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Immunoglobulin V_H Gene Diversity and Somatic Hypermutation during SIV infection of Rhesus Macaques

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Abstract

B cell functional defects are associated with delayed neutralizing antibody development in pathogenic lentivirus infections. However, the timeframe for alterations in the antibody repertoire and somatic hypermutation (SHM) remains unclear. Here, we utilized the SIV/rhesus macaque (RM) model to investigate the dynamics of immunoglobulin V_H gene diversity and SHM following infection. Three RMs were infected with SIVmac239 and V_H1, V_H3 and V_H4 genes were amplified from peripheral blood at 0, 2, 6, 24 and 36 weeks post-infection for next-generation sequencing. Analysis of over 3.8 million sequences against currently available RM germline V_H genes revealed a highly biased V_H gene repertoire in outbred RMs. SIV infection did not significantly perturb the predominant IgG1 response, but overall immunoglobulin SHM declined during the course of SIV infection. Moreover, SHM at the AID deamination hotspot, WRC, rapidly decreased and was suppressed throughout SIV infection. In contrast, a transient increase in mutations at the APOBEC3G deamination hotspot, CCC, coincided with a spike in APOBEC3G expression during acute SIV infection. The results outline a timetable for altered V_H gene repertoire and IgG SHM in the SIV/RM model and suggest a burst of APOBEC3G-mediated antibody SHM during acute SIV infection.

INTRODUCTION

Restoring immune function in HIV-1 infected individuals requires a detailed understanding of dysfunctional components of the immune response. HIV-1 infection causes significant functional T cell defects, as exemplified by CD4⁺ T cell depletion and chronic CD8⁺ T cell activation. In addition, HIV-1 infection is associated with significant humoral immune

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dysfunction. Immunoglobulin (Ig) levels are elevated and this has been linked to polyclonal B cell activation (Lane et al. 1983). In addition, many B cell phenotypic perturbations have been described in HIV-infected individuals (Moir and Fauci 2009). These B cell perturbations during HIV-1 infection may in part explain why neutralizing antibody responses (NAb) develop late during infection and do not temporally correlate with control of viremia.

Antibodies are composed of IgH (heavy) and IgL (light) chains that are combinatorially assembled in the bone marrow. For IgH, this involves linking V_H , D_H and J_H genes, whereas IgL is assembled from V_L and J_L genes. Successful V(D)J rearrangement results in mature, naïve cells that patrol various compartments for foreign antigens. Subsequently, naïve B cells that have encountered antigen could undergo class-switching from IgM to IgG and become memory cells to efficiently respond to antigen re-exposure. Notably, peripheral B cells from HIV+ individuals show an immature/transitional CD10+ phenotype (Malaspina et al. 2006), and CD27+ memory B cells are gradually depleted (De Milito et al. 2001; Nagase et al. 2001). These B cell perturbations may alter the Ab repertoire during HIV infection. Since specific V_H genes have been associated with reactivity to certain antigens, an altered V_H repertoire may differentially impact humoral immunity to a variety of pathogens and vaccines (Bekker et al. 2006; Kroon et al. 1999; Malaspina et al. 2005). Alterations in the Ab repertoire may also predispose HIV-infected individuals to opportunistic infections (Hart et al. 2007; Janoff et al. 1993; Pitzurra et al. 2003).

Upon encountering antigen, B cells rapidly proliferate and Ab genes mutate to increase their affinity to the cognate antigen through somatic hypermutation (SHM). Ig SHM is primarily driven by Activation Induced Deaminase (AID), an enzyme which deaminates deoxycytidines to deoxyuridines in Ig genes (Muramatsu et al. 2000; Revy et al. 2000). During Ig SHM, AID catalyzes C→T (forward strand) or G→A (reverse strand) mutations. This is followed by other error-prone repair processes which result in additional mutations (Peled et al. 2008). While Ig SHM primarily occurs in germinal centers (GCs) in secondary lymphoid organs, dysregulated IgG SHM, particularly in the V_H3 complementarity determining regions (CDR), has been detected in peripheral blood of HIV-1-viremic individuals (Bowers et al. 2014). However, the timeframe for SHM perturbations during HIV-1 infection remains unknown.

AID belongs to a diverse family of deaminases that include APOBEC3 (Conticello et al. 2007). APOBEC3 could potentially restrict retroviruses by inhibiting reverse transcription and instigating G→A mutations in retroviral reverse transcripts (Malim 2009). Interestingly, APOBEC3 improved retrovirus NAb responses in a mouse model of retrovirus infection (Santiago et al. 2008; Smith et al. 2011; Tsuji-Kawahara et al. 2010). We recently obtained evidence for a direct mechanism for this genetic link (Halemano et al. 2014). Similar to WT mice, APOBEC3-deficient mice exhibited normal levels of V_H mutations at preferred sites of AID deamination, WRC (Pham et al. 2003; Rogozin and Kolchanov 1992; Zheng et al. 2005) (W =Weak base=A or T; R =purine=A or G). However, APOBEC3-deficient mice showed significantly lower levels of TYC mutations (Y =pyrimidine=C or T) – the deamination hotspot for mouse APOBEC3 (Langlois et al. 2009; Petit et al. 2009; Yu et al. 2004) – in V_H genes associated with the retrovirus-specific B cell response. These data

suggest that APOBEC3 may edit Ig genes during retrovirus infection in vivo. While mice encode only one APOBEC3 gene, humans and RMs express 7 APOBEC3 proteins (A3A, A3B, A3C, A3D, A3F, A3G and A3H) that together mutate retroviral reverse transcripts in the preferred YC context (pYrimidine=C or T) (Hultquist et al. 2011; Jern et al. 2009; Schmitt et al. 2011). However, it remains unclear if primate APOBEC3 genes could edit Ig genes, and if mutations in APOBEC3 sequence hotspots are dysregulated during pathogenic lentivirus infections.

In general, HIV-1 specific NAb neutralize prior, but not contemporary HIV-1 strains in an infected individual (Richman et al. 2003; Wei et al. 2003). Thus, the humoral immune response may not have the capacity to cope with the rapid pace of HIV-1 evolution. The slow NAb response to newly-evolved HIV-1 strains may be exacerbated by an altered Ab repertoire and impaired SHM. However, understanding Ab repertoire diversity and SHM defects may require the longitudinal tracking of Ig genes. This undertaking is logistically difficult in humans due to the rarity of samples pre- and post-infection relative to a defined transmission time point. In contrast, the timing, route and dose of infection can be controlled and samples at defined stages of the disease can be obtained in SIV-infected rhesus macaques (RMs). Importantly, the SIV/RM model recapitulates many aspects of both T and B cell dysfunction documented in HIV-1 disease (Das et al. 2011; Kuhrt et al. 2010; Titanji et al. 2010; Zhang et al. 2007). However, to date, it remains unknown how B cell dysfunction during the course of SIV infection of RMs influenced Ab repertoire diversity and SHM.

Quantifying Ab repertoire diversity and SHM rates in RMs would require tools that capture the unique RM Ig repertoire. Here, we utilized available RM V_H germline sequences (Andris et al. 1997; Bible et al. 2003; Helmuth et al. 2000; Olivieri and Gambon-Deza 2015; Sundling et al. 2012) primarily mined from the RM genome sequence (Gibbs et al. 2007) and adopted a next-generation sequencing (NGS) strategy to quantify Ig SHM. We provide, to our knowledge, the first longitudinal analysis of IgG V_H gene distribution and SHM rates of RMs infected with SIV using an NGS strategy. The data provides a timetable for alterations in V_H gene repertoire and SHM and suggest that A3G may drive early IgG SHM in pathogenic lentivirus infections.

MATERIALS AND METHODS

Ethics statement

The RMs used in the study were obtained from certified vendors and housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International-approved animal facility at the University of Kansas Medical Center (KUMC). RMs were housed in compliance with standard cage dimensions as certified by AAALAC International and with additional sources of enrichment including objects for perching and other manipulanda. RMs were given 5038 primate chow with access to water ad libitum. All RMs were handled in accordance with the recommendations in the NIH Guide for the Care and Use of Laboratory Animals through an Animal Care Use Protocol (ACUP) approved by the KUMC Institutional Animal Care and Use Committee (IACUC) with protocol number ACUP 2009-1789. The SIVmac239 stock was obtained from the NIH AIDS Reagent

Program. All infections and blood collections were performed with the RMs under ketamine anesthesia using appropriate efforts to minimize suffering.

Viral stock and inoculation

SIVmac239 virus stocks were prepared in CEMx174 cells. RMs were inoculated i.v. with 10^4 TCID₅₀ of virus stock. RMs 2H2 and 8G5 were 2 years 8 months old and RM 4440 was 2 years 6 months old at the time of inoculation.

Processing of blood samples

EDTA-treated blood was collected weekly for 4 weeks, then at 2-week intervals until 4 months and monthly intervals until 40 to 42 weeks, and sent overnight by courier to the University of Colorado Denver. PBMCs were obtained using Ficoll-Hypaque gradients, and cryopreserved at 10^6 cells/ml.

Plasma viral load

For each time point, 1 ml of plasma was pelleted using ultracentrifugation (Beckman SW55Ti, 250,000×g, 2 h) and RNA was extracted using the QIAamp viral RNA extraction kit (Qiagen). Quantitative reverse-transcriptase PCR was performed using SIV gag primers and probe as previously described (Hofmann-Lehmann et al. 2000). Standard curves were prepared using six 10-fold dilutions of SIV gag RNA of known concentration. The assay had a limit of detection of 100 copies/ml.

Quantification of RM IgG

ELISAs were performed at room temperature and with 100 µl/well volumes unless otherwise indicated. Mouse-adsorbed goat anti-human κ-light chain and goat anti-human λ-light chain (MyBiosource) were coated into Immulon-4 HBX plates (Thermo Scientific) at 100 ng/well each and incubated overnight at 4°C. Plates were blocked with 200 µl/well SuperBlock (Pierce) for 1 hr and washed once with phosphate buffered saline (PBS) with 0.05% Tween-20 (PBS-T). Serial 2-fold dilutions of purified RM IgG (NIH Nonhuman Primate Reagent Resource/NPRR) in PBS, starting at 0.2 ng/µl, were added to the plate to produce a standard curve. Serial 2-fold dilutions of plasma in PBS, starting at 1:5000, were added to the plate and incubated for 2 hrs at 37°C. After 5 washes with PBS-T, 1:6000 HRP-conjugated mouse anti-human IgG (G18-145, BD Pharmingen; this mAb cross-reacts with RM IgG as noted in the NIH NPRR) was added and incubated for 1 hr. Following 6 PBS-T washes, 100 µl/well of TMB substrate (BioFX Laboratories) was added and incubated in the dark for 10 min. The reaction was stopped with 0.3N sulfuric acid and absorbances were read at 450 nm in a Victor X5 plate reader (Perkin Elmer).

Flow cytometry

PBMCs were thawed and incubated in Fc receptor block (Innovex Biosciences) for 20 min at 4°C. Cells were resuspended in 1 ml PBS per 1×10^6 cells and stained with 1 µl/ml Live/Dead Aqua viability stain (Invitrogen) for 30 min at 4°C. Cells were resuspended in FACS buffer (PBS, 1% fetal bovine serum) and stained with CD3-V450 (Clone SP34-2, BD Horizon), CD4-APC-H7 (L200, BD Pharmingen), CD8-Alexa Fluor 700 (RPA-T8, BD

Pharmingen), CD20-Alexa Fluor 700 (2H7, BD Pharmingen). The stained cells were analyzed in an LSR-II flow cytometer (BD Biosciences) and analyzed using the Flowjo software (Treestar).

Quantitative PCR for A3G expression

Total RNA was extracted from RM PBMCs using the RNeasy kit (Qiagen). cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen). The cDNA was used for triplicate quantitative PCR (qPCR) reactions (25 µl) with RM A3G (rhA3G) specific primers: 5'-GCCATTAAAGCCTTGAACA-3' (sense), 5'-GAGCCTGGTTGCGTAGAAAG-3', and probe: 5'-[6-FAM]AACCTTGGGTCAGTGGACAG[TAMRA]-3'. GAPDH was used as the housekeeping gene using specific primers: 5'-CCCATGTTTCGTCATGGGTGT-3' (sense), 5'-TGGTCATGAGTCCTTCCACGAT-3' (antisense), and probe: 5'-[6-FAM]CTGCACCACCAACTGCTTAGCACCC[TAMRA]-3'. All qPCR were performed in a CFX-96 real-time PCR machine (Biorad). Briefly, 5 µl of cDNA was mixed with 12.5 µl of 1× TaqMan Gene Expression Master Mix (Applied Biosystems), 10 pmol of primers and 5 pmol of probe and subjected to 40 cycles of 15 s denaturation at 95°C and 45 s of annealing/elongation at 60.5°C and 64.5°C for rhA3G and GAPDH, respectively. Cloned cDNA sequences were used as standards for the absolute quantification. The rhA3G qPCR assay had a limit of detection of 5 copies per reaction, efficiency of 99% and R² value of 99%.

Amplification of IgH sequences

Total RNA was extracted from RM PBMCs (10⁶ cells) using the RNeasy kit (Qiagen), cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen) according to the manufacturer's instructions. Amplicons were produced using Phusion Hi-Fidelity DNA Polymerase (New England Biolabs) and 10 pmole of V_H1, V_H3 or V_H4 forward and IgG or IgM reverse primers. Three previously described human V_H1, V_H3, and V_H4 forward primers were found to be highly conserved in the leader regions (Tiller et al. 2008) were also highly conserved in counterpart RM V_H1, V_H3, and V_H4 genes. These primers were modified to include Illumina MiSeq adapters and barcodes. The reverse primer was located 180 bp within the IgG constant region to include the SNPs required for determination of IgG subclass. To increase diversity in the sample, a four-nucleotide random sequence (NNNN) was added 5' of the 6 bp barcode (or index) sequence. The primers were purchased from Eurofins MWG Operon. The sequences (5'-3') are shown below:

V_H1_forward:

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC
CGATCT NNNN INDEX AGGTGCCCCACTCCCAGGTGCAG

V_H3_forward:

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC
CGATCT NNNN INDEX AAGGTGTCCAGTGTGARGTGCAG

V_H4_forward:

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTC
CGATCT NNNN INDEX ATGGGTCTGTCCCAGGTGCAG

IgHG_reverse:

CAAGCAGAAGACGGCATAACGAGATGTGACTGGAGTTCAGACGTGTGCTCTT
CCGAT CT NNNN INDEX GMGCCTGAGTTCCACGACACG

IgHM_reverse:

CAAGCAGAAGACGGCATAACGAGATGTGACTGGAGTTCAGACGTGTGCTCTT
CCGAT CT NNNN INDEX GGGAATTCTCACAGGAGACGA

Amplification was performed in a S1000 thermo-cycler (Biorad) using the following conditions: 98°C for 30 min, 35 cycles of 98°C for 10 s, 62°C for 15 s, and 72°C for 25 s. Amplicons were gel-extracted using the QIAquick gel extraction kit (Qiagen). Amplicons were quantified by Quant-IT PicoGreen dsDNA assay kit (Invitrogen) and mixed at equimolar amounts for paired-end 2×250 bp sequencing on the Illumina MiSeq.

Illumina sequencing and quality scores

FASTQ output files from the MiSeq were sorted into samples using 6-bp barcodes. The quality score (Q score) for each base was used to filter out low scoring segments of sequence. Using custom Perl scripts, segments of 10 bases (10-base sliding window) were tested at time. If the average Q score of the segment fell below 20, which corresponds to 1 error in 100 bases, the segment was discarded along with the preceding (reverse read) or succeeding (forward read) bases. If the length of a quality-filtered sequence was less than 100 bp, the sequence read was considered too short and also discarded. Afterwards, gene-specific primer sequences were removed from the ends of sequences called 'Read1' (forward). Subsequently, the filtered Read1 sequences, which include majority of the V_H region, were collapsed into unique sequences while keeping track of the count numbers. The Read2 sequences were used for IgG subclass classification. Read1 sequences that occurred only once were excluded from further analysis to decrease error rates based on optimization studies (Supplementary Fig. 1). Raw NGS sequence data were deposited in the Sequence Read Archive Bioproject PRJNA267125.

Calculation of V_H repertoire changes

For each RM, the percentage of each V_H1, V_H3 and V_H4 gene relative to the total number of V_H1, V_H3 and V_H4 counts were computed, respectively. The 'percent change' for each infection time point (2, 6, 24 and 36 wpi) was computed relative to the pre-infection (0 wpi) time point. For example, if the percentage of V_H3.58 among the total V_H3 counts at 0 wpi was 0.18%, and at 2 wpi, it was 0.40%, the calculated change was $[100 \times (0.40\% - 0.18\% \text{ divided by } 0.18\%) = +122\% \text{ change}]$. Note that the change in representation could either be a negative or positive value depending on whether the V_H gene was downregulated or upregulated. To evaluate overall gene repertoire perturbations within the V_H1, V_H3 and V_H4 clades, the absolute percent change values relative to 0 wpi were obtained for each RM at 2, 6, 24 and 36 wpi. The absolute percent change from each RM served as biological replicates for each V_H gene per time point. Repeated-measures ANOVA was used to evaluate if there

were significant changes in the gene repertoire within the V_H1, V_H3 and V_H4 clades (Graphpad Prism).

Mutational frequencies

Unique sequences were matched and aligned against all RM V_H genes mined from the RM genome (Olivieri and Gambon-Deza 2015; Sundling et al. 2012), King's College RM Ig Gene Database, and IMGT Database using USEARCH (Edgar 2010), then classified according to the nomenclature in Supplementary Table 1. Custom Perl scripts were used to compare each unique sequence to the aligned reference sequence in order to determine the mutation frequency and to identify mutations as AID-type or APOBEC3-type based on their dinucleotide or trinucleotide context (Supplementary Fig. 2). For some analyses, we utilized IgM sequences from each RM as the reference, similar to retroviral quasispecies comparisons that we recently described (Barrett et al. 2014). To quantify the frequencies of AID and APOBEC3-type mutations, we utilized the total number of bp analyzed as denominator. To quantify the percentage of AID and APOBEC3-type deaminations, the counts were normalized to the number of G→A and C→T mutations.

Phylogenetic analysis

Human and RM V_H nucleotide sequences were aligned and unrooted phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei 1987) excluding gaps as implemented in the CLUSTAL_X interface (Thompson et al. 1997). Clades supported by >90% bootstrap after 1000 replicates were considered significant.

Statistical analysis

All analyses were performed using Prism 5.0 (GraphPad). Comparisons between two groups at 2 time points were performed using a paired 2-tailed Student's t test. For analysis of longitudinal data with time as an independent variable, repeated measures ANOVA was performed followed by Tukey's multiple comparison posttest. Data for each RM served as biological replicates for inferring longitudinal changes in distribution and mutation rates across well-represented V_H genes. Comparisons of multiple groups were performed using Kruskal-Wallis ANOVA with Dunn's multiple comparison posttest. P values less than 0.05 were considered significant.

RESULTS

RM V_H germline reference sequences

For this longitudinal study, we focused on V_H sequences since the sequence length provides more information for molecular classification. The analysis of Ig V_H repertoire diversity and SHM in humans and mice are made possible by the availability of annotated germline V_H sequences in the Immunogenetics (IMGT) database (<http://www.imgt.org>) (Lefranc et al. 2009). However, the number of functional RM germline V_H sequences in the IMGT database (n=23) was disproportionately low compared to the human sequences (n=53; Supplementary Table 2). Mining of the currently available RM IgH locus (Gibbs et al. 2007) revealed 61 RM V_H genes (Sundling et al. 2012), but functional V_H germline sequences (n=89) from several studies (Andris et al. 1997; Bible et al. 2003; Helmuth et al. 2000) were

also compiled in the King's College RM Ig Gene Database (http://www.kcl.ac.uk/immunobiology/Mac_ig/).

Preliminary analyses of RM V_H genes from these multiple sources revealed substantial sequence identities. To consolidate the currently available set of RM V_H sequences, we utilized known allelic variations in human V_H genes as a guide. In the IMGT human V_H compilation, the maximum pairwise difference between human V_H alleles averaged $3.01 \pm 2.23\%$ (mean $\pm$ SD; Supplementary Table 2). We considered RM V_H gene sequences that differed by $>7.5\%$ from genome-mined sequences as distinct V_H genes, as a $2 \times$ SD difference could reliably distinguish the human V_H genes. This resulted in the inclusion of 7 additional V_H genes (Supplementary Table 1). Phylogenetic analyses showed that these 7 V_H genes were distinct from other V_H genes but belonged to one of the 7 major primate V_H clades, V_H1 to V_H7 (Fig. 1). Similar to humans, the RM V_H3 clade is the most diverse with 39 genes, followed by V_H4 (n=12) and V_H1 (n=8).

Humoral immunity during SIV infection of RMs

To investigate the dynamics of V_H gene distribution and SHM during SIV infection, we infected 3 RMs with SIVmac239. Blood samples were obtained prior to and at regular intervals following SIV infection. Viral loads showed the characteristic peak in viremia before 4 weeks post-infection (wpi) and the establishment of a viral load set-point shortly after (Fig. 2A). Gradual inversion of the CD4:CD8 ratio was observed, indicating progressive CD4⁺ T cell depletion (Fig. 2B, upper). We observed an ~ 2 -fold decrease in B cell percentages during acute infection in line with other studies (Das et al. 2011; Kuhrt et al. 2010; Titanji et al. 2010; Zhang et al. 2007), but these differences did not reach statistical significance, possibly due to low numbers of RMs analyzed (Fig. 2B, lower). Of note, IgG levels gradually increased (Fig. 2C), consistent with polyclonal B cell activation. However, none of the plasma samples at any time point neutralized the parental SIVmac239, consistent with previous reports (Sato et al. 2008; Yeh et al. 2010).

IgG1 is the predominant IgG subclass independent of SIV infection

Analogous to humans, RM IgG can be classified into 4 subclasses: IgG1, IgG2, IgG3 and IgG4 (Scinicariello et al. 2004). The relative proportion of these IgG subclasses during SIV infection remains unknown. While it is feasible to quantify IgG subclass distribution by qPCR, this would require the independent validation of each qPCR assay and necessitate absolute quantifications to obtain distribution information. We therefore pursued a paired-end NGS strategy to determine the relative transcript abundance of RM IgG subclasses (Fig. 3A). Conserved reverse primers were designed to capture single nucleotide polymorphisms in the C γ region that would distinguish the 4 IgG subclasses. Since 5' V_H sequences were not conserved across RM V_H clades, separate forward primers were designed in the conserved leader regions within V_H1, V_H3 and V_H4 (Fig. 3A) (Sundling et al. 2012), respectively. V_H1, V_H3 and V_H4 are the most prevalent V_H clades in RMs (Margolin et al. 1997), as well as in SIV-specific antibodies (Kuwata et al. 2011; Kuwata et al. 2013) (Supplementary Fig. 3). IgG V_H genes were PCR-amplified from RNA extracted from 10^6 unstimulated PBMCs prior to infection and at 2, 6, 24 and 36 wpi. V_H sequences with stop codons (3.1%) were considered non-functional and excluded from subsequent analyses.

Over 3.8 million sequence reads were analyzed (Fig. 3A). Prior to SIV infection, majority (>90%) of sequence reads were IgG1, independent of V_H clade (Fig. 3B). IgG2 was lower but significantly represented (7.1%), whereas IgG3 and IgG4 each represented less than 0.3% of IgG transcript reads. This IgG subclass distribution remained consistent throughout SIV infection, although transient perturbations in IgG1/IgG2 ratios were observed during chronic SIV infection (Supplementary Fig. 4, arrows). In general, however, IgG1 dominated the V_H transcript pool before and during SIV infection.

Dynamics of V_H gene distribution during SIV infection

We next evaluated the relative proportions of individual V_H genes within the V_H1, V_H3 and V_H4 clades. There was substantial bias in V_H gene distribution in uninfected RM IgG1 transcripts, since a relative minority of V_H genes accounted for majority of observed sequences. Remarkably, V_H gene usage bias was consistent across the 3 RMs studied (Fig. 3C). Two V_H1 genes (V_H1.23 and V_H1.16) accounted for >80% of V_H1 genes, and 6 V_H3 genes and 4 V_H4 genes accounted for majority (>60%) of V_H3 and V_H4 genes, respectively. These findings demonstrate a biased IgG1 V_H gene repertoire that is conserved between outbred RMs.

We next evaluated whether IgG1 V_H gene distribution was altered during the course of the SIV infections. Increasing, decreasing and ‘blip’ patterns were observed in several V_H genes, but these trends were not consistent (Supplementary Fig. 5). To determine if there were overall changes in V_H gene distribution within the V_H1, V_H3 and V_H4 clades, the absolute percent changes at 2, 6, 24 and 36 wpi relative to the 0 wpi time point were calculated and analyzed by repeated measures ANOVA as described in the Materials and Methods (‘Calculation of V_H repertoire changes’). These analyses revealed that: (1) significant perturbations in V_H3 gene distribution occurred at 2 wpi relative to later post-infection time points; (2) V_H1 gene distribution was altered at 2 wpi but remained stable thereafter; and (3) V_H4 gene distribution was relatively unchanged from baseline (Fig. 3D). Thus, the data showed alterations in the gene distribution of IgG1 V_H3 and V_H1, but not V_H4, particularly during acute SIV infection. We also evaluated IgG2 V_H gene usage in time points when IgG2 accounted for >25% of the total IgG transcripts (Supplementary Fig. 4, arrows). The sharp increase in V_H1 IgG2 at 24 wpi was >70% due to V_H1.23, the most prevalent V_H1 gene. However, the sharp increase in V_H3 IgG2 at 6 wpi was >90% due to a less prevalent V_H3 gene, V_H3.27. Thus, blips in IgG1/IgG2 subclass distribution could be due to the induction of a specific V_H gene.

SIV infection decreased overall V_H SHM frequencies

The identification of germline counterparts of RM V_H sequences provided an opportunity to determine if SIV infection could influence overall Ig SHM. To gain an overall perspective of IgG SHM during SIV infection, we consolidated all IgG sequences regardless of subclass designation. To quantify SHM, we first removed sequence reads that were found only once in the dataset to minimize NGS errors (Supplementary Fig. 1). As we previously described for mice (Halemano et al. 2014), we ‘collapsed’ the remaining data into unique sequences per RM, since the expansion of specific B cell clones (and corresponding V_H sequences) may bias the SHM frequencies obtained. SHM was quantified as the number of mutations

relative to germline, divided by the number of nucleotides analyzed per V_H gene $\times 100\%$. Prior to infection, the average SHM frequency per IgG V_H gene was 6.43% and were not significantly different between the RMs studied (Fig. 4A).

We next evaluated longitudinal SHM rates up to 36 wpi in 41 different V_H genes that were well-represented in the 3 RMs. This analysis involved 1.3 million sequence reads with an average of 6,331 reads per IgG V_H gene per time point (Supplementary Table 3). Of note, the average number of reads obtained per time point were not significantly different from each other (Supplementary Table 3), suggesting that the number of B cell lineages sampled were equivalent despite a modest reduction in B cell percentages during acute SIV infection (Fig. 3B). For each V_H gene, the mean SHM frequency between the SIV-infected RMs at each time point was computed. In other words, data from individual RMs were used as biological replicates to obtain a single SHM value per V_H gene at 0, 2, 6, 24 and 36 wpi. We then tested whether each V_H gene showed a similar pattern of increased or decreased SHM during SIV infection using repeated measures ANOVA. This analysis revealed a progressive decline in IgG V_H SHM (Fig. 4B). The lowest SHM levels were observed at 24 wpi (Fig. 4B), corresponding to a 22% reduction in SHM. At 24 wpi, nearly all V_H genes had a decrease in SHM relative to baseline (Fig. 4C).

Decreased SHM frequencies were observed in V_H4 , and to a lesser extent, V_H3 genes at various time points following infection (Fig. 4D). By 36 wpi, SHM frequencies returned to baseline levels (Fig. 4B), primarily due to increased SHM in V_H1 genes (Fig. 4D). The data indicated that overall IgG V_H SHM was impaired during the first 6 months of SIV infection.

Decreased mutations at AID deamination hotspots during SIV infection

The progressive decrease in Ab SHM during SIV infection could be due to the dysregulation of various factors in the SHM machinery. Previously, it was shown that patients with established (chronic) HIV-1 infection exhibited decreased V_H3 mutations that fit the preferred AID deamination context, WRC (Bowers et al. 2014). However, it remains unknown if this phenomenon extends to SIV infection of RMs and if so, at which infection timeframe WRC mutations decline. To fill this knowledge gap, we evaluated mutations that fit the preferred deamination context for AID, WRC (Pham et al. 2003; Rogozin and Kolchanov 1992; Zheng et al. 2005). Deamination in the WRC context of either DNA strand results in 8 possible $C \rightarrow T$ or $G \rightarrow A$ mutations (Supplementary Fig. 2). To calculate WRC frequencies, the number of WRC mutations were counted, and divided by the number of base pairs analyzed $\times 100\%$. This analysis revealed that the frequency of WRC deaminations were similar between the uninfected RMs (Fig. 5A).

We next evaluated the frequency of WRC mutations during the course of SIV infection. RM V_H genes exhibited decreased percentages of WRC mutations beginning at 2 wpi, and this was maintained during the course of SIV infection (Fig. 5B). This was further exemplified in Fig. 5C, which showed that the majority of V_H genes had reduced WRC mutation frequencies at 6 wpi. These data revealed that reduced mutational frequencies at WRC sites contributed to the decline in overall SHM rates during the course of SIV infection. Decreased WRC mutational frequencies were most pronounced in the V_H3 and V_H4 genes (Fig. 5D).

Temporal correlation between A3G expression and A3G-type V_H mutations during acute SIV infection

Our recent study demonstrating that mouse APOBEC3 could edit Ig genes during retrovirus infection (Halemano et al. 2014) raised the possibility that the primate APOBEC3 proteins may also edit IgG genes during HIV/SIV infection. Interestingly, the APOBEC3 proteins deaminate deoxycytidines with a distinct sequence context from AID. Primate A3A, A3D, A3F and A3H preferentially mutate in the YTC context, whereas A3G proteins prefer to mutate in the CCC context (Supplementary Fig. 2) (Beale et al. 2004; Bishop et al. 2004; Harris et al. 2003; Hultquist et al. 2011; Liddament et al. 2004; Refsland et al. 2012; Schmitt et al. 2011; Schmitt et al. 2009; Wiegand et al. 2004). We therefore investigated the frequency of APOBEC3-type mutations in V_H genes during the course of SIV infection. Prior to infection, RMs had similar YTC and CCC mutation frequencies (Kruskall-Wallis ANOVA, $p=0.3931$ and 0.2818 , respectively). Mutations in the YTC context remained unchanged (Fig. 6A). Surprisingly, we observed a significant increase in the frequency of CCC mutations at 2 wpi (Fig. 6B), particularly for the V_{H3} genes, with a 2.4-fold increase relative to baseline (Fig. 6C).

It was previously shown that RM PBMC A3G levels were transiently induced during the first 2 weeks of SIV infection (Mussil et al. 2011). Consistent with these findings, we detected a 4 to 6-fold induction of A3G mRNA levels during the first 2 weeks of SIV infection (Fig. 6D). At 6 wpi, the frequency of A3G-type mutations returned to baseline, while A3G expression levels were suppressed from 3 to 20 wpi. A3G expression increased again at 24 to 36 wpi (Fig. 6D), but V_H CCC mutations did not during these time points (Figs. 6B and 6D). Thus, there was a temporal correlation between a spike in A3G expression and a burst in A3G-type CCC mutations in Ig V_H genes during acute, but not chronic, SIV infection.

Transient changes in the sequence context of V_H deaminations during acute SIV infection

The previous analyses revealed quantitative changes in mutations at different sequence contexts, but do not provide insights on qualitative changes in deamination profiles. Of note, mutations (G→A and C→T) due to AID, A3G and other APOBEC3 members were also associated with the less stringent dinucleotide contexts RC, CC and TC, respectively (Supplementary Fig. 2). To determine if there were qualitative changes in the dinucleotide context of deamination mutations, we tallied the dinucleotide context of 576,147 G→A and C→T mutations, which accounted for ~30% of all V_H mutations throughout SIV infection (Fig. 7A). As expected, over half of G→A and C→T mutations fit the AID dinucleotide hotspot RC at baseline (Fig. 7B), but CC mutations were common, accounting for an average of 11% (range: 2 to 26%) of deaminations (Fig. 7B). By 2 wpi, CC mutations significantly increased to 17% (range: 5 to 35%) of deaminations (Fig. 7B), the percentage of RC mutations significantly decreased (Fig. 7C) and TC mutations had no change (Fig. 7D). Subsequently, both CC and RC deamination profiles returned to baseline (Figs. 7B–C). No significant changes in TC mutations were observed during SIV infection (Fig. 7D). Thus, based on the relative proportions of RC versus CC mutations, acute SIV infection likely altered the deamination profile of V_H genes.

Mutational profiles using pre-infection IgM as reference sequences

Ig SHM rates for humans, mice and RMs are routinely calculated using reference sequences from a representative genome sequence. However, with the exception of laboratory mice (which are inbred), it is theoretically possible that genomic DNA polymorphisms in V_H genes may influence mutational rate calculations. We therefore investigated the impact of potential allelic polymorphisms by obtaining IgM sequences from each RM by NGS. The rationale is that IgM is induced during the primary Ab response and should not have undergone extensive SHM. In other words, IgM sequences should have higher sequence identity to the germline compared to IgG. We focused our analyses on V_{H3} genes as it is the most diverse and accounted for majority of V_H genes in our mutational analyses in Figs. 4–7. We obtained 11,555 unique IgM V_{H3} sequences from the pre-infection time point, translating to an average of 168 unique IgM reference sequences per V_{H3} gene per RM. At the pre-infection time point, significant mutational frequencies were observed in IgM sequences, suggesting that we captured not only potential germline polymorphisms, but also memory IgM+ B cells that have undergone SHM. However, as expected, when compared against the standard RM V_H gene reference database, these IgM sequences had significantly lower SHM relative to IgG sequences (Fig. 8A). We next utilized the pre-infection IgM sequences of each RM as reference sequences to evaluate IgG mutational frequencies for that particular RM. In effect, this approach would cancel any IgG mutation found in the pre-infection IgM repertoire, and would therefore provide a more conservative estimate of IgG SHM. Using this approach, AID-type WRC V_{H3} mutations declined (Fig. 8B), but A3G-type CCC mutations spiked at 2 wpi (Fig. 8C), similar to our results using the standard RM V_H gene database (Figs. 5D and 6C, respectively). Moreover, the proportion of deamination mutations in the CC context increased whereas RC mutations decreased by 2 wpi (Fig. 8D), similar to Fig. 7B and 7C, respectively.

DISCUSSION

HIV-1 infection is characterized by humoral immune dysfunction that is mirrored during SIV infection of RMs. However, acute HIV-1 infection studies are logistically difficult, thus highlighting the importance of the SIV-RM model in understanding early pathogenic events relevant to HIV-1 infection. Notably, HIV-1 infection is associated with altered V_{H3} gene usage and reduced SHM in the CDR regions compared to uninfected individuals (Bowers et al. 2014). However, the impact of SIV infection on the overall RM Ab repertoire remains unknown. Here, we utilized RM V_H genes mined from published sources (Andris et al. 1997; Bible et al. 2003; Helmuth et al. 2000; Sundling et al. 2012) as well as pre-infection IgM sequences to quantify V_H gene repertoire changes and mutation rates. Using the SIV-RM model, we now provide a potential timetable for SHM changes during pathogenic lentivirus infection.

Our analysis of SHM mutation rates suggested a decrease in AID-associated SHM that begins during acute SIV infection. Although the magnitude of this decrease in SHM can be interpreted as numerically modest, the biological impact of these changes may be compounded by additional B cell perturbations already documented in the SIV/RM model. We speculate on possible mechanisms for the observed decrease in AID-associated IgG

SHM. Acute SIV and HIV-1 infection were associated with decreased numbers of GCs (Levesque et al. 2009; Zhang et al. 2007), the primary sites for AID activity. Thus, the lack of SHM may be a direct consequence of early GC dysfunction. SIV infection of follicular helper T cells (Folkvord et al. 2005; Petrovas et al. 2012; Xu et al. 2013), which prime GC B cells to proliferate, may potentially alter cytokine production by these cells and stall Ab SHM processes. In addition, unusually high levels of B cell activation may result in premature expansion of class-switched B cells with lower SHM levels. Although we controlled for the contribution of B cell or plasma cell expansion in this study by analyzing 41 V_H genes and focusing on nonredundant IgG sequences per RM time point to capture as much V_H diversity as possible, we could not exclude that B cell lineage bias may have contributed to our analyses. The decline in AID-associated IgG SHM may also be a direct effect of HIV-1/SIV on AID function. AID was up-regulated in B cells following co-incubation with HIV-1 (Perise-Barrios et al. 2012), especially if host CD40L was incorporated in virions (Imbeault et al. 2011). The consequence of HIV-1 induced AID expression on SHM remains to be determined, but AID overexpression may lead to reduced activity due to sequestration of AID into inactive cytoplasmic complexes (Hasler et al. 2012). Further studies would be required to confirm if AID function is indeed impaired during pathogenic lentivirus infections.

The results from this study enrich current models of B cell dysfunction in pathogenic lentivirus infections. It was therefore surprising that CCC or CC mutations significantly increased during acute SIV infection, despite the fact that overall SHM frequencies were on a downward trend. These CCC or CC mutations could be due to coldspot AID activity, but AID hotspot mutation frequencies (WRC or RC) actually decreased at 2 wpi. While we cannot entirely rule out that some of these mutations may be due to other APOBEC3 members, these deaminases preferentially mutate in the YTC or TC context. In contrast, A3G mutates retroviral transcripts preferably in the CCC (or CC) context (Bishop et al. 2004; Harris et al. 2003; Liddament et al. 2004; Wiegand et al. 2004; Yu et al. 2004). Moreover, there was a marked induction of A3G expression during acute SIV infection. A3G is endogenously expressed in activated B cells, and A3G may have generated CCC mutations in single-stranded Ig gene substrates following co-incubation with nuclear tonsil B cell lysates in vitro (Pham et al. 2008). Our studies with APOBEC3-deficient mice also provided evidence that mouse APOBEC3 could edit V_H genes in vivo (Halemano et al. 2014). Thus, the simplest explanation for our results is that A3G directly mutated multiple IgG V_H genes during acute SIV infection. Interestingly, we did not observe a correlation between A3G induction and V_H-CC mutations during chronic SIV infection. One possibility is that the cell types where A3G was induced during chronic SIV infection were not B cells, but due to limited blood samples available for the various analyses, we were unable to analyze A3G levels in purified B cells at various time points. Alternatively, during chronic SIV infection, mechanisms supporting A3G-mediated SHM may have been impaired, or inhibitory mechanisms may have been induced. Moreover, it is feasible that a different mutational profile will be obtained in lymphoid compartments (Briney et al. 2014), particularly in germinal centers which are the primary sites of SHM. Studies are ongoing to evaluate how A3G function in B cells is regulated and correlate with IgG SHM patterns in lymphoid tissues of RMs with acute versus chronic SIV infection (Connick et al. 2014).

A spike in A3G-mediated SHM during acute SIV infection may provide an initial boost to diversify the Ab repertoire. We speculate that some of these A3G-mutated B cells may be used to initiate virus-specific Ab responses. Several SIV-specific rhMAbs (Kuwata et al. 2013) harbored CC mutations relative to germline (K. Guo, T. Kuwata, M. Santiago, unpublished), but it remains to be determined if these mutations emerged during acute SIV infection. It will be of interest to determine if spikes in V_H-CC mutations occur in other viral infections. Finally, the transient nature of the A3G-deamination mutations suggested a tightly regulated mechanism. One potential mechanism driving A3G induction and V_H-CC mutational spike is the type I Interferon (IFN) response. IFN α could regulate APOBEC3 activity in vivo (Harper et al. 2013; Mehta et al. 2012; Pillai et al. 2012) and IFN α exhibited rapid, transient expression during acute HIV-1 and SIV infection (Malleret et al. 2008; Rahmberg et al. 2013; Stacey et al. 2009). Further understanding of mechanisms that regulate A3G-mediated SHM during acute SIV infection may provide approaches to augment vaccine regimens that require eliciting antibodies with high levels of SHM, such as the highly sought-after broadly-neutralizing antibodies against HIV-1 infection (Burton et al. 2012; Klein et al. 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Phylogeny of human versus RM V_H germline genes. RM V_H genes mined from the RM genome sequence (Sundling et al. 2012) were consolidated with sequences compiled in IMGT and the King's College RM Ig gene databases. Database sequences that differed by >7.5% in nucleotide sequence relative to the mined germline sequences from the RM genome were considered as distinct genes (arrows and grey circles). RM V_H gene names were adapted from (Sundling et al. 2012). The 7 major V_H clades were conserved between human and RMs. Trees were generated by neighbor-joining method and evaluated using 1000 bootstrap replicates.

Fig. 2. Profiles of SIVmac239-infected RMs. (A) Plasma viral load as measured by quantitative PCR. (B) T cell levels. The average value for SIV-infected RMs (n=3) at the indicated timeframes were computed and shown $\pm$ SEM. (C) Total plasma IgG levels were determined by ELISA. Dots correspond to means and error bars correspond to SD.

Fig. 3.

IgG subclass and V_H gene distribution in RMs evaluated by NGS. (A) NGS strategy. Forward primers were positioned at leader (*) and 5' V_H sequences conserved within the V_H1, V_H3 and V_H4 clades, respectively, whereas reverse primers were positioned at C_γ regions conserved between the IgG subclasses. The 250-bp paired-end reads would not form a contig due to the length, but V_H sequences can be matched to the corresponding C_γ sequence. Both reads yielded an average of 240 bp. The number of reads obtained by NGS for each V_H dataset are shown. (B) IgG1 subclass distribution in uninfected RMs. Independent of V_H clade, IgG1 accounted for over 90% of C_γ reads. Error bars correspond to SD. (C) Baseline V_H gene distribution in uninfected RMs. V_H1, V_H3 and V_H4 genes that accounted for majority (>60%) of read counts were shown in color. (D) Overall changes in V_H gene usage. The average net change from Figure S5 were computed. Error bars correspond to SEM. Data were analyzed using repeated measures ANOVA followed by Tukey's multiple comparison posttest. *, $p<0.05$.

Fig. 4.

Impaired antibody SHM during SIV infection. SHM were calculated dividing the number of mutations relative to germline to the number of bp analyzed $\times 100\%$. The analyses were limited to 41 different V_H genes with sequence data in at least 2 RMs for the 5 time points. The average SHM per RM was calculated for each V_H gene. (A) Baseline (or pre-infection, 0 wpi) total IgG SHM rates in uninfected RMs. Each dot corresponds to a V_H sequence and lines correspond to means. Data were analyzed using a Kruskal-Wallis ANOVA followed by Dunn's multiple comparison posttest. (B) Box-and-whiskers plot showing median and 25–75 percentiles as hinges. Error bars correspond to 10–90 percentile ranges. (C) Paired analysis of SHM in 41 V_H genes at 0 and 24 wpi. Data were analyzed using a paired 2-tailed Student's *t* test. (D) Comparison of SHM in the 3 major V_H clades. Values correspond to mean $\pm$ SEM. Data in panels B and D were analyzed using repeated measures ANOVA followed by Tukey's multiple comparison posttest. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Fig. 5.

Mutations at the AID deamination hotspot. Mutation frequencies were calculated by dividing the number of WRC mutations to the total number of bp analyzed $\times 100\%$. The analyses were limited to 41 different V_H genes with sequence data in at least 2 RMs per time point. The average WRC mutational frequencies per RM were calculated for each V_H gene. (A) Baseline WRC mutation frequencies in 4 uninfected RMs. Each dot corresponds to a V_H sequence and lines correspond to means. Data were analyzed using Kruskal-Wallis ANOVA followed by Dunn's multiple comparison posttest. (B) Box-and-whiskers plot (25–75th percentile) showing median WRC mutational frequencies at defined time points following SIV infection and 10–90 percentile as error bars. The data were analyzed using repeated measures ANOVA, and groups were compared using Tukey's multiple comparison test. (C) Paired analysis of SHM in 41 V_H genes at 0 and 6 wpi. Data were analyzed using a paired 2-tailed Student's *t* test. (D) Comparison of WRC mutational frequencies in the 3 major V_H clades. The data were analyzed using repeated measures ANOVA followed by Tukey's multiple comparison posttest. Values correspond to mean $\pm$ SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Fig. 6.

Mutations at APOBEC3-deamination hotspots. (A–C) Mutation frequencies were calculated by dividing the number of mutations at APOBEC3 deamination hotspots to the total number of bp analyzed $\times 100\%$. To facilitate longitudinal analyses, the dataset was limited to 41 different V_H genes that were represented in at least 2 RMs per time point. The average APOBEC3-type mutations per RM were calculated for each V_H gene. Box-and-whiskers (25–75th percentile) plot showing median (A) TYC and (B) CCC mutational frequencies at defined time points following SIV infection and Tukey error bars. (C) CCC mutations in 24 different V_{H3} genes. Values correspond to mean $\pm$ SEM. The data were analyzed using repeated measures ANOVA followed by Tukey's multiple comparison posttest; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. (D) A3G mRNA expression levels in PBMCs during SIV infection. A3G/GAPDH copies were normalized to 1 at the pre-infection time point per RM.

Fig. 7.

Dinucleotide contexts of G→A and C→T mutations during the course of SIV infection. The box-and-whiskers plot corresponds to the median and 25–75 percentile values, with 10–90 percentiles as error bars. The data corresponds to the aggregate analysis of 41 different V_H genes. (A) Percent G→A and C→T mutations, normalized to the number of mutations $\times$ 100%. The percentage of (B) CC; (C) RC and (D) TC mutations relative to the number of G→A and C→T mutations were computed. These contexts correspond to dinucleotide deamination hotspots for A3G, AID and other APOBEC3 proteins, respectively. The data were analyzed using a repeated measures ANOVA followed by Tukey's multiple comparison posttest; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Fig. 8.

IgG V_{H3} mutational analyses using individualized IgM repertoire sequences. (A) Comparison of IgG versus IgM mutation frequencies. The average mutation frequency for each V_{H3} gene relative to the standard RM gene database was computed. The resulting values were compared using a 2-tailed paired Student's t test. Mutational frequencies at (B) WRC and (C) CCC sites were evaluated using the corresponding IgM repertoire for each RM. The box-and-whiskers (25–75th percentile) plots show medians at defined time points following SIV infection and Tukey error bars. The data were analyzed using repeated measures ANOVA followed by Tukey's multiple comparison posttest. (D) Dinucleotide contexts of G→A and C→T mutations using IgM reference sequences customized for each RM. The percentage of (left) RC and (right) CC mutations relative to the number of G→A and C→T mutations were computed. The data were analyzed using a paired 2-tailed Student's t -test. For all statistical tests, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.