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An Automated Fluorescence-Based Method to Isolate Bone Marrow-Derived Plasma Cells from Rhesus Macaques Using SIVmac239 SOSIP.664

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Simian immunodeficiency virus (SIV) infection of Indian rhesus macaques (RMs) is one of the best-characterized animal models for human immunodeficiency virus (HIV) infection. Monoclonal antibodies (mAbs) have shown promise for prevention and treatment of HIV infection. However, it has been difficult to isolate mAbs that potently neutralize the highly pathogenic SIVmac239 strain. This has been largely due to the low frequency of circulating B cells encoding neutralizing Abs. Here we describe a novel technique to isolate mAbs directly from bone marrow-derived, Ab-secreting plasma cells. We employed an automated micromanipulator to isolate single SIVmac239 SOSIP.664-specific plasma cells from the bone marrow of a SIVmac239-infected RM with serum neutralization titers against SIVmac239. After picking plasma cells, we obtained 44 paired Ab sequences. Ten of these mAbs were SIV specific. Although none of these mAbs neutralized SIVmac239, three mAbs completely neutralized the related SIVmac316 strain. The majority of these mAbs bound to primary rhesus CD4+ T cells infected with SIVmac239 and induced Ab-dependent cellular cytotoxicity. This method is a first step in successful isolation of antigen-specific bone marrow-derived plasma cells from RMs.

INTRODUCTION

Monoclonal antibodies (mAbs) are becoming the leading class of newly introduced drugs to the biopharmaceutical market. Over 90 therapeutic mAbs have been approved for a variety of targets and diseases in recent years.[1] Although the field of infectious disease has so far been underrepresented, mAbs are a promising approach for treatment and prophylaxis. In the context of HIV infection, for which there is no vaccine, many potent human immunodeficiency virus (HIV)-neutralizing mAbs (nmAbs) have been isolated, and some are currently being tested in human clinical trials.

The simian immunodeficiency virus (SIV)-infected Indian rhesus macaque (RM) model is an excellent method of testing nmAb efficacy *in vivo*. Indeed, a number of human nmAbs have been tested in RMs infected with chimeric SIV/HIV strains. The simian/human immunodeficiency virus (SHIV) model has been criticized because the pathogenesis of this chimeric virus may not mimic that of HIV or SIV. To address the shortcomings of the SHIV-infected RM model, several groups have attempted to isolate mAbs against SIV isolates and pathogenic clones that might more closely resemble HIV infection. To date, it has proven difficult to isolate tier 3 SIV-specific nmAbs, and only one group has reported consistent isolation of different SIV-specific nmAbs.[2]

Many different technologies have facilitated isolation of antigen-specific mAbs, including single-cell sorting with fluorescent bait, B cell immortalization, and B cell culture.[3] However, these methods require probes, high-throughput screening of recombinant mAb libraries, or maintenance of thousands of sorted immortalized B cells or antibody (Ab)-secreting cells in culture. In fact, Gorman et al.[2] isolated the first SIVmac239 nmAbs after screening ~5,700 cultured CD20+ immunoglobulin D (IgD)− IgM− total B cells and 118 mAbs from sorted gp140 FT+ CD20+ IgG+ IgM− memory B cells.

The generation of B cells encoding high-affinity Abs is usually associated with extensive selection and proliferation in the germinal center. Although terminally differentiated plasma cells in the bone marrow are responsible for sustaining the majority of circulating Ig,[4–7] memory B cells have been the most exploited source to isolate SIV-specific mAbs because they can be easily sorted based on their surface Ig specificity. Thus, isolation of Abs from plasma cells

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represents another potential source of mAbs. However, there are several limitations associated with working with these cells. Although the plasma cell phenotype from RMs has been defined previously as CD138+ CD31+ B cells,[8] there is selective loss of these critical phenotype-defining markers following freezing, limiting our ability to use previously frozen bone marrow samples. In addition, antigen-specific plasma cells cannot be easily isolated using traditional flow cytometry because they do not have membrane-bound Ig. To date, only two groups have successfully isolated antigen-specific bone marrow-derived plasma cells from mice, rabbits, and rats.[9,10]

Based on the methodology described by Clargo et al.,[9] we established a fluorescence-based platform to isolate SIVmac239 SOSIP.664-specific plasma cells from r10051, a SIVmac239-infected Indian RM with high neutralizing titers against this pathogenic clone. Here we used an automated micromanipulator linked to a fluorescence microscope to identify antigen-specific plasma cells without performing direct functional assays on cultured cells. Typically, HIV nmAbs have high levels of somatic hypermutations (SHMs), insertions or deletions (indels), and often long variable heavy (VH)-chain CDR3s. Plasma cells from a RM making neutralizing Abs should have accumulated such characteristics necessary for SIV neutralization because of repeated antigen exposure and multiple selection cycles. Using the ALS CellCelector, we picked plasma cells and subsequently isolated RNA encoding the VH and variable light (VL) chains of 44 complete Ab pairs. Ten of these 44 mAbs were SIV specific and targeted different areas of the viral Envelope (Env) glycoprotein. Although none of these mAbs neutralized SIVmac239, three potently neutralized 100% of the related tier 1 SIVmac316 strain. In addition, these mAbs bound to primary rhesus CD4+ T cells infected with SIVmac239, and seven induced Ab-dependent cellular cytotoxicity (ADCC), which could have implications for their *in vivo* utility.

RESULTS

Development of a Fluorescence-Based Screening Platform for Isolation of SIVmac239-Specific Plasma Cells

We previously reported the serum neutralization titers of our cohort of 34 Indian RMs at the Wisconsin National Primate Research Center.[11] There we identified a conventional progressor, r10051, that developed SIVmac239-neutralizing titers of 1:2,157 80 weeks post-infection. After 2 additional years on antiretroviral therapy, its serum neutralization titers remained elevated (1:1,610). Additionally, we detected SIVmac239-specific Ab-secreting cells in the bone marrow by Ig enzyme-linked immunospot ELISpot (Figure 1A). Although approximately 7% of the total bone marrow cells were Ab-secreting cells, 20% of these Ab-secreting cells were reactive against the SIVmac239 gp140 FT protein.[12]

To identify bone marrow-derived SIVmac239-specific Ab-secreting cells from r10051, we selected the recently available SIVmac239 SOSIP.664 trimer as our screening tool because of its native-like structure.[13] Unlike other SIV subunits (i.e., gp140), SOSIP.664 contains quaternary epitopes that facilitate proper formation of the Env spike apex, mimicking the native Env protein. This soluble protein is usually purified using an affinity column with PGT145, an Ab that recognizes a quaternary epitope at the trimer apex and allows exclusion of non-trimeric Env protein. We verified by ELISA that our SIVmac239 SOSIP.664 had the correct trimeric conformation (Figure S1). Thus, to isolate SIVmac239 SOSIP.664-specific plasma cells using our newly developed fluorescence method, we first coated 24-well plates (with imprinted 50 × 50 μm nanowells) with streptavidin overnight and then added biotinylated SIVmac239 SOSIP.664 the following day (Figure 1B). Bone marrow aspirates were obtained from both femora and both humeri of r10051 at different time points during SIV infection and while this animal was on antiretroviral therapy (ART). To prevent false positives with our fluorescence-based method, we pre-incubated total bone marrow cells with different mouse anti-Fcγ receptor (FcγR) I–III Abs to block the FcγRs present on the surface of certain types of cells, including macrophages and dendritic cells. These cells were then resuspended in medium containing recombinant a proliferation-inducing ligand (APRIL), interleukin-6 (IL-6), and mouse anti-monkey IgG fluorescein isothiocyanate (FITC) and added to the SIVmac239 SOSIP.664 plates.[8,14,15] After a 12-h incubation period, we used the fluorescence microscope of the ALS CellCelector to visualize fluorescent halos around cells that had secreted SOSIP.664-specific Abs (Figure 1C). We then used the micromanipulator to pick single cells with 30-μm oil-filled glass capillaries and transferred them into 96-well plates containing lysis buffer for subsequent PCR amplification. This technique allowed us to functionally screen 10 million bone marrow cells in less than a day. Of the picked bone marrow-derived cells, we amplified 46 unique VH chains and 56 VL chains, which resulted in a total of 44 complete Ab pairs. Ten of these wells were positive for multiple kappa and lambda chains, indicating that more than one B cell had been picked using the ALS CellCelector. In these instances, we selected both light chains and verified their binding specificities in functional assays.

Non-human Primate Bone Marrow-Derived Ab Genetics

We next analyzed V-gene use, VH CDR3 length, percent divergence from the germline, and presence of indels of the 44 VH/VL Ab pairs isolated from the bone marrow of r10051. IGHV4-2*01 was used by over 40% of the total amplified mAbs, which was also true for the 10 SIVmac239 SOSIP.664-specific mAbs (Figure 2A). Of note, we identified three sets of two Ig V-region genes that were clonally related but had unique VH CDR3s. We then analyzed the amino acid length distributions of the plasma cell-derived VH CDR3 sequences. VH CDR3 length ranged from 9–23 amino acids, indicating considerable VH CDR3 length diversity (Figure 2B). Although the average SHM of IgGs isolated from healthy donors is 5% nucleotide divergence from the germline,[16] the total mAbs isolated in this study diverged from their putative germline predecessors by 14.0% ± 4.7%. Interestingly, the four most mutated mAbs were all SIV specific and diverged from their germline sequences by up to 24.7% (Figure 2C). Previous studies have suggested that development of neutralization breadth is usually associated with extensive SHM in the antigen-binding site of the V-gene in conjunction with in-frame indels.[17] Kepler et al.[17] determined that HIV-infected patients have higher numbers of indels compared with non-HIV-infected individuals, and 25% of the HIV

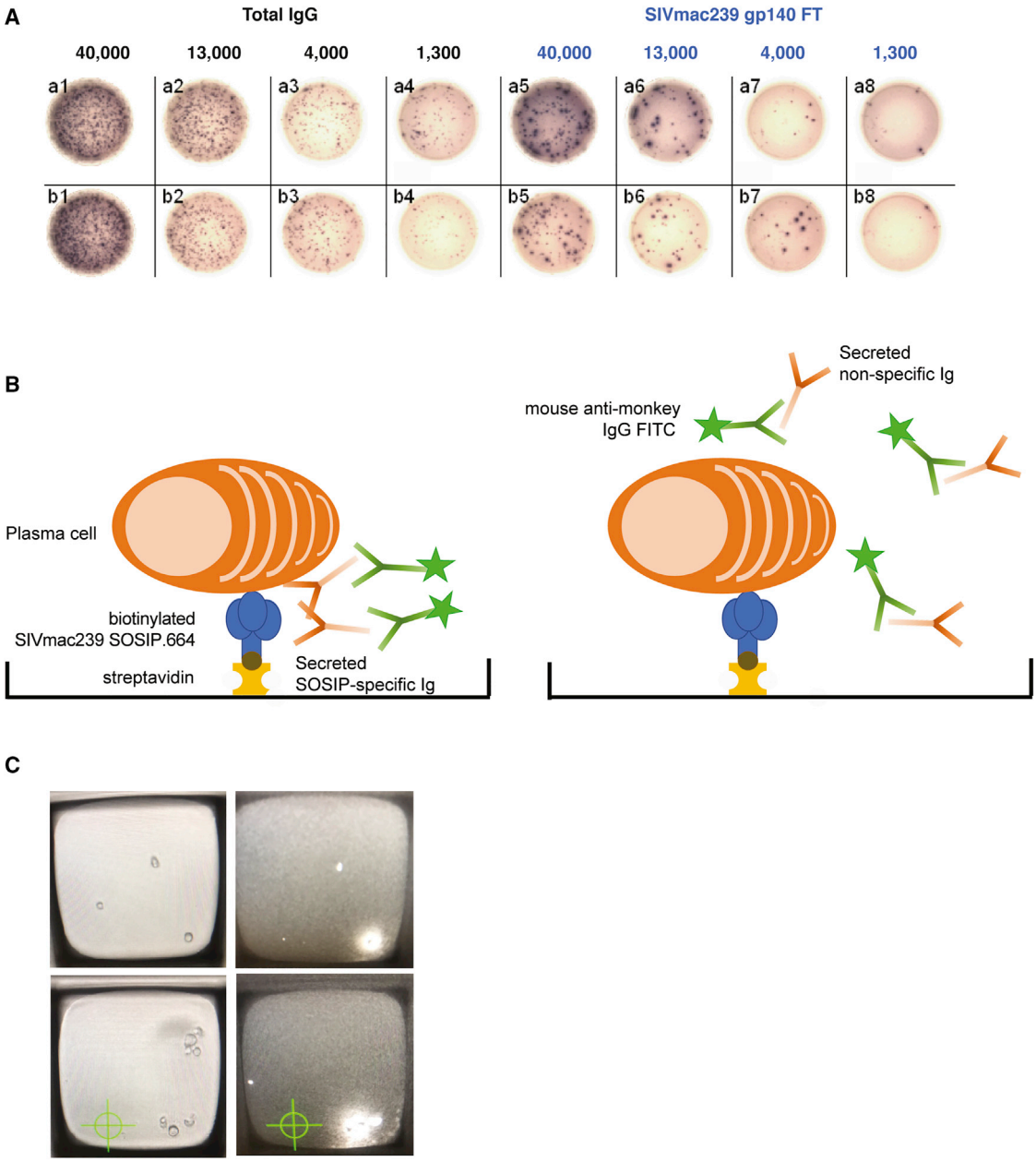

Figure 1. Characterization and Isolation of Abs from the Bone Marrow of a SIVmac239-Infected RM

(A) We detected Ab-secreting cells in total bone marrow cells isolated from r10051 by IgG ELISpot. Twenty percent of these cells were specific for SIVmac239. The four columns on the left correspond to the total Ab-secreting cell population at different concentrations (40,000, 13,000, 4,000, and 1,300 cells/well); the four columns on the right indicate SIV-specific responses at the same frequency. Each dilution is shown in duplicate. (B) Schematic representation of the fluorescence-based platform. We coated nanowells with streptavidin (yellow) and subsequently added the SIVmac239 SOSIP.664 trimer (blue). Total bone marrow cells were added to the wells in medium containing APRIL, IL-6, and mouse anti-monkey IgG FITC (green), which binds to the Abs secreted by the plasma cells. Left: SIVmac239-specific Abs bind the SOSIP.664 trimer. Right: the secreted Abs do not bind SOSIP.664 and diffuse away. (C) ALS CellCelector image of plasma cells using bright-field view on the left and the fluorescent field on the right. The green cursor in the lower image shows the picking diameter of the capillary.

broadly nmAb genes contain indels, a 7-fold higher rate compared with 13,000 V-genes extracted from NCBI GenBank.[17] In our study, 37% of the total plasma cell-derived mAbs and 40% of the SIV-specific mAbs contained indels.

Plasma Cell-Derived Ab Binding and Neutralization Activity

To evaluate the Abs expressed by the plasma cells of r10051, we first determined their epitope specificity by ELISA with different Env subunits and mutants (Table 1), as we described previously.[11]

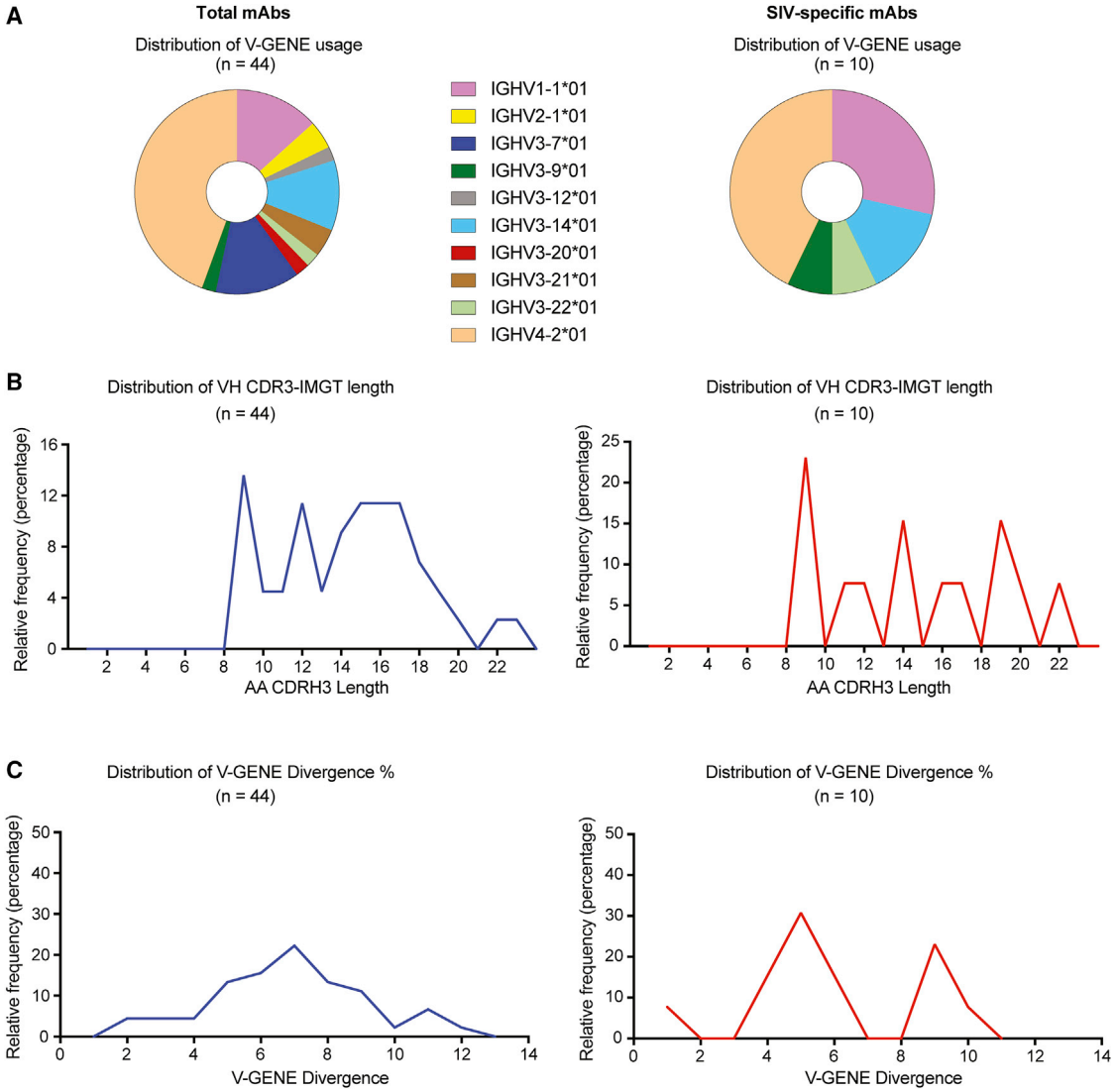

Figure 2. Characteristics of the Total and SIVmac239-Specific VH Chains Isolated from Bone Marrow-Derived Plasma Cells

(A) V-gene distribution. (B) Histogram representation of VH CDR3 length distribution. (C) Histogram representation of the percentage of nucleotide difference from the germline. Top: all 44 isolated mAbs, including SIV-specific mAbs. Bottom: only the 10 SIV-specific mAbs.

Of the 44 mAbs, 10 were SIVmac239 Env specific (22.7% of the total isolated mAbs; Table S1; GenBank: MT_678464–MT_678483). Four of these mAbs targeted the gp41 fusion protein, and four additional mAbs bound the variable (V) loops. Interestingly, we identified one mAb that only bound SOSIP.664 trimer but not gp140, and another mAb that bound gp140 but did not bind gp41 or the V loops.

We then tested the neutralization activity of the isolated mAbs against SIVmac239 and SIVmac316.[18,19] SIVmac316 is a tier 1 strain sensitive to neutralization that was isolated from alveolar macrophages of a SIVmac239-infected RM.[20] Although none of the mAbs neutralized SIVmac239, two V1V2V3-specific mAbs and one V4-specific mAb exhibited 100% neutralization of SIVmac316, with 50% inhibitory concentration (IC_{50}) values ranging from 2–19 ng/mL.

Binding to Infected Primary CD4+ T Cells and Induction of ADCC Activity

Plasma cells reside in the bone marrow and produce large quantities of circulating Abs.[21] We therefore examined the potential effector role of these non-neutralizing mAbs *in vivo*. First, we analyzed the capacity of these mAbs to bind to primary rhesus CD4+ T cells infected with SIVmac239 (Figure 3). All but two of the plasma-cell-derived mAbs isolated in this study, P1A05 and P2H04, bound to infected CD4+ T cells, albeit with varying affinities. In fact, our V4-specific mAb, P2F06, bound CD4+ T cells similarly as 5L7 IgG1, a

Table 1. Functional Characteristics of the Bone Marrow-Derived SIV-Specific Abs Isolated from r10051

	Env Specificity	Binding to Infected CD4+ T Cells at 100 μg/mL	SIVmac239 IC_{50} (μg/mL)	SIVmac316 IC_{50} (μg/mL)	SIVmac316 V_{max}
P1A05	gp140	N/B	>50	>50	N/D
P1B05	V1V2V3	>40%	>50	0.019	100%
P1D06	V1V2V3	>40%	>50	>50	N/D
P1F04	gp41	<20%	>50	>50	N/D
P1H06	V1V2V3	>40%	>50	0.006	100%
P2C02	SOSIP.664	<20%	>50	>50	N/D
P2F06	V4	>40%	>50	0.002	100%
P2H02	gp41	<20%	>50	>50	ND
P2H04	gp41	NB	>50	>50	ND
P2H06	gp41	<20%	>50	>50	ND
5L7 (Pos Ctl)	V4	>40%	>50	0.003	100%

IC_{50}, inhibitory mAb concentration at which 50% virus neutralization was attained; V_{max}, maximum percentage neutralization; N/B, non-binding; N/D, not determined; Pos Ctl, Positive control.

non-neutralizing V4-specific mAb that protected a single macaque from six successive SIVmac239 intravenous challenges when present at serum concentrations greater than 200 μg/mL.[22] 5L7 IgG1 also lowered peak and set point viremia and increased the number of challenges required to achieve infection at lower concentrations. Not surprisingly, the mAbs that targeted the V loops bound to higher proportions of SIVmac239-infected CD4+ T cells than gp41-specific mAbs.

To determine whether mAbs might have a potential effect *in vivo*, we measured their ADCC activity. We employed a luciferase-based assay that measures natural killer (NK) cell-mediated ADCC activity independent of neutralization or complement activity.[23] As expected, P1A05, P2H04, and P2C02 had no detectable ADCC activity (Figure 4). mAbs P1A05 and P2H04 did not bind to SIVmac239-infected CD4+ T cells, and P2C02, which only binds the SOSIP.664 trimer, bound weakly. Six of the 10 SIV-specific bone marrow-derived mAbs efficiently recruited NK cells and induced ADCC activity similar to 5L7, with 50% ADCC activity ($ADCC_{50}$) values of close to 0.1 μg/mL. Surprisingly, P2F06, the only V4-specific mAb tested besides 5L7, had a 10-fold lower $ADCC_{50}$ than 5L7 of 0.03 μg/mL.

DISCUSSION

Here we developed a fluorescence-based platform to isolate SIV-specific mAbs from an Indian RM with high serum neutralization titers against SIVmac239 using the trimeric SIVmac239 SOSIP.664 Env protein. Utilizing this methodology, we picked bone marrow-derived plasma cells and isolated 44 rhesus VH/VL paired sequences. Ten of these 44 mAbs were SIV specific and targeted different parts of the trimeric Env glycoprotein, including the V loops and the gp41 fusion protein. Although none of these mAbs neutralized SIVmac239, three of them exhibited 100% neutralization of the tier 1 SIVmac316 strain. In addition, the majority of these mAbs bound to primary rhesus CD4+ T cells infected with SIVmac239 and induced ADCC activity against SIVmac239-infected cells, suggesting that these mAbs could have a role *in vivo*.

The scientific community has commonly used memory B cells as the main source of cells for mAb isolation. However, plasma cells contribute to the serum neutralization responses detected in conventional functional assays.[4–7] Unfortunately, these cells cannot be sorted using fluorescent bait because they do not express surface Ig. To date, only two studies have isolated antigen-specific plasma cells from bone marrow of different rodents,[9,10] and no studies have focused on isolation of antigen-specific long-lived plasma cells from bone marrow of RMs. Clargo et al.[9] utilized an automated micromanipulator to identify bone marrow-derived mAbs that targeted tumor necrosis factor alpha (TNF-α), a highly conserved molecule. Automated micromanipulators systems, like the ALS CellCelector, have been used primarily to isolate circulating tumor cells.[24–27] However, Ogunniyi et al.[28] employed this methodology in combination with V(D)J gene amplification to evaluate human humoral responses present in blood and mucosal tissue from HIV-infected patients. After characterization of the samples, they verified the phenotype of eight Env-specific mAbs, two of which partially neutralized two HIV-1 tier 1 and 2 strains.

There are multiple factors that might affect the likelihood of isolating a SIVmac239-specific nmAb. The most relevant include the levels of neutralizing titers against SIVmac239 in r10051, which might influence our ability to isolate nmAbs. Use of a relevant native-like probe, such as SOSIP.664, would theoretically enhance our ability to isolate nmAbs. The number of screened B cells or cloned Abs is also a factor. In our case, the limited number of mAbs screened in this study was probably the main reason why we did not identify such a nmAb. It is worth noting that, although the described method has some throughput limitations compared with other methods, it might be possible to increase the number of screened cells with a pre-enrichment step. Unfortunately, other high-throughput methods (e.g., flow cytometry) are not suitable for isolating antigen-specific plasma cells. Although Martinez-Murillo et al.[8] characterized the phenotype and function of fresh bone marrow plasma cells from RMs, they also

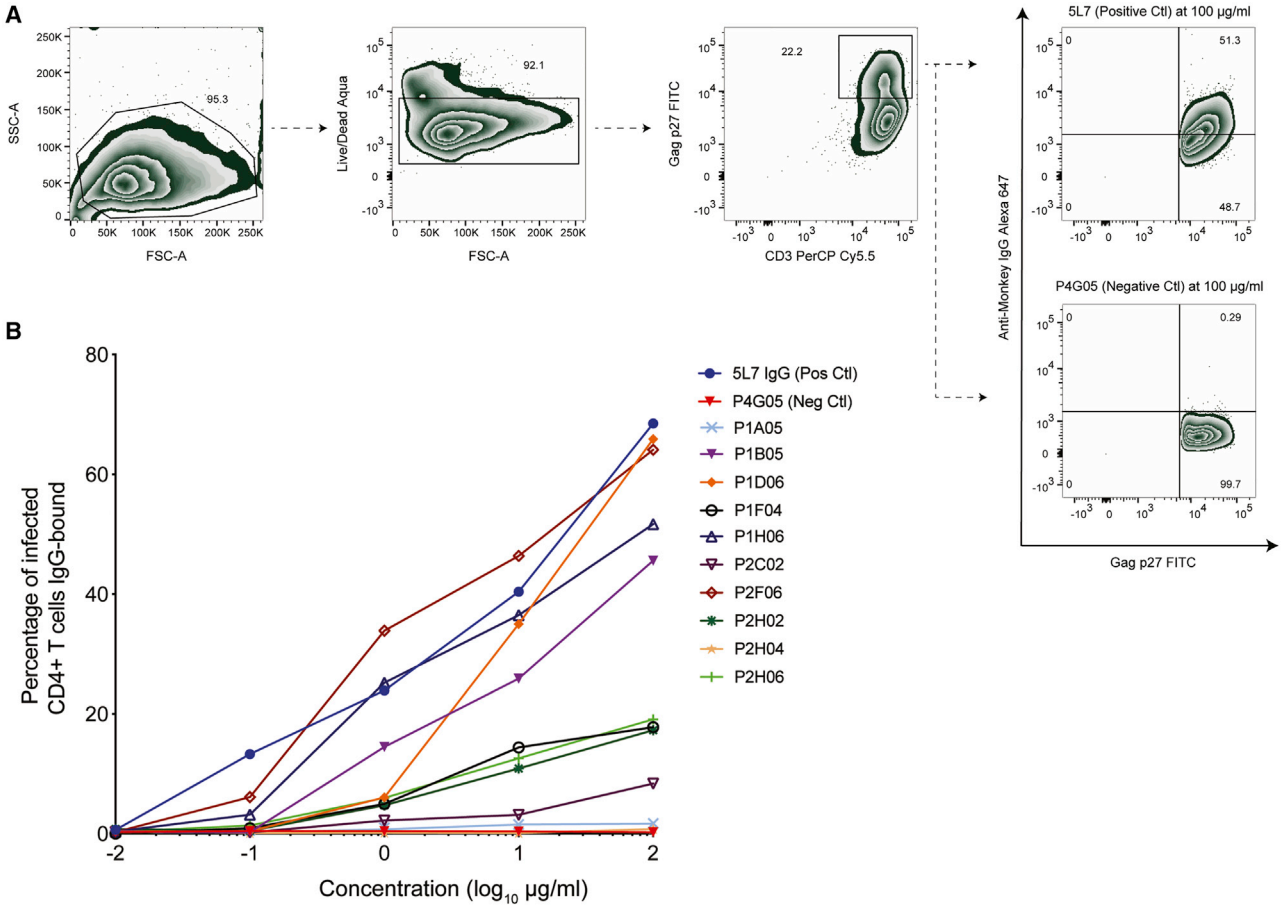

Figure 3. Binding of Isolated mAbs to SIVmac239-Infected Rhesus CD4+ T Cells

CD4+ T cells were first infected with SIVmac239 and then cultured for 48 h. The infected target cells were mixed with the SIV-specific mAbs at the indicated concentrations, starting at 100 µg/mL, and subsequently stained with a panel of fluorophore-conjugated mAbs for detection purposes. (A) The gating strategy used was as follows: lymphocytes/live/CD3+/Gag p27+ cells bound by rhesus IgG. A representative analysis is shown. (B) Titration curves of bone marrow-derived mAbs using SIVmac239-infected primary rhesus CD4+ T cells.

described the striking 10-fold difference in frequency between fresh and frozen rhesus CD138+ CD31+ cells, also called bone marrow-derived plasma cells. This finding indicates that there is selective loss of cells expressing CD138 and CD31 markers after freezing, which limits our ability to use this technique to enrich for plasma cells. Contrarily, it might be possible to further enrich for bone marrow-derived plasma cells by removing unwanted cells that are phenotypically defined and are not adversely affected by freeze/thaw cycles (e.g., T cells or monocytes) using magnetic bead-based negative selection. The platform described in this study is an alternative strategy to isolate antigen-specific bone marrow-derived plasma cells from RMs.

We recently evaluated the frequency of circulating plasmablasts and the genetics of the Abs expressed by these cells in r10051, the same SIV-infected macaque utilized in this study.[13] Interestingly, the frequencies of SIV-specific circulating plasmablasts and long-lived plasma cells in this particular RM were both 20%. Although the two subsets of Ab-secreting cells analyzed in our studies originated from the same receptor-dependent activated B cells, they followed distinctive differentiation pathways.[21] Thus, we compared the mAbs derived from plasmablasts and plasma cells to determine whether there are differences between mAbs derived from different cell types (Table 2). In r10051, IGHV4-2*01 was the most represented V-gene independent of specificity or cell type. Interestingly, these mAbs do not seem to differ in other relevant Ab characteristics, including VH CDR3 average length and length distribution as well as V-gene nucleotide divergence, and are comparable with those identified in human HIV-specific broadly nmAbs.[29] However, these characteristics are not shared with the three recently isolated SIVmac239 nmAbs, which exhibit minimal deviation from their germline sequences.[2] Although our mAbs did not neutralize SIVmac239, they were capable of neutralizing the related tier 1 SIVmac316 strain. The discrepancy between binding and neutralization activity of a mAb has been described previously in mAbs targeting the HIV-

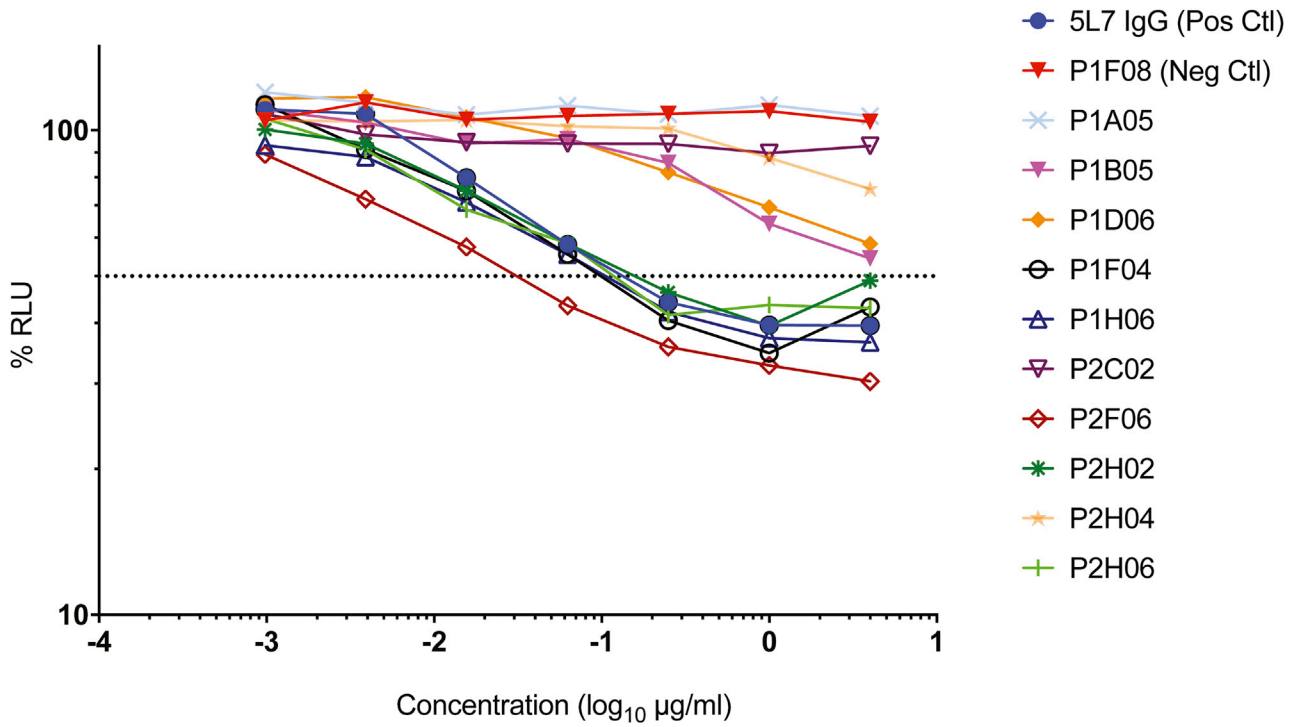

Figure 4. ADCC Activity of Isolated mAbs against SIVmac239-Infected Target Cells

Four-fold serial dilutions of the SIV-specific mAbs were added to a mixture of NK cells and CEM.NKR CCR5+ Luc+ target cells infected with SIVmac239 at an effector-to-target (E:T) ratio of 10:1. The dashed line indicates 50% RLU or 50% ADCC activity against SIVmac239-infected cells. RLU loss is proportional to the loss of infected cells during incubation and represents ADCC activity.

1 CD4-binding site (CD4bs).[30] Even though CD4bs-specific mAbs can access their target epitope with high affinity, some mAbs do not induce the conformational changes required to facilitate viral neutralization, which is associated with a low entropy of binding.[30] It is possible that our mAbs require a higher level of entropy for neutralization and that the unique characteristics associated with HIV breadth and neutralization potency might not be applicable to SIVmac239 nmAbs.

Even though our SIV-specific mAbs do not neutralize SIVmac239, they might still be able to exert pressure on viral populations *in vivo* via neutralization-independent mechanisms. Although the mechanism of protection described in the 5L7 IgG1 study remains unknown,[22] ADCC activity was associated with preventing infection from six escalating dose challenges in one RM and reducing peak and set point viremia in the other SIVmac239-infected RMs. Typically, a non-neutralizing Ab response against HIV has a limited *in vivo* effect on the virus and viral loads because the Abs have limited interactions with functional Env trimers.[31] In contrast, we isolated functional non-neutralizing SIV-specific mAbs that efficiently bind to the Env trimer glycoprotein, to primary infected CD4+ T cells and are also capable of inducing ADCC activity at low concentrations. Although further studies are necessary to determine the behavior and efficacy of these mAbs *in vivo*, these functional mAbs might help clarify the *in vivo* role of non-neutralizing mAbs in preventing SIVmac239 acquisition and controlling viral replication.

In conclusion, our data show that it is possible to isolate SIV-specific plasma cell-derived VH/VL chains from RMs using a fluorescence-based platform. Although this technique facilitated the isolation of SIV-specific plasma cell-derived mAbs from an Indian RM, it could also be utilized to identify Abs specific for other pathogens, highlighting the applicability of this method of Ab isolation.

MATERIALS AND METHODS

Research Animals and Ethics Statement

The Wisconsin National Primate Research Center (WNPRC) housed the Indian RM (*Macaca mulatta*) used in this study and provided care in strict accordance with the Weatherall Report guidelines (i.e., housing, feeding, environmental enrichment, and steps to minimize suffering).[32] WNPRC adhered to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures were executed in accordance with approved protocols by the University of Wisconsin Graduate School Animal Care and Use Committee (Animal Welfare Assurance A3368-01).

Table 2. Comparison between SIV-Specific Plasmablast-Derived and Plasma Cell-Derived mAbs from r10051

	Plasmablasts (n = 7)	Plasma Cells (n = 10)
V-gene usage	IGHV4-2*01 (42.9%)	IGHV4-2*01 (40%)
Presence of indels	42.9%	40%
VH CDR3 length distribution	11–22 aa	9–22 aa
VH CDR3 length average and standard deviation	14.7 ± 5.2 aa	14.5 ± 4.7 aa
V-gene divergence % distribution	11.5–21.2%	10.8–24.7%
V-gene divergence % average and standard deviation	16.1 ± 4.0%	17.0 ± 5.0%

aa, amino acids.

Plasma Cell Isolation Using the ALS CellCelector

To isolate SIVmac239 SOSIP.664-specific plasma cells, we used an automated fluorescence micromanipulator, which allowed us to isolate antigen-specific Ab-secreting cells. The micromanipulator was housed in a sealed biosafety cabinet at 37°C with 5% CO_2. Nanowells were coated with streptavidin overnight and then incubated with biotinylated SIVmac239 SOSIP.664 trimer for 1 h. We then overlaid processed bone marrow aspirates from Indian RMs that had developed a neutralizing Ab response against SIVmac239. These aspirates were placed in medium containing a fluorescently labeled mAb against rhesus IgG and incubated at 37°C for 12 h. Localized fluorescent halos were identified surrounding SIV-specific plasma cells because of their secreted SOSIP-specific Abs. These plasma cells were then picked using a 30-μm glass microcapillary tip and placed into lysis buffer for later Ig amplification.

Env-Specific ELISpot

ELISpot assays were conducted as described previously using total bone marrow cells to calculate total and SIV-specific Ab-secreting cell frequencies.[33] ELISpot assays were performed in duplicate with 40,000, 13,000, 4,000, or 1,300 cells per well and according to the manufacturer's instructions (MABTECH). First we activated the plate membrane by adding 50 μL of 70% ethanol for a maximum of 2 min, followed by a washing step with sterile water. We then coated the ELISpot plates overnight with a purified anti-human IgG mAb at 15 μg/mL to capture the rhesus Ig produced by the Ab-secreting cells present in the bone marrow. The following day, we washed the ELISpot plates with sterile 1× PBS and incubated them with R10 medium (RPMI 1640, 10% heat-inactivated fetal bovine serum (FBS), antibiotic and antimycotic) as a blocking step for 30 min. We then removed the medium and added the total bone marrow cell suspension to the ELISpot plates. After 16- to 24-h incubation at 37°C, we washed the plates with PBS and added the MT78/145-biotin Ab at 1 μg/mL to detect the total Ab-secreting bone marrow cell frequency or biotinylated SIVmac239 SOSIP.664 at 5 μg/mL to determine SIV-specific bone marrow Ab-secreting cells. After 2-h incubation, the plates were washed and incubated with alkaline phosphatase (ALP)-conjugated streptavidin. Last, we added 100 μL of the substrate solution (5-Bromo-4-chloro-3-indolyl phosphate [BCIP]/nitro blue tetrazolium [NBT]-plus) and developed the plates until distinct spots emerged. The plates were imaged, and spots were enumerated using an AID ELISpot reader.

Monoclonal Ab Amplification, Cloning, and Purification

Picked bone marrow-derived plasma cells were immediately frozen on dry ice for subsequent amplification, cloning, expression, and purification of the VH and VL chains, as described previously.[11,34] Briefly, the VH and VL genes were produced by reverse-transcriptase PCR using random hexamers followed by two additional nested PCRs. The nested PCRs utilized 5′ V gene-specific primers and 3′ primers that bound to the heavy, kappa, or lambda constant regions and incorporated compatible ends with Gibson assembly subcloning. After cloning the VH and VL genes into the rhesus IgG1 expression vector (InvivoGen), the heavy and light chains of each Ab were transfected using the suspension-adapted Expi293 expression system (Thermo Fisher Scientific). The following day, the reaction was boosted by adding enhancers. The Ab-containing supernatant was harvested after 5 additional days. All Abs were purified using protein A columns (GE Healthcare).

ELISA

The binding characteristics of the mAbs isolated in this study were determined by ELISA. All mAbs were screened against SIVmac239 SOSIP.664 at 1 μg/mL. When positive, we tested the SIVmac239 SOSIP.664-binding mAbs against other SIVmac239 subunits: gp120 (Immune Technologies), gp41 (ImmunoDX), and gp140 FT, SIVmac239 ΔV1/V2/V3 gp140 FT, and SIVmac239 ΔV4 gp140 FT (based on Mason et al.[12]), as described previously.[9] Briefly, the plates were coated with the SIV Env subunits at 5 μg/mL. To test reactivity against SIVmac239 SOSIP.664, we coated the plates with 100 μL of streptavidin. The following day, the streptavidin-coated wells were washed with 1× PBS with Tween 20 and incubated with biotinylated SIVmac239 SOSIP.664 for 1 h at 37°C. Following this incubation, all plates were washed and blocked with 5% powdered skim milk in PBS for 1 h at 37°C. All isolated mAbs, diluted at 1 μg/mL, were then added to the appropriate wells. After 1 h at 37°C, the plates were washed again and incubated with goat anti-human IgG horseradish peroxidase (HRP) (Southern Biotech). Last, all plates were washed and developed using 3,3′,5,5′-tetramethylbenzidine (TMB) (MilliporeSigma). The reaction was stopped using TMB Stop solution and read at 450 nm to determine the extent of binding.

Pseudovirus Neutralization Assay

We used replication-incompetent SIVmac239 and SIVmac316 pseudoviruses in a TZM-bl assay to evaluate the neutralization capabilities of our SIV-specific mAbs against SIVmac239 and SIVmac316, as described previously.[11,35] Briefly, we incubated the diluted SIV-specific mAbs mixed with pseudovirus at 37°C for 1 h before adding them onto TZM-bl cells. We tested mAb concentrations starting at 100 μg/mL, followed by six 4-fold dilutions. IC_{50} was defined as the Ab concentration at which 50% virus neutralization (in relative light units [RLUs]) was attained after comparing the virus control wells

and subtracting background RLU. V_{max} was defined as the maximum percentage of neutralization observed.

Flow Cytometry Analysis of mAb Binding to Infected CD4+ T Cells

To assess the ability of the isolated mAbs to bind native Env conformations present on SIVmac239-infected rhesus CD4+ T cells, we employed a flow cytometry-based binding assay, as described previously.[11] Briefly, we isolated CD4+ T cells from rhesus PBMCs using a nonhuman primate CD4+ T cell isolation kit (Miltenyi Biotec). These cells were later stimulated with phytohemagglutinin-P (Sigma-Aldrich), IL-2 (Roche), and anti-CD3/CD28/CD49d mAbs (NIH AIDS Reagent Program clone 6G12, Becton Dickinson [BD] clone L293, and BD clone 9F10, respectively) for 48 h in R10 medium (RPMI 1640, 10% heat-inactivated FBS, and antibiotic/antimycotic). CD4+ T cells were infected with SIVmac239 by spinoculation and cultured for 48–72 h. Following removal of dead cells and debris by Ficoll density centrifugation, cells were stained with the isolated mAbs at the indicated concentrations. After washing, cells were stained with the following fluorophore-conjugated mAbs and amine-reactive dye: anti-monkey IgG Alexa Fluor 647 (clone SB108a, Southern Biotech), anti-human CD4 BV605 (clone OKT4, BioLegend), anti-human CD8 BV785 (clone RPA-T8, BioLegend), and aqua live/dead dye (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, Invitrogen). Cells were washed, fixed, and permeabilized (Cytofix/Cytoperm and Perm/Wash buffer, BD) and then stained intracellularly with CD3 PerCP-Cy5.5 (clone SP34-2, BD Biosciences) and anti-SIVmac Gag p27 (clone 55-2F12, NIH AIDS Reagent Program, FITC-conjugated at WNPRC). Following a final wash in Perm/Wash buffer, we acquired at least 35,000 events per sample on a specially ordered BD LSR II flow analyzer. Data were analyzed with FlowJo 9.9.6. The ability of mAbs to bind infected cells is reported as a function of the proportion of infected cells (live CD3+ Gag p27+ cells) bound by rhesus IgG (Alexa Fluor 647+ cells) for a given mAb concentration using GraphPad Prism software.

ADCC Assay

The ADCC activity of the isolated mAbs against SIVmac239-infected cells was assessed using a previously described luciferase-based ADCC assay.[23] Briefly, CEM.NKR CCR5+ Luc+ lymphoblasts were infected with SIVmac239 by spinoculation (2 h, 1,800 × *g*, 23°C, 250 ng p27 antigen per 500,000 cells), washed twice in R10 medium, and then cultured in R10 medium for 4 days at 37°C. A rhesus CD16-expressing NK cell line (parent line KHYG-1) was maintained in a separate flask in R10 medium supplemented with 10 U/mL IL-2 (Roche) and 1 μg/mL cyclosporine A (Sigma). On day 4 post-spinoculation, NK cells and infected lymphoblasts were mixed to an effector-to-target (E:T) ratio of 10:1 in R10 medium supplemented with 10 U/mL IL-2. A mixture of NK cells and uninfected lymphoblasts at an E:T ratio of 10:1 was also prepared as a negative control. Cell mixtures (10:1 E:T ratio, total cell concentration of 1.5×10^6 cells/mL) were aliquoted into round-bottom 96-well plates (75 μL per well), and serially diluted mAbs were added to the cells (25 μL of 4× mAb per well). Following an 8-h incubation period at 37°C, cells were lysed in lysis buffer containing luciferase substrate (BrightGlo Luciferase Assay System, Promega) and transferred to white-bottom 96-well plates for luminescence quantification on a microplate reader (PerkinElmer). Following subtraction of the background signal (luminescence for wells containing NK cells and uninfected lymphoblasts), percent RLU values were computed by dividing the luminescence for a mAb-treated well by the average luminescence for wells lacking mAbs and multiplying by 100. Because the percent RLU value is directly proportional to the number of remaining infected lymphoblasts in a given well, lower percent RLU values upon addition of mAbs indicate ADCC activity.

Statistical Analyses

The averages and standard deviations included in this manuscript were performed using Microsoft Excel. A fitting sigmoidal dose-response curve (GraphPad Prism v.7) was utilized to determine the 50% ADCC and neutralization titers.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.08.004.

AUTHOR CONTRIBUTIONS

Conceptualization, D.I.W., N.P.-L., M.J.R., and B.C.R.; Writing – Original Draft, N.P.-L.; Writing – Review & Editing, D.I.W., N.P.-L., B.C.R., and M.J.R.; Investigation, D.I.W., N.P.-L., M.J.R., B.C.R., G.S., R.A., D.R.B., and E.G.R.; Resources, D.I.W.; Methodology, D.I.W., N.P.-L., M.J.R., and B.C.R.; Supervision, D.I.W.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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