{196-203}$HW8. These epitopes are restricted by Mafa-A1*063, an MHC class I molecule expressed in all four animals. CD8 T cell responses to $Nef_{103-111}$RM9 are routinely detected 2 to 4 weeks after SIVmac239 infection, and responses to $Nef_{196-203}$HW8 are detected 8 weeks after SIVmac239 infection (13, 15–17). We found that no animals tested positive for a response against either Nef epitope (Fig. 3B), and all animals tested positive for a response against the $Env_{338-346}$RF9 epitope that is present in SIVmac239Δnef. Animal cy0689 appeared to have an elevated response to $Nef_{103-111}$RM9 at day 21, but this value did not exceed the positive threshold calculated for this animal at this time point (see Materials and Methods) and is therefore not considered a positive response. These data provide further evidence that there was no full-length *nef* sequence replicating in these four animals.

DISCUSSION

Unlike other studies of live attenuated SIV vaccines, we challenged seven vaccinated animals both mucosally and intravenously with a high dose of a minimally heterologous virus. Even though there is no absolute definition of a heterologous virus, we found that all seven animals were protected from mucosal challenge, but only four animals were protected from intravenous challenge. These results were independent of the specificity of vaccine-elicited CD8 T cells or host MHC genetics. Similar studies that sequentially challenge vaccinated animals with a high dose of virus are rare (18) because a subset of animals is typically infected during the initial SIV challenge. Nonetheless, challenging animals serially but by different routes can help discern whether vaccine-elicited immune responses can offer protection at one site of exposure but not another one.

It is possible that serial inoculation of the animals in our study affected the differential protection observed with intravenous challenge. Notably, serial exposures are used in models that use rapidly repeated low-dose SIV challenges. While serial challenges may be a concern, this approach is necessary when assessing if host

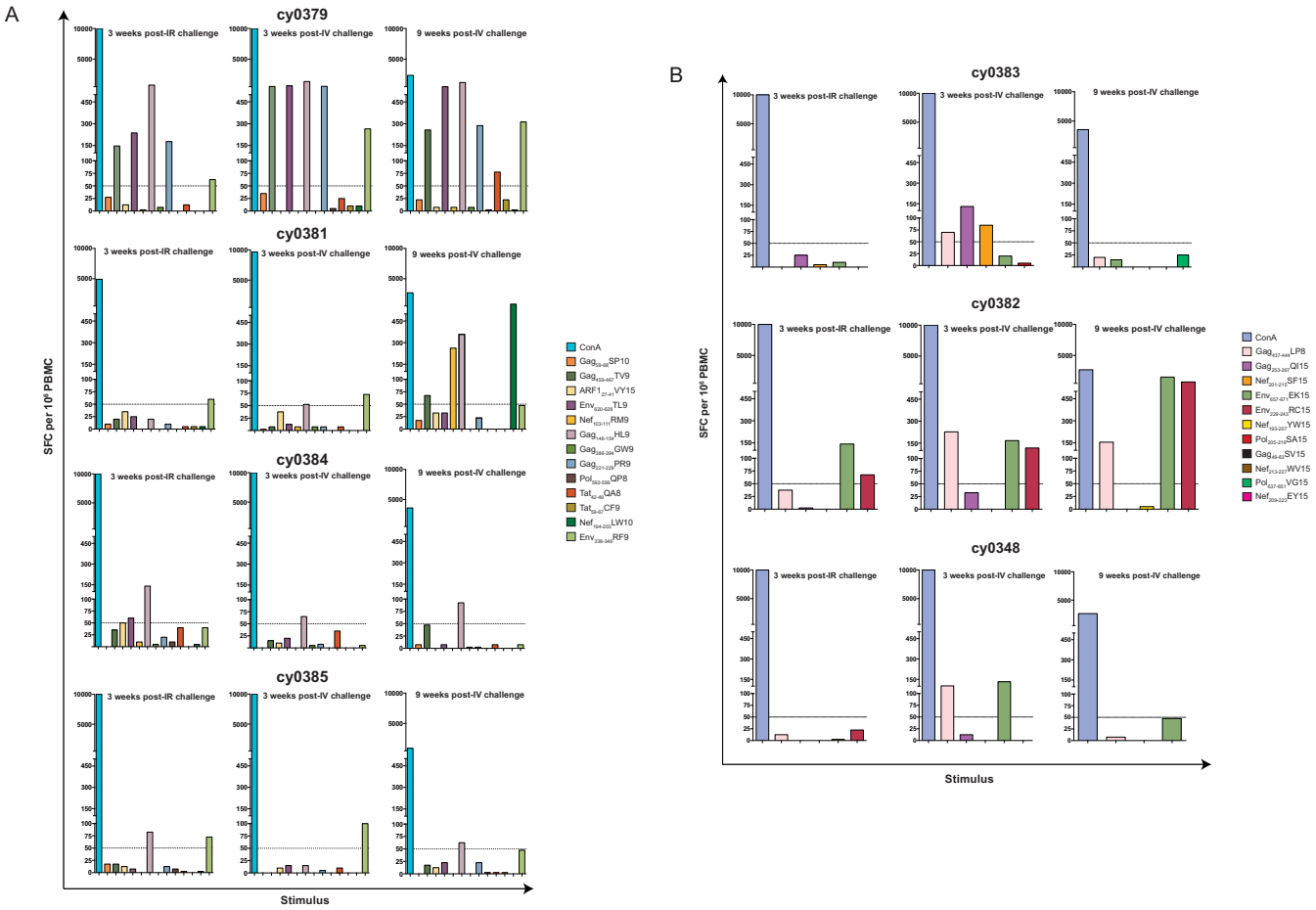

FIG 2 Minimal differences in T cell responses elicited following intrarectal and intravenous SIVmac239 challenges. IFN-γ ELISPOT assays were performed by using peptides for epitopes that could be targeted in animals that were M3 positive (A) and M3 negative (B). T cell responses were measured 3 weeks after intrarectal (IR) challenge, 3 weeks after intravenous challenge, and 9 weeks after intravenous challenge, with concanavalin A (ConA) being used as a positive control at all time points. Of note, at the time of the assay, it was unknown that the $Nef_{194–203}$LW10 peptide contained the $Nef_{196–203}$HW8 epitope used for Fig. 3B. A response was considered positive if the number of SFCs per 10^6 PBMCs exceeded our threshold of the no-stimulation average plus 2 times the standard deviation for nonstimulated wells or 50 SFCs per 10^6 PBMCs, whichever was greater. This threshold of 50 SFCs per 10^6 PBMCs is represented with a horizontal dotted line. Each bar color represents a different peptide and shows the average of data for two duplicate wells following subtraction of the average for the nonstimulated wells.

immune responses in a single individual protect from different types of virus exposures.

Unfortunately, there was no clear reason why three of the m3KOΔnef-vaccinated animals had replication of full-length *nef* after intravenous challenge with SIVmac239, whereas four animals did not. Four of the seven animals were M3 positive and MHC identical, but we found that M3-positive animals were in both outcome groups. Although the numbers of animals are small, this suggests that host MHC genetics did not play a role in the susceptibility of vaccinated MCMs to intravenous challenge with SIVmac239. Furthermore, there were no overt differences in the specificity of the T cell responses 3 weeks after intrarectal and intravenous challenges, as measured by IFN-γ ELISPOT assays (Fig. 2 and data not shown), suggesting that the specificity of T cells in the periphery did not determine whether animals were able to prevent the replication of viruses with full-length *nef* sequences after intrarectal, but not intravenous, challenge. Together, these observations are consistent with data from previous studies suggesting that control of SIV replication may be independent of the CD8 T cell response, especially among animals that do not have protective MHC alleles (19, 20). It is alternately possible that persistent replication of the m3KOΔnef virus in some animals offered the maintenance of effective host immune responses, but this hypothesis is difficult to test in the small cohort of animals in this study. Future studies will need to address the immunological reasons for this differential outcome.

Overall, it is not that surprising that a single-vaccine approach offers better protection from mucosal challenge than from intravenous challenge. In fact, animals vaccinated with SIVmac239Δnef and then subjected to rapidly repeated low-dose challenges with SIVsmE660 were less susceptible to pathogenic infection than a separate vaccinated cohort that was challenged intravenously with SIVsmE660 (2, 21). Our study is unique, however, for two reasons. First, we challenged the same cohort of animals both mucosally and intravenously with the same virus. Second, our mucosal challenge used a dose of SIV with >10 times the number of copies of virus particles as those used in previous rapidly repeated low-dose challenges (21), yet none of the vacci-

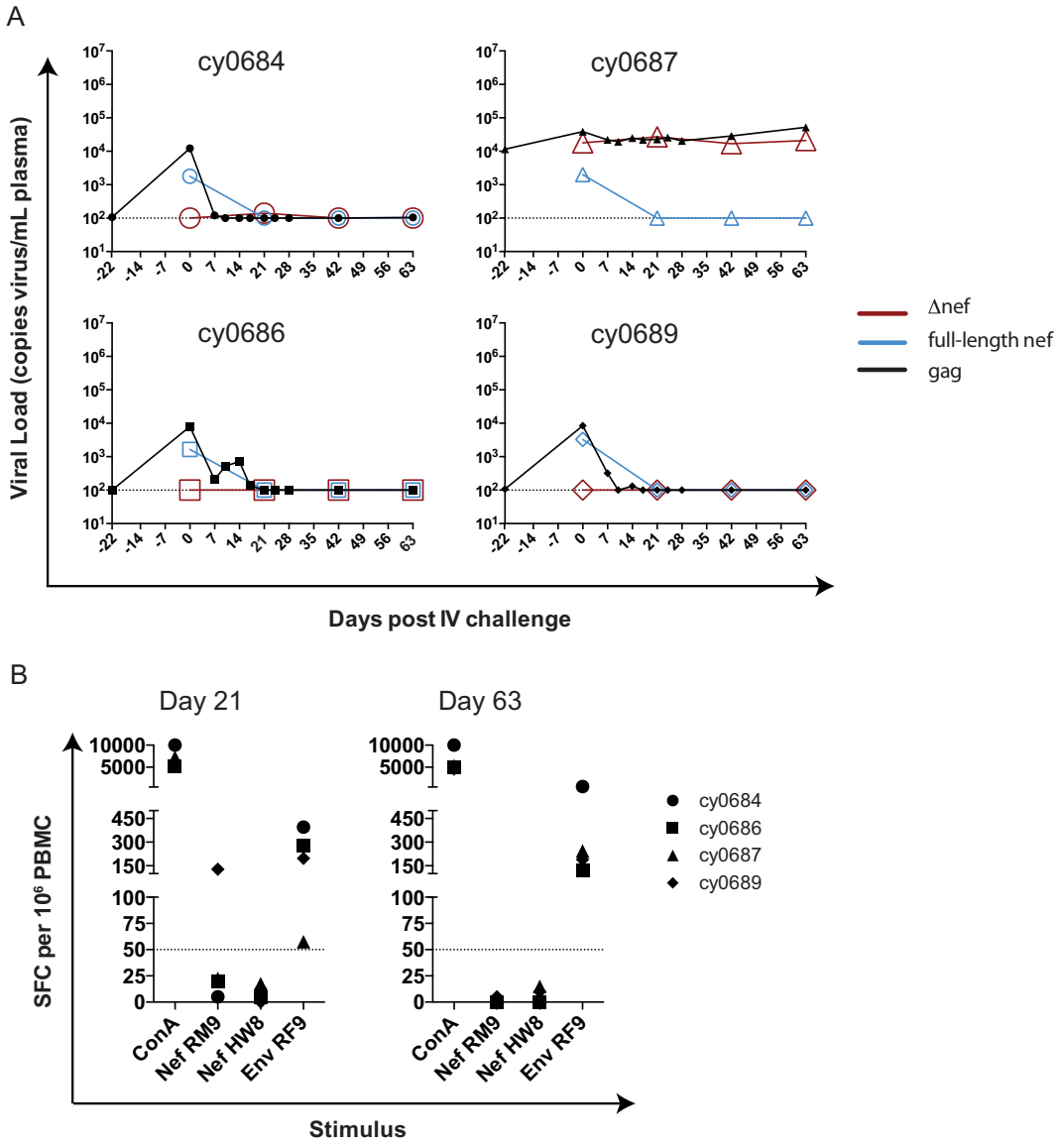

FIG 3 Animals vaccinated with SIVmac239Δnef are resistant to intravenous challenge with SIVmac239. (A) *gag* qRT-PCR was performed on plasma samples at the indicated times following intravenous SIVmac239 challenge, and data were graphed. Full-length *nef* and Δ*nef* qRT-PCRs were performed at select time points for each animal. The limit of detection of the assay is shown with a horizontal dotted line. (B) IFN-γ ELISPOT assays were performed with two epitopes ($Nef_{103-111}$RM9 and $Nef_{196-203}$HW8) at days 21 and 63 post-intravenous challenge. An additional epitope present in SIVmac239Δnef, $Env_{338-346}$RF9, is shown as a positive control, as is concanavalin A (ConA). A response was considered positive if the number of SFCs per 10^6 PBMCs exceeded our threshold of the no-stimulation average plus 2 times the standard deviation of data for nonstimulated wells or 50 SFCs per 10^6 PBMCs, whichever was greater. This threshold of 50 SFCs per 10^6 PBMCs is represented with a horizontal dotted line. Each shape represents a different animal and is the average of data for two duplicate wells following subtraction of the average for the nonstimulated wells.

nated animals in our study became infected with a dose of virus that readily infects unvaccinated animals (14).

Even though the sample size for this study is small, our data suggest that immune responses elicited by strains of live attenuated SIV may protect from a high-dose mucosal challenge with SIV, even if they do not offer similar protection from intravenous challenge. We build upon previous vaccine studies because we show that vaccine-elicited immunity from mucosal exposure does not necessarily protect the same animal from intravenous exposure. Since mucosal transmission accounts for most HIV transmission globally, these results suggest that an intravenous challenge may be an inappropriately high bar for evaluating vaccine performance, and this difference should be a definite point to consider in future vaccine studies.

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We declare that we have no competing interests.

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loads were measured by A.M.W. and G.L.-B. as part of virology services at the WNPRC, which is managed by T.C.F. A.B., S.L.O., A.J.B., and M.S.S. performed IFN-γ ELISPOT assays. S.L.O. conceived of the study. M.S.S. and S.L.O. wrote the manuscript.

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