ABSTRACT

AMOS, JOSHUA DANIEL. African Green Monkeys Evolved towards Rapid Development of a gp120-focused B cell Response during Acute Simian Immunodeficiency Virus Infection. (Under the direction of Dr. Sallie R. Permar).

The initial phases of acute human immunodeficiency virus type 1 (HIV-1) infection immediately following the transmission event are critical for development of protective envelope (Env)-specific antibodies that might thwart establishment of the pool of latent HIV-1-infected CD4+ T cells and development of virally-induced immune hyperactivation. However, the initial systemic HIV-1 Env-specific antibody response targets nonneutralizing epitopes of gp41 and is polyreactive and ineffective at controlling acute-phase viremia. African-origin, natural simian immunodeficiency virus (SIV) hosts do not typically progress to AIDS and rarely exhibit postnatal virus transmission to their infants, despite high milk virus RNA loads. This is in contrast to SIV-infected rhesus macaques (RMs), Asian-origin non-natural SIV hosts, which sustain pathogenic infections and exhibit higher rates of postnatal virus transmission. This distinction in the frequency of postnatal transmission may be attributable to the strong gp120-focused, neutralizing antibody responses we have previously observed in breast milk of chronically-infected African green monkeys (AGMs), a natural SIV host species. In this study, we compared the initial systemic Env-specific B cell responses of SIV-infected AGMs to that of RMs during acute infection. AGMs developed higher magnitude plasma gp120-specific IgA and IgG responses during acute infection compared to RMs. These responses were coincident with the presence of higher levels of circulating Env-specific memory B cells in the peripheral blood of acutely SIV-infected AGMs compared to RMs. These findings indicate that AGMs, likely as a result of evolutionary adaptation to the virus, display distinct initial Env-specific B cell responses

during acute SIV infection which may result in a more functional SIV-specific humoral response that contributes to the rarity of postnatal transmission observed in this natural SIV host species.

African Green Monkeys Evolved towards Rapid Development of a gp120-focused B cell Response during Acute Simian Immunodeficiency Virus Infection

by
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DEDICATION

This work is dedicated to my wife, Cara, who has given devoted support to my many endeavors; and to my three young children, Luke, Sadie, and Ellie, who fill each day with love, laughter, and adventure.

BIOGRAPHY

Joshua Daniel Amos was born on November 29, 1982 and is the son of Martin Daniel Amos and Donna Louise Smith. He spent most of his childhood in rural Hillsborough, and initially attended Orange High School before graduating from Ashbrook Senior High School upon relocation to Gastonia, North Carolina. Josh then attended East Carolina University and graduated with a Bachelor of Science degree in Biology with a concentration in research skills. Upon graduation in 2005, he accepted a research assistant position at the University of Texas M.D. Anderson Cancer Center in Houston, Texas where he spent two and a half years under the direction of Dr. Marsha Frazier studying DNA methylation patterns in genes associated with increased risk of lung cancer. In 2008, Josh decided to return to North Carolina to pursue his longstanding interests in infectious diseases research and accepted a position as a research analyst under the direction of Dr. Tony Moody at the Duke Human Vaccine Institute (DHVI) in Durham. In 2011, Josh accepted a lab manager position in the laboratory of Dr. Sallie Permar, another DHVI investigator who specializes in the study of neonatal viral pathogens and their associated immune responses. It was there that he manifested his interests in advancing his immunological education and research skills. In 2012, he was accepted into the Master of Science degree program in Immunology at North Carolina State University where he is now working to complete the requirements for his degree.

ACKNOWLEDGMENTS

I would like to graciously thank my advisor, Dr. Sallie Permar, for her continued mentorship and support. She has shown me the true value of a relentless and unyielding work ethic. I would also like to thank my committee members, Drs. Sue Tonkonogy and Scott Laster, for their continued support and mentorship throughout my graduate program. I must also express my sincere gratitude to all of those who contributed to this work, including Dr. Tony Moody and Thad Gurley for insightful discussions, and Jonathon Himes, David Martinez, Glenn Overman, Carrie Ho, and Tatenda Mahlokozera for providing expert technical assistance and advice.

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LIST OF ABBREVIATIONS

AGM:	African Green Monkey
AIDS:	Acquired Immunodeficiency Syndrome
APC:	Allophycocyanin
BnAbs:	Broadly Neutralizing Antibodies
BV:	Brilliant Violet
CBC:	Complete Blood Count
ELISA:	Enzyme-linked Immunosorbent Assay
Env:	Envelope
FITC:	Fluorescein Isothiocyanate
FMO:	Fluorescence Minus One
gp41:	Envelope glycoprotein 41
gp120:	Envelope glycoprotein 120
gp140:	Envelope glycoprotein 140
gp160:	Envelope glycoprotein 160
HIV:	Human Immunodeficiency Virus
HIV-1:	Human Immunodeficiency Virus Type 1
HIV-2:	Human Immunodeficiency Virus Type 2
HRP:	Horseradish Peroxidase
Ig:	Immunoglobulin
mAbs:	monoclonal antibodies

MPER:	Membrane Proximal External Region
NHP:	Nonhuman Primate
OD:	Optical Density
PBMCs:	Peripheral Blood Mononuclear Cells
PBS:	Phosphate-buffered Saline
PE:	Phycoerythrin
PerCP:	Peridinin Chlorophyll Protein
qRT-PCR:	Quantitative Real-Time PCR
RM:	Rhesus Macaque
RNA:	Ribonucleic acid
SIV:	Simian Immunodeficiency Virus
SM:	Sooty Mangabey
sp400:	Immunodominant Region of gp41
T/F:	Transmitted/Founder
V1/V2:	Variable Loop 1/Variable Loop 2
V3:	Variable Loop

1. INTRODUCTION

The development of a preventative human immunodeficiency virus type 1 (HIV-1) vaccine remains a global priority [1]. A major goal of a safe and effective HIV-1 vaccine is to induce broadly neutralizing antibodies (BnAbs) capable of neutralizing HIV-1 strains across all genetic subtypes [2]. However, to date there is no immunogen formulation that consistently induces BnAbs in humans and they typically arise naturally in only a minority (~20%) of HIV-1 infected individuals many years after infection [3-6]. In addition, the appearance of autologous neutralizing antibody responses against the transmitted/founder (T/F) HIV-1 strain(s) is also delayed, emerging months after primary HIV-1 infection [7-10]. Notably, autologous and broadly neutralizing antibody responses are predominantly targeted against Envelope (Env) gp120 epitopes, including the gp120 CD4 binding site [11-15] and two peptide-glycan epitopes in the V1V2 loop [16-18] and V3 region [19-21] of gp120, although neutralizing antibodies against the membrane proximal external region (MPER) of gp41 have also been isolated [22, 23].

The initial systemic and mucosal antibody responses against T/F HIV-1 Env target the gp41 protein [24, 25] and first appear in the blood of HIV-1-infected individuals approximately 13 days after detectable viremia [24]. This autologous Env gp41-specific response has been shown to be polyspecific, nonneutralizing and ineffective at controlling viremia [24, 26, 27]. Moreover, the development of an autologous Env gp120-specific antibody response is further delayed and first appears in blood approximately 28 days after detectable plasma virus [24]. Recombinant monoclonal antibodies (mAbs) isolated from

circulating plasmablasts/plasma cells of HIV-1-infected individuals during acute infection have also been shown to be primarily targeted against Env gp41 and exhibit polyspecificity with host and environmental antigens, including commensal bacteria [27]. Further investigation has revealed that this initial gp41-specific antibody response may be due to the presence of a preexisting pool of memory B cells primed by commensal bacterial antigens in the terminal ileum that are cross-reactive with Env gp41 [28].

African-origin primates, such as African green monkeys (AGMs) and sooty mangabeys (SMs), have been endemically infected with species-specific strains of simian immunodeficiency virus (SIV) for thousands of years and are collectively referred to as natural SIV hosts [29-31]. They sustain nonpathogenic SIV infections that do not typically progress to simian AIDS and rarely transmit the virus to their infants despite high levels of blood and milk virus replication [32-36]. This is in contrast to SIV-infected Asian-origin primates, non-natural SIV hosts, and HIV-1-infected humans, which develop pathogenic lentiviral infections that progress to immunodeficiency syndromes and readily transmit the virus to their infants [37]. Unique and defining features of nonpathogenic lentiviral infections during chronic infection include resolution of immune activation [38-41], downmodulation of type I interferon responses [42], lack of B cell dysfunction [43], absence of progressive depletion of circulating and mucosal CD4+ T cells [38], preservation of mucosal Th17 cell levels [44, 45], and absence of microbial translocation in the intestine [46, 47]. However, natural SIV hosts experience several virus-induced immune perturbations during acute infection similar to that of progressive SIV/HIV-1 infections, including high levels of virus replication and immune activation, rapid depletion of mucosal CD4+ T cells,

and upregulation of type I interferon responses [48]. Several reports have demonstrated that adaptive immune responses, including humoral responses, may play only a limited role, if any, in viral containment and the lack of disease progression in natural SIV hosts [49-51]. Although only low-level autologous neutralizing antibody titers have been identified in the plasma of chronically SIV-infected SMs [52], strain-specific autologous neutralizing responses of variable potency have been characterized in the plasma of SIV-infected AGMs, indicating that there may be variability in the potency of autologous neutralizing antibody responses among natural SIV hosts. [53]. Moreover, we previously described a lack of hypergammaglobulinemia in addition to robust gp120-binding and strong autologous virus neutralizing antibody responses in the plasma and milk of chronically SIV-infected AGMs [43, 54], factors which indicate a lack of B cell dysfunction and may contribute to the rarity of postnatal transmission in natural SIV hosts. These findings prompted us to investigate the development of the early B cell responses to SIV Env during acute infection, which may set the stage for more functional antibody responses during SIV chronic infection. In this study, we compared the kinetics of the initial systemic SIV Env-specific B cell subpopulations and antibody responses in AGMs to that of rhesus macaques (RMs), a non-natural SIV host species. Comparing the development of the initial systemic B cell responses to SIV Env could help elucidate the presence of a preexisting B cell repertoire in natural hosts, which may have evolved under the influence of evolutionary pressures from endemic SIV infection, that is capable of generating a humoral immune response against SIV Env epitopes that is distinct from non-natural SIV hosts. Characterization of this response may be of functional

importance for the design of new and effective vaccine strategies to elicit protective antibody responses against HIV-1 infection.

2. MATERIALS AND METHODS

Study animals and specimen collection. Six female AGMs and four female RhMs were intravenously inoculated with SIVsab92018ivTF and SIVmac251, respectively, as previously described [43]. Blood was collected prior to infection and once per week at 1-6, 15-26, and 52-64 weeks post infection. Peripheral blood mononuclear cells (PBMCs) and plasma were isolated by density-gradient centrifugation using Ficoll-Paque (GE Healthcare, Waukesha, WI). Animals were maintained according to the *Guide for the Care and Use of Laboratory Animals* [55].

Viral Load Quantification. Quantitative real-time PCR (RT-PCR) was performed to determine the SIVsab92018ivTF and SIVmac251 plasma RNA loads as previously described [43, 54].

SIV Envelope/peptide production. SIVmac251 and SIVsab92018ivTF gp120 and gp140 *env* genes [56] and the V1/V2 regions of gp120 were codon-optimized and cloned into pcDNA3.1 expression plasmids (Invitrogen, Carlsbad, CA) using standard molecular technology. Recombinant Env proteins and V1/V2 peptides (SIVsab92018ivTF V1/V2: METDTLLLWVLLLWVPGSTGDVKLTPMCIKMQCTELEPTTASKTSKTTAAPEVVSSS FNATAIEKEMSKEGAMNCSFAMAGYRRDVKKNYSTVWYDQEVQCEPGTQGNRSG VKNCYMIHCNTSVIKEAGLNDIFEAQKIEWHELEVLFQGPG and SIVmac251 V1/V2: METDTLLLWVLLLWVPGSTGDQVKLSPLCITMRCNKSETDRWGLTKSITTTASTTST

TASAKVDMVNETSSCIAQDNCTGLEQEQMISCKFNMTGLKRDKKKEYNETWYSAD LVCEQGNNTGNESRCYMNHCNTSVIQESGLNDIFEAQKIEWHELEVLFQGPG) were produced in 293F cells by transient transfection and purified using Galanthus nivalis lectin-agarose (Vector Laboratories, Burlingame, CA) and nickel-nitrilotriacetic acid (Ni-NTA) agarose column chromatography, respectively, as previously described [57]. The following peptides were obtained commerically: SIVsab92018ivTF V3 (KTVLPVTIMAGLVFHSQKYNT) and sp400 (HIV-1 gp41 immunodominant region, RVTALEKYLEDQARLNIWGCAFRQICHTTVPWKFNNTPDWNN), SIVmac251 sp400 (RVTAIEKYLKDQAQLNAWGCAFRQVCHTTVPWPNASLTPDWNN) (all from CPC Scientific, Sunnyvale, CA) and SIVmac251 V3 (KTVLPVTIMSGLVFHSQPVNE) (Primm Biotech, Milano, Italy).

Plasma IgG Depletion and SIV Env/peptide-specific IgM, IgG, and IgA ELISAs. IgG was depleted and the non-IgG fractions were purified from the plasma of uninfected and SIV-infected AGMs and RMs as described elsewhere [58]. 384-well plates were coated with 3 μg/mL of SIVsab92018ivTF or SIVmac251 Env gp140, gp120, V1/V2, V3, or sp400 proteins/peptides and then blocked using 4% whey protein, 15% goat serum, and 0.5% Tween 20 diluted in phosphate-buffered saline (PBS). Env and peptide-specific IgG or IgM/IgA titers were measured in duplicate by incubation with serial 3-fold dilutions of plasma or IgG-depleted plasma, respectively. Env or peptide-specific antibodies were detected by incubation with horseradish peroxidase (HRP)-conjugated goat polyclonal anti-monkey IgG or IgM (Rockland Immunochemicals, Gilbertsville, PA), HRP-conjugated

mouse anti-human IgA (GA112) (Invitrogen, Carlsbad, CA), or biotinylated mouse anti-rhesus IgA (10F12) (NIH Nonhuman Primate Reagent Resource, Boston, MA) followed by addition of streptavidin-HRP (Thermo Fisher Scientific, Waltham, MA). SureBlue Reserve TMB substrate was then added followed by TMB stop solution (KPL, Gaithersburg, MD) and the ODs were measured at 450 nm using a Spectramax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA). The SIV Env or peptide-specific antibody titer was calculated as the inverse of the lowest dilution of non-depleted or IgG-depleted plasma which had an average OD greater than two times the OD of the superblock-negative control.

SIV Env-specific memory B cell phenotyping. PBMCs were stained with a panel of fluorochrome conjugated antibodies reactive with the following cell surface markers: CD20 peridinin chlorophyll protein (PerCP)-Cy5.5 (L27) and CD3 allophycocyanin (APC)-Cy7 (SP34-2) (both from BD Biosciences, San Jose, CA), CD16 Brilliant Violent (BV)570 (3G8) and CD14 BV605 (M5E2) (both from Biolegend, San Diego, CA), CD8 Alexa Fluor 700 (3B5) (Invitrogen, Carlsbad, CA), CD27 phycoerythrin (PE)-Cy7 (O323) (eBioscience, San Diego, CA), surface IgA fluorescein isothiocyanate (FITC) (polyclonal) (Jackson Immunoresearch, West Grove, PA), and surface IgD PE (polyclonal) (Southern Biotech, Birmingham, AL). For SIV Envelope-specific memory B cell phenotyping, SIVsab92018ivTF or SIVmac251 gp140 proteins were labeled with Alexa Fluor 647 and BV421 and gp120 proteins were labeled with PE-CF594 using fluorochrome labeling kits (Invitrogen, Carlsbad, CA). The optimal titers for all antibodies in the panel were determined and used accordingly for all flow experiments. Aqua vital dye (Invitrogen,

Carlsbad, CA) was used as a live/dead cell discriminator. Stained cells were acquired on an LSRII flow cytometer (BD Biosciences, San Jose, CA), and flow cytometry data were analyzed using FlowJo software (TreeStar, Ashland, OR). Automated complete blood counts (CBCs) were used to quantitate the absolute number of lymphocytes per microliter of blood. The absolute numbers of Env-specific memory B cell subsets per µL of blood were calculated by multiplying the percentage of the gated population by the absolute lymphocyte count.

Statistical analyses. GraphPad Prism 5 (GraphPad Software, La Jolla, CA) was used to perform all statistical analyses. The comparisons of systemic SIV Env-specific antibody titers and memory B cell frequencies and numbers between species were performed with the paired nonparametric Mann-Whitney U test, whereas comparisons in the same species were performed with the paired Wilcoxon signed-rank test.

3. RESULTS

Rapid development of systemic IgA responses against Env gp140 and gp120 in AGMs compared to RM during acute SIV infection.

The first detectable systemic antibody response against HIV-1 Env occurs approximately 13 days after the onset of viremia and first appears as an IgM response directed against gp41 followed by Ig class switching to IgG and IgA. This response is followed by systemic IgG responses against gp120, which first appear approximately 28 days after detectable viremia in HIV-1-infected individuals and is targeted initially against the V3 loop [24]. To date, production of the gp41 protein using recombinant DNA technology remains a technical challenge. In order to probe Env-specific B cell responses in SIV-infected AGMs and RMs, we utilized trimeric gp140 and monomeric gp120 Envs derived from the autologous challenge viruses, SIVsab92018ivTF and SIVmac251, respectively. Thus, we first compared the kinetics of the initial systemic IgM, IgG, and IgA responses against Env gp140 and gp120 in the plasma of AGMs and RMs prior to and during the first six weeks of acute SIV infection. IgM responses against Env gp140 developed with faster kinetics in SIV-infected AGMs compared to RMs, increasing 2-2.5-fold from their baseline by week 2 post infection ($p = 0.02$). This strong gp140-specific IgM response remained 1 or 2 logs higher in AGMs than RMs through weeks 3 and 4 post infection ($p = 0.03$ and $p = 0.01$, respectively), but was similar to that in RMs when evaluated at weeks 6 and 15 post infection (Fig. 1A). In contrast, gp120-specific IgM responses developed with similar kinetics and magnitude during acute infection in AGMs and RMs (Fig. 1B). Interestingly,

systemic IgG responses against gp140 developed with similar kinetics during acute infection in each species, but were 1-2 logs higher in the plasma of SIV-infected RMs than AGMs at week 2 ($p = 0.05$), week 3 ($p = 0.01$), and week 4 post infection ($p = 0.01$), and continued to rise through week 15 post infection ($p = 0.01$) (Fig. 1C). Although systemic IgG responses against gp120 also developed with similar kinetics in both species during acute infection, AGMs exhibited slightly higher magnitude plasma gp120-specific IgG titers at week 6 ($p = 0.01$) and week 15 post infection ($p = 0.01$) compared to RMs (Fig. 1D). Notably, IgA responses against gp140 ($p = 0.01$) and gp120 ($p = 0.02$) were first detectable in the plasma of SIV-infected AGMs by week 2 post infection, but remained below the level of detection in the plasma of SIV-infected RMs until week 3 or 4 post infection. (Fig. 1E and 1F). Moreover, in contrast to systemic gp140-specific IgA titers which were similar magnitude in AGMs and RMs at weeks 3, 4, 6, and 15 post infection, plasma gp120-specific IgA responses were at least 2 logs higher magnitude in AGMs than RMs at early time points (week 3, $p = 0.01$; week 4, $p = 0.02$) and remained higher magnitude at weeks 6 ($p = 0.01$) and 15 post infection ($p = 0.04$) (Fig. 1F).

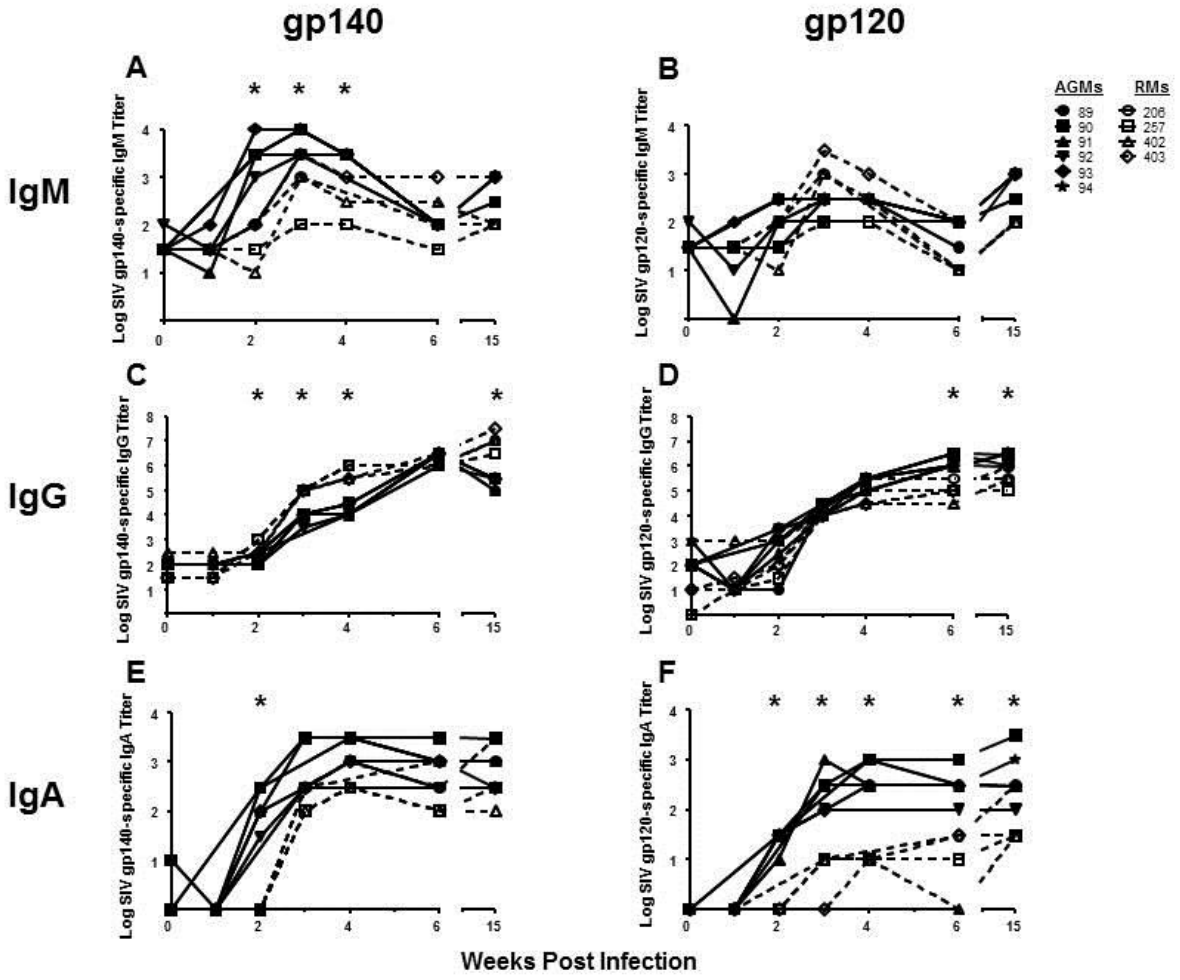

Figure 1. Earlier development of systemic IgA/IgM binding responses against Env gp120 during acute SIV infection in AGMs compared to RMs. Comparison of the initial gp140/gp120-specific IgM (A and B), IgG (C and D), and IgA (E and F) responses in AGMs (closed symbols, solid lines) and RMs (open symbols, dotted lines). Autologous Env gp140 and gp120 proteins were used for evaluation of AGM (SIVsab92018 T/F) and RM (SIVmac251) gp140/gp120-specific antibody responses. Each symbol represents an individual animal. * $p < 0.05$ by the Mann-Whitney test.

Given the differential development of the acute systemic Env gp140- and gp120-specific antibody responses in SIV-infected AGMs and RMs, we next investigated the epitope specificity of the initial systemic Env-specific antibody response by evaluating plasma IgM, IgG, and IgA responses in each species against the species-specific SIV epitopes: sp400, the immunodominant region of HIV-1 gp41 [24], and the V1/V2 and V3 regions of gp120. IgM responses against gp41 sp400, V1/V2, and V3 developed with similar kinetics and magnitude during acute infection of each species, all of which also maintained similar IgM titers at week 15 post infection (Fig. 2A-2C). Additionally, gp41 sp400-specific IgG responses developed with similar kinetics in each species during the first six weeks of infection, but were approximately 2 logs higher in RMs than AGMs at week 15 post infection ($p = 0.005$) (Fig. 2D), corresponding to the high magnitude responses against gp140. IgG responses against the V1/V2 loops remained at baseline levels through the first four weeks of infection, increasing modestly at week 6 post infection and reaching their highest levels at week 15 post infection in each species (Fig. 2E). Interestingly, V3-specific IgG responses developed more rapidly in SIV-infected RMs than AGMs, increasing above baseline levels at week 3 post infection ($p = 0.05$) and maintaining higher levels at weeks 4 ($p = 0.01$), 6 ($p = 0.06$, trend towards significant), and 15 post infection ($p = 0.01$) (Fig. 2F). While IgA responses against gp41 sp400 developed with similar kinetics and magnitude through the first 6 weeks of infection in each species, they were significantly higher in SIV-infected RMs than AGMs at week 15 post infection ($p = 0.01$) (Fig. 2G). IgA responses against V1/V2- and V3 were largely undetectable through the first 6 weeks of infection in each species. However, at week 15 post infection, V1/V2-specific IgA responses trended higher in SIV-infected AGMs

than RMs ($p = 0.09$), whereas V3-specific IgA responses trended higher in SIV-infected RMs than AGMs ($p = 0.09$) (Fig. 2H and I).

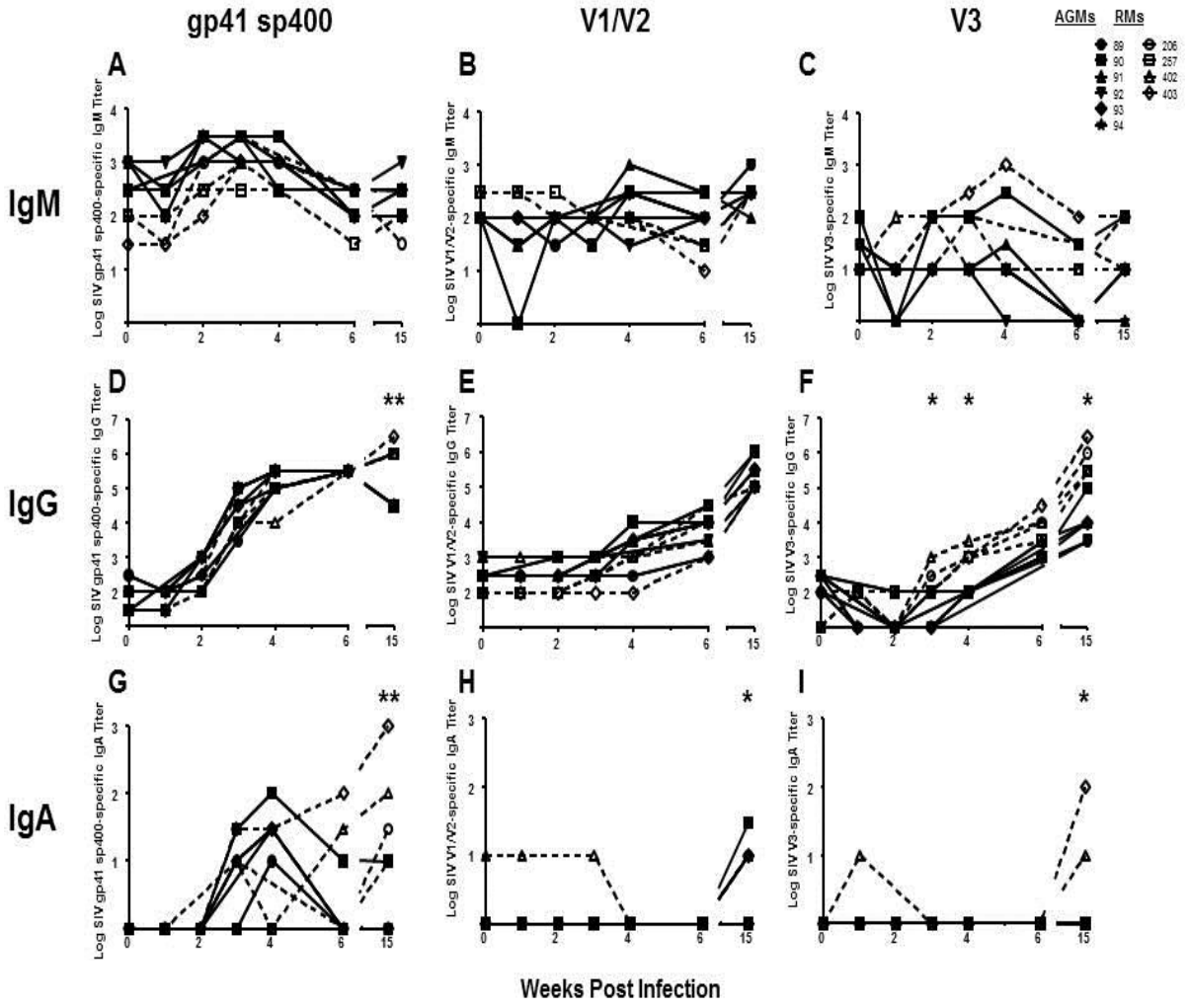

Figure 2. Epitope specificity of the initial Env-specific IgM, IgG, and IgA responses in the plasma of SIV-infected AGMs and RMs during acute infection. Comparison of the IgM (A-C), IgG (D-F), and IgA (G-I) responses against the immunodominant region of gp41, sp400, V1/V2 and V3 regions of gp120 in AGMs (closed symbols, solid lines) and RMs (open symbols, dotted lines). Each symbol represents an individual animal. * $p < 0.05$; ** $p < 0.005$ by the Mann-Whitney test.

Higher absolute numbers of Env gp140-specific memory B cells in the peripheral blood of SIV-infected AGMs compared to that of SIV-infected RMs during acute infection.

We next sought to compare the frequencies and absolute numbers of circulating Env gp140-specific memory B cells of SIV-infected AGMs and RMs during acute and chronic infection. Gp140-specific memory B cells were phenotypically defined by selecting CD20+ and CD3- lymphocytes that did not express IgD and were reactive with Env gp140 conjugated to two different fluorochromes, Alexa Fluor 647 and BV421 (dual gp140 positive) (Fig. 3). Representative dot plots and dual gp140 positive memory B cell frequencies at preinfection and 4, 15, and 52 weeks post infection are shown for AGMs and RMs (Fig. 3). Circulating dual gp140+ memory B cells trended towards significantly higher proportions in SIV-infected AGMs compared to RMs at week 4 post infection (median increase from baseline, 1.76%; range, 0.94 to 4.72%; $p = 0.06$), but were present at similar levels at weeks 15 and 52 post infection in each species (Fig. 4A). Moreover, there was an associated increase in the absolute numbers of circulating dual gp140+ memory B cells at four weeks post infection in SIV-infected AGMs (median increase from baseline, 2.66; range, 1.84 to 7.67 dual gp140+ B cells/μl; $p = 0.01$). We next investigated the relationship between the kinetics of the Env-specific B cell response and SIV replication. Intravenous inoculation of strain-specific SIVs in each species resulted in similar viral kinetics during acute infection, with SIV RNA loads reaching their peaks 14 days after infection, but lower overall viral loads in AGMs, as previously reported [43] (Fig. 4C). Analysis of the relationships between frequencies or absolute numbers of dual gp140+ memory B cells and SIV RNA loads at weeks 4, 15, and 52 revealed no significant correlations between viral load

and circulating antigen-specific B cells in either AGMs (Fig. 4D and E) or RMs (Fig. 4F and G).

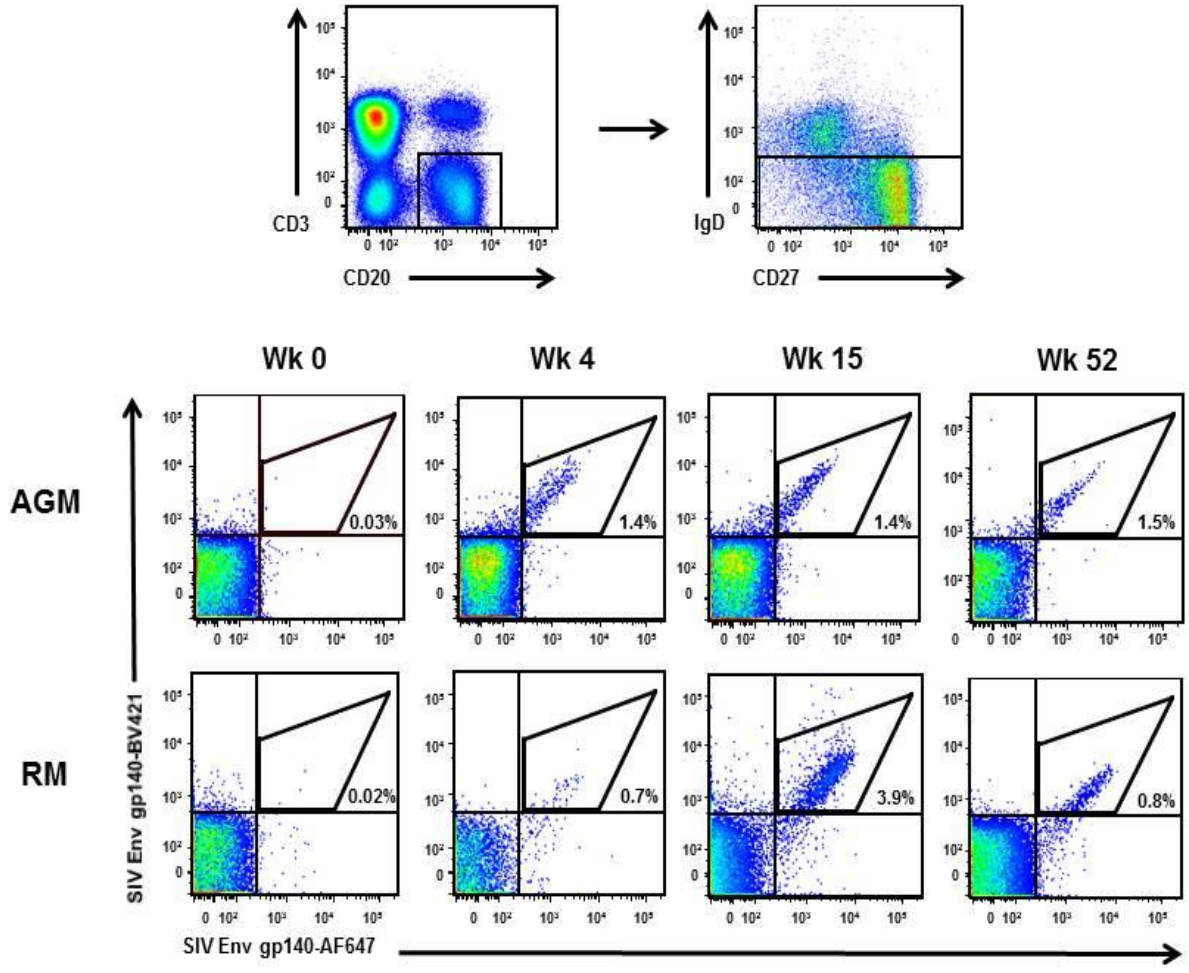

Figure 3. SIV Env gp140-specific B cells in the peripheral blood of AGMs and RMs during acute and chronic infection. Gating strategy used to identify Env gp140-specific B cells was as follows: gating of lymphocytes on forward scatter versus side scatter dot plot followed by selection of live CD14-/CD16- lymphocytes and then CD3-, CD20+ B cells. IgD- memory B cells were then selected on a CD27 vs IgD dot plot and dual Env gp140 positive B cells were gated as shown. Representative dot plots of dual Env gp140 positive B cells prior to and at 4, 15, and 52 weeks following SIV infection from each species are shown. Numbers adjacent to gates represent the frequency of dual gp140 positive B cells.

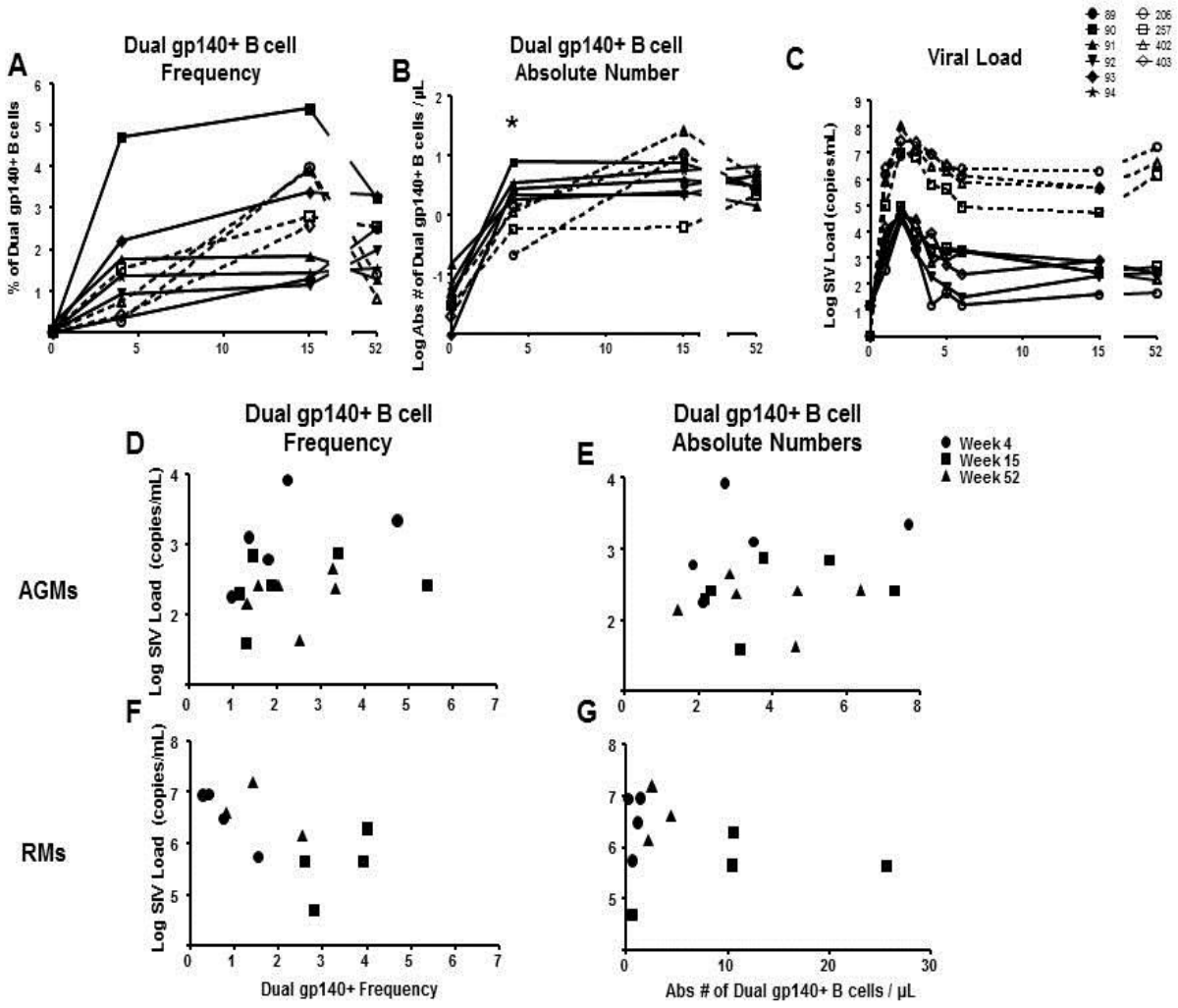

Figure 4. Increased numbers of dual gp140 positive B cells in peripheral blood of SIV-infected AGMs than RMs during acute infection. Frequencies (A) and absolute numbers (B) of dual gp140 positive B cells prior to and during SIV infection in AGMs and RMs. (C) Viral load in AGMs and RMs during acute and chronic SIV infection. AGMs are represented by closed symbols and solid lines and RMs by open symbols and dotted lines. Each symbol represents an individual animal. * $p < 0.05$ by the Mann-Whitney test. (D-G) Correlations of dual gp140 positive B cell frequencies and absolute numbers with viral loads in AGMs (D and E) and RMs (F and G) at weeks 4 (closed circles), 15 (closed squares), and 52 (closed triangles).

Env-specificity and isotype of the circulating gp140-specific memory B cells during acute and chronic SIV infection of AGMs and RMs.

Given the distinct kinetics in the development of plasma gp120-specific IgA responses in acutely SIV-infected RMs and AGM, we next evaluated characteristics of the acute and chronic SIV gp140-specific memory B cell response on the basis of reactivity with monomeric gp120 and cell surface expression of IgA. Quadrant gating was used to distinguish each subpopulation as either reactive or nonreactive with gp120 (gp120+ or gp120-, respectively) and positive or negative for surface expression of IgA (IgA+ or IgA-, respectively), and fluorescence minus one (FMO) controls at each time point were used to set gates (Fig. 5A). A gating legend and representative dot plots at weeks 4, 15, and 52 post infection are shown for AGMs and RMs (Fig. 5A). The frequencies of gp140-specific memory B cells that were gp120+/IgA- trended higher at week 4 ($p = 0.06$) and were significantly higher at week 15 ($p = 0.009$) and week 52 post infection ($p = 0.02$) in SIV-infected AGMs compared to RMs (Fig. 5B and C), potentially reflecting high levels of circulating gp120-specific IgG-producing memory B cells in AGMs consistent with strong gp120-specific IgG responses in plasma at these time points. An associated increase in the absolute numbers of the gp120+/IgA- subpopulation was also observed at week 4 ($p = 0.01$) and week 52 post infection ($p = 0.04$) in AGMs compared to RMs (Fig 5D and E). Interestingly, while the frequencies of the gp120+/IgA+ subpopulation were similar in each species at weeks 4 and 15 post infection, the frequency of this population was significantly higher in SIV-infected RMs than AGMs at week 52 post infection ($p = 0.04$) (Fig. 5B and C). However, there were no significant differences in the absolute numbers of the gp120+/IgA+

subpopulation between species at either time point (Fig. 5D and 5E). Additionally, the frequency ($p = 0.02$) and absolute number ($p = 0.02$) of the gp120-/IgA+ subpopulation were also higher in RMs than AGMs at week 52 post infection ($p = 0.02$) (Fig. 5B-E). While the frequency of the gp120-/IgA- subpopulation was similar at each time point between species (Fig. 5B and C), absolute numbers of this population were higher at week 4 post infection in SIV-infected AGMs compared to RMs ($p = 0.03$) (Fig. 5D and E). Thus, we found noteworthy differences in gp120 reactivity and IgA expression of circulating gp140-specific memory B cells in AGMs and RMs using this methodology.

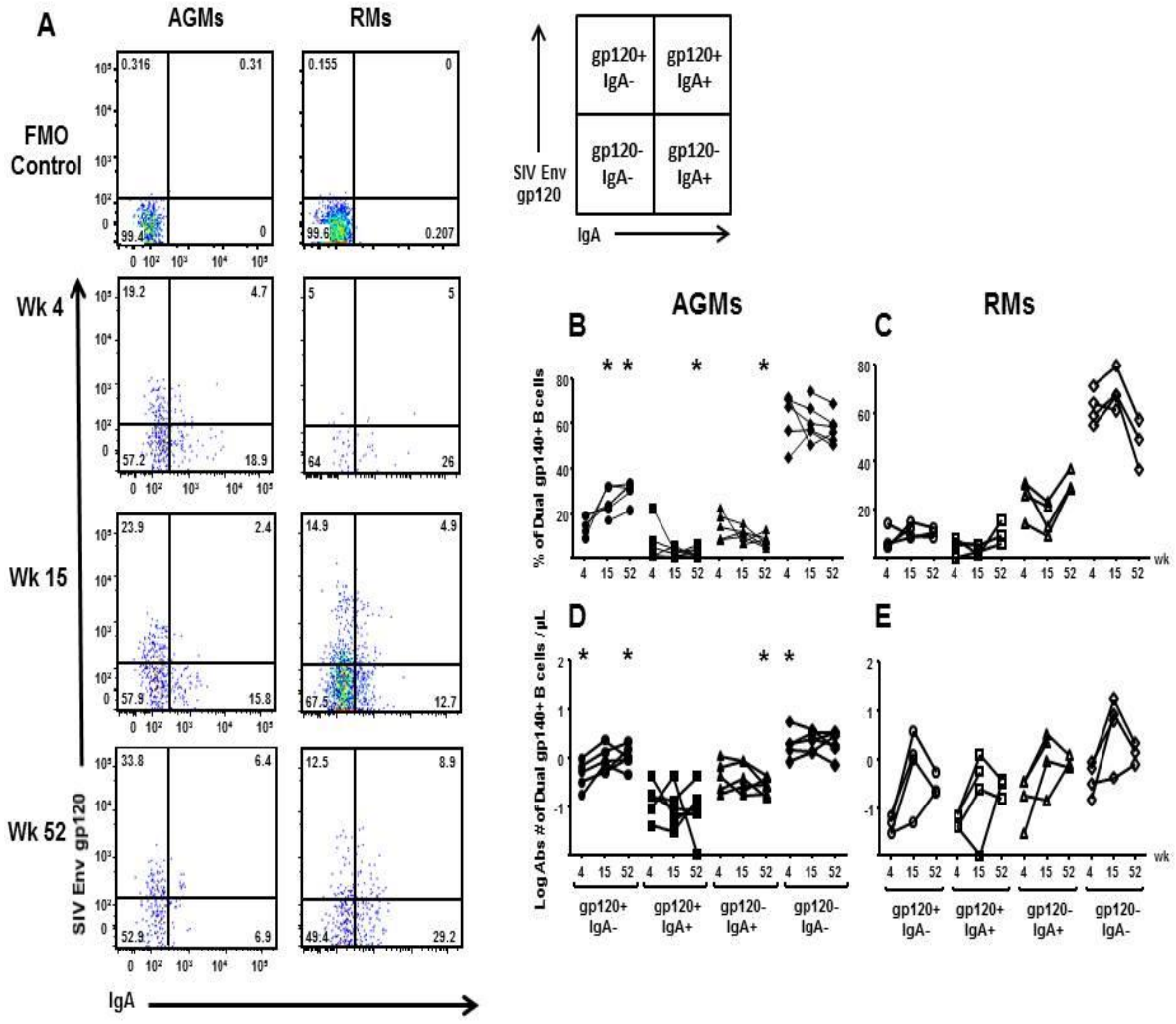

Figure 5. Specificity and antibody isotype of circulating dual gp140 positive B cells in SIV-infected AGMs and RMs during acute and chronic infection. (A) A gating legend and representative dot plots of IgA vs Env gp120 staining at weeks 4, 15, and 52 weeks following infection, including fluorescence minus one (FMO) controls at week 4, in each species are shown. Gating at each time point is based on FMO controls. Numbers within each quadrant represent the frequency of gp120±/IgA± B cells. Frequencies and absolute numbers of gp120+, IgA-, gp120+, IgA+, gp120-, IgA+, and gp120-, IgA- B cells in peripheral blood prior to and during SIV infection of AGMs (B and C; closed symbols) and RMs (D and E; open symbols) at weeks 4, 15, and 52 weeks post infection are graphed. * $p < 0.05$ by the Mann-Whitney test.

4. DISCUSSION

The rarity of postnatal SIV transmission and lack of progression to AIDS in natural hosts provides a unique setting to study the immunological mechanisms that may have evolved over thousands of years to contribute to disease resistance. We previously identified limited B cell dysfunction, lack of hypergammaglobulinemia, and the presence of strong gp120-focused and autologous virus neutralizing antibody responses in the milk and plasma of chronically SIV-infected AGMs compared to that of non-natural SIV hosts, RMs [43, 54]. In the present study, we report an earlier and higher magnitude systemic gp120-specific antibody responses and Env-specific memory B cell populations during acute SIV infection in AGMs compared to that in RMs, implicating that differences in the pre-existing or acute antibody response to SIV may contribute to the development of distinct virus-specific B cell repertoire in these natural SIV host species.

The earliest HIV-1-specific B cell response can be detected as virion-antibody immune complexes about 8 days after detectable plasma virus. The first free antibodies to HIV-1, occurring approximately 13 days after onset of viremia, are anti-gp41 IgM antibodies which are then followed by an anti-gp120 response that targets the V3 region an additional 15 days later [24]. In the natural SIV host, AGMs, we observed earlier development and higher magnitude gp140-specific IgM and IgA binding responses in the plasma of SIV-infected AGMs compared to RMs, with these responses first increasing significantly above base line levels at two weeks post infection in AGMs. Given the similar kinetics of the gp120-specific and gp41 sp400-specific IgM responses during the course of acute infection

in AGMs and RMs, it is plausible that the targets of the high magnitude gp140-binding IgM responses in AGMs are against quaternary epitopes present on the trimeric Env spike of SIV virions and not to other gp41 epitopes, as the initial anti-HIV-1 gp41 antibody response has been shown to be primarily targeted to the immunodominant region of gp41 [24]. In fact, HIV-1/SIV-associated quaternary epitopes have been described as a primary target of autologous and broadly neutralizing antibodies in HIV/SIV infections [59, 60] unlike that of nonneutralizing gp41 epitopes [24]. The IgA response against gp140 was about one log higher than that against gp120 at week 2 post infection in AGMs, which may also suggest that a fraction of the Env-specific IgA response is also targeted to quaternary gp140 epitopes, especially given the limited sp400-specific IgA response in AGMs at the same time point.

The notably higher magnitude IgA response to gp120 at week 2 post infection in AGMs compared to RMs was of particular interest given that this response was undetectable in RMs at the same time point. Trama et. al. [28] recently reported that the initial plasma anti-HIV-1 gp41 antibody response may be attributable to a preinfection pool of memory B cells that are primed by bacterial antigens to cross-react with gp41 Env. Likewise, the rapidity of the gp120-specific IgA response in AGMs may be the result of a pool of IgA-secreting gp120-reactive memory B cells that are primed in the intestinal mucosa by commensal antigens to cross-react with gp120 Env. That this acute gp120-specific IgA response, as well as the convalescent gp120-specific IgG response, persisted at significantly higher magnitudes in AGMs compared to RMs throughout chronic SIV infection may be of functional importance. The rapid development of the acute gp120-specific antibody responses in AGMs are also consistent with our previous observations of gp120-dominant

IgA and IgG binding responses in the milk and plasma of chronically SIV-infected AGMs [43]. While IgG responses against the variable loops were detected in AGMs following acute SIV infection, the epitope specificity of the acute anti-gp120 IgA response remains yet unknown, as we were not able to detect IgA responses against the V1/V2 or V3 loops during acute infection of AGMs. Therefore, initial gp120-specific IgA responses may be directed against other gp120 epitopes, including conformational epitopes such as the CD4 binding site.

In this study, we also identified higher frequencies and absolute numbers of gp140-specific memory B cells in the peripheral blood of SIV-infected AGMs than RMs at four weeks post infection, potentially contributing to the distinct development of the Env-specific IgM and IgA responses during acute infection in AGMs. However, there were no differences in the frequency or absolute number of circulating dual gp140+ memory B cells that expressed gp120-reactive IgA molecules between AGMs and RMs at week 4 post infection, suggesting that the presence of a systemic gp120-specific IgA-producing memory B cell population alone likely did not account for the strong acute Env-specific IgA response observed in AGMs. However, the gp120-reactive non-IgA expressing memory B cell population, likely primarily made up of IgG-producing B cells, was present in higher proportions and numbers during convalescent infection, consistent with the high plasma gp120-specific IgG responses at the same time points. Analysis of the gp140-specific memory B cell populations at earlier time points may shed light on the timing of the first appearance of the gp120-specific memory B cell population in AGMs, but we were unable to perform this analysis due to limited cell availability. As Ig-secreting plasmablasts are a

major constituent of antibody-secreting cells, they contribute significantly to the total plasma antibody content. Therefore, it is conceivable that SIV Env-specific IgA-secreting plasmablasts also account for the elevated levels of gp120-specific IgA responses during acute infection in AGMs. Yet, a previous study reported that only a small fraction of HIV-1-specific plasmablasts account for the abnormally high levels of circulating plasmablasts in HIV-1 viremic individuals [61]. The lack of correlation of viral loads with dual gp140-specific memory B cell frequencies and absolute numbers at weeks 4, 15, and 52 post infection indicates that viral loads did not strongly influence the kinetics of the gp140-specific memory B cell population in the blood of AGMs and RMs throughout SIV infection, further implicating differences in the pre-existing B cell repertoire that drive the differences in acute infection to SIV between the two species.

We previously described strong gp140-specific, as opposed to gp120-specific, plasma and milk IgG responses in chronically SIV-infected RMs compared to AGMs [43]. Accordingly, there were also stronger gp140-specific IgG responses between 2-4 weeks post infection and again at 15 weeks post infection in SIV-infected RMs compared to AGMs. This finding is remarkable as there were no differences between the kinetics and magnitude of the gp120-specific IgG responses between species until weeks 6 and 15 post infection when these responses were significantly elevated in SIV-infected AGMs. Therefore, RMs appear to have a more gp41 or quaternary gp140 epitope-focused antibody response compared to AGMs following SIV infection. Moreover, given that gp41 sp400-specific IgG responses developed with similar magnitude and kinetics in each species through the first six weeks of infection, it is plausible that the higher magnitude gp140-specific IgG response in

RMs are attributable to binding interactions with other gp41 epitopes. The fact that plasma IgG responses against both gp140 and gp41 sp400 were stronger in RMs, but not AGMs, at week 15 indicates that the macaque humoral immune response, like that of acutely HIV-1-infected humans, may be geared towards targeting nonneutralizing gp41 epitopes during the early stages of virus infection, resulting in an inability to control virus replication and viral-induced immune dysregulation effects in non-natural SIV/HIV-1 hosts.

In conclusion, there is an earlier development and higher magnitude IgM and IgA response to the Env gp120 molecule during acute SIV infection of natural SIV hosts AGMs compared to that in RM, a response that is not targeted to the variable loops, but is coincident with the presence of high levels of circulating gp140-specific memory B cells during acute infection in AGMs. These findings could indicate evolutionary adaptation, such as distinct shaping of the pre-existing B cell response, in natural SIV hosts to mount more functional humoral immune responses during the initial phases of acute SIV infection compared to primate species that did not evolve with this viral pathogen. These potentially more functional systemic antibodies may then transudate into the breast milk of natural SIV hosts where they could play an important role in the protection of infants from postnatal virus acquisition. In fact, we have previously described strong autologous virus neutralization responses in breast milk of chronically SIV-infected AGMs [43, 54], a response that could impede autologous maternal virus variants from initiating infection in the infant gastrointestinal tract. Identifying the functional roles of the rapidly-elicited and high magnitude systemic and mucosal gp120-specific antibody responses that evolved in natural SIV hosts may help to inform maternal or infant vaccine strategies for the prevention of

mother-to-child transmission of HIV-1 and/or ways to better contain immune activation and dysfunction in HIV-1-infected humans.

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