AIDS virus-neutralizing antibody induction reciprocal to a PI3K gain-of-function disease

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Running title: SIV neutralization post-PI3K diminution
Abstract

HIV and simian immunodeficiency virus (SIV) infections are known for impaired neutralizing antibody (NAb) responses. While sequential virus-host B cell interaction appears to be basally required for NAb induction, driver molecular signatures predisposing to NAb induction still remain largely unknown. Here we describe SIV-specific NAb induction following a virus-host interplay reciprocal to a congenital human antibody-dysregulating disease. Screening of seventy neutralization-resistant SIVmac239-infected macaques found nine NAb-inducing animals, with seven selecting for a specific CD8+ T-cell escape mutation in viral nef before NAb induction. This mutation reduced aberrant Nef interaction-mediated drive of B-cell maturation-limiting phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin complex 2 (mTORC2). In vivo imaging cytometry depicted preferential Nef perturbation of cognate Envelope-specific B cells, corroborating cognate B-cell maturation post-mutant selection up to NAb induction. Results exemplify a NAb induction pattern extrinsically reciprocal to human PI3K gain-of-function antibody dysregulation, shaped through two-front, sequential virus interaction with both wings of adaptive immunity.

Keywords
neutralizing antibody; B cells; CD8+ T cells; PI3 kinase; inborn errors of immunity; HIV/SIV
Introduction

Virus-specific neutralizing antibody (NAb) responses by B cells are induced by a tight cooperation of adaptive immune cells (Kumar et al., 2010; Shulman et al., 2014; Giltin et al., 2014; Wang et al., 2014) and often play a central role in clearance of acute viral infections (Junt et al., 2007). In contrast, persistence-prone viruses such as human immunodeficiency virus type 1 (HIV-1), simian immunodeficiency virus (SIV) and lymphocytic choriomeningitis virus (LCMV) variously equip themselves with B cell/antibody-inhibitory countermeasures (Moir et al., 2001; Mattapallil et al., 2005; Sommerstein et al., 2015; Sammicheli et al., 2016; Fallet et al., 2016; Mason et al., 2016) and NAb responses are impaired (Levesque et al., 2009; Mikell et al., 2011). These viruses successfully suppress elicitation of potent NAb responses particularly in acute infection (Hunziker et al., 2003; Tomaras et al., 2008) and establish viral persistence, posing considerable challenge for developing protective strategies.

Reciprocal to this detrimental acute-phase NAb absence, we and others have previously described that acute-phase passive NAb infusion can trigger an endogenous T-cell synergism resulting in robust SIV/SHIV (simian/human immunodeficiency virus) replication control (Haigwood et al., 1996; Yamamoto et al., 2007; Ng et al., 2010; Iseda et al., 2016; Nishimura et al., 2017). This shows that virus-specific NAbs not only confer sterile protection but also can evoke T-cell-mediated non-sterile viral control, suggesting the importance of inducing endogenous NAb responses during an optimal time frame as well as humoral-cellular response synergisms. Therefore, identifying mechanisms driving NAb induction against such viruses is an important step to eventually design NAb-based HIV control strategies.

As an approach, analysis of in vivo immunological events linked with NAb induction against difficult-to-neutralize SIVs in a non-human primate model can provide important insights into this goal. HIV-specific NAb induction patterns and correlates, such as antibody-NAb coevolution (Moore et al., 2012), autoimmune-driven induction (Moody et al.,
2016) and natural killer cell-related host polymorphisms (Bradley et al., 2018) have been variously reported to date; the wealth of knowledge on this topic collectively, and interestingly, rather suggests that pathways to NAb induction against neutralization-resistant viruses including HIV/SIV can be highly redundant and there may be as-yet-unknown in vivo mechanisms that can robustly drive NAb induction. For example, the neutralization resistance of certain SIV strains apparently is not explanatory by the aforementioned, posing such models as attractive analytic targets of NAb induction mechanisms.

In the present study, we examined virus-specific antibody responses in rhesus macaques infected with a highly neutralization-resistant SIV strain, SIVmac239. This virus is highly pathogenic, shows persistent viremia and hosts lack NAb responses throughout infection (Kestler et al., 1991). Yet, upon performing a large-scale screening of SIVmac239-infected rhesus macaques for up to 100 weeks, we noticed a subgroup inducing NAbs in the chronic phase. Interestingly, these animals commonly selected for a specific CD8+ cytotoxic T lymphocyte (CTL) escape mutation in the viral Nef-coding gene before NAb induction. The mutant Nef, compared with wild-type (WT) Nef, manifested a decrease in aberrant interaction with phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin complex 2 (mTORC2), resulting in decreased downstream hyperactivation of the canonical B-cell negative regulator Akt (Omori et al., 2006; Limon et al., 2014). Nef was graphically identified via machine learning-comprising imaging cytometry to preferentially target Env-specific B cells in vivo, and in those macaques inducing NAbs, the NAb induction was linked with sustained Env-specific B-cell responses after the mutant Nef selection. Results describe a B-cell functional boosting reciprocal to human PI3K gain-of-function antibody dysregulation, and suggest that intervening PI3K/mTORC2 signaling can potentially result in harnessing NAb induction against difficult-to-neutralize viruses.
Results

Identification of macaques inducing SIV\textsubscript{mac239}-neutralizing antibodies

We performed a retrospective antibody profile screening in rhesus macaques infected with NAb-resistant SIV\textsubscript{mac239} \((n = 70)\) (Figure 1A) and identified a group of animals inducing anti-SIV\textsubscript{mac239} NAb responses \((n = 9)\), which were subjected to characterization. These NAb inducers showed persistent viremia with no significant difference in viral loads compared with a profile-clarified naïve, major histocompatibility complex class I (MHC-I) haplotype-balanced subgroup of NAb non-inducers \((n = 19)\) representatively utilized for comparison hereafter (Figure 1B and Figure 1-figure supplement 1). Plasma SIV\textsubscript{mac239}-NAb titers measured by 10 TCID\textsubscript{50} SIV\textsubscript{mac239} virus-killing assay showed an average maximum titer of 1:16, being induced at an average of 48 weeks post-infection (p.i.) (Figure 1C). Two of the nine NAb inducers showed detectable NAb responses before six months p.i., while the remaining seven induced NAbs after six months. Anti-SIV\textsubscript{mac239} neutralizing activity was confirmed in immunoglobulin G (IgG) purified from plasma of these NAb inducers (Figure 1-figure supplement 2). SIV Env-binding IgGs developed from early infection in NAb inducers as well as NAb non-inducers (Figure 1-figure supplement 3); titers were not higher but rather lower at year 1 p.i. in the NAb inducers (Figure 1-figure supplement 3B and 3C), consistent with other reports (Havenar-Daughton et al., 2016). NAb inducers and non-inducers showed similar patterns of variations in viral env sequences (Burns et al., 1993), mainly in variable regions 1, 2 and 4 (V1, V2 and V4) (Figure 1-figure supplement 4).

Selection of a viral nef mutation (Nef-G63E) precedes chronic-phase SIV\textsubscript{mac239}-specific NAb induction

Viral nonsynonymous polymorphisms outside env were next assessed. Strikingly, we found selection of a viral genome mutation resulting in G (glycine)-to-E (glutamic acid) substitution at residue 63 of Nef (Nef-G63E) in seven of the nine NAb inducers (Figure 2A). This 63rd residue is in the unstructured N-terminus of Nef, flanked by conserved α-helices
(Figure 2B). This region is conserved in HIV-2/SIV but not in HIV-1, occasionally being deleted in laboratory HIV-1 strains (Geyer et al., 2001). AlphaFold2-based structure prediction did not derive palpable change except for low-probability disruption of the alpha helices (data not shown). The Nef G63E mutation was not observed in two early NAb inducers (with detectable NAbs before 6 months p.i.) but selected in all of the remaining seven inducing detectable NAb responses after 6 months. In contrast, this mutation was found only in two of the nineteen NAb non-inducers, including one rapid progressor (Figure 1-figure supplement 1) and selection was significantly enriched in the NAb inducers (Figure 2C). Analysis of Nef-G63E mutation frequencies in plasma viruses showed that Nef-G63E selection preceded or at least paralleled NAb induction (Figure 2D).

Nef-G63E is a CD8+ T-cell escape mutation

These seven NAb inducers selecting this mutation elicited CD8+ T-cell responses specific for a 9-mer peptide Nef62–70 QW9 (QGQYMNTPW) (Figure 3A). This Nef62–70 QW9 epitope is restricted by MHC-I molecules Mamu-B*004:01 and Mamu-B*039:01 (Evans et al., 1999; Sette et al., 2012). Possession of these accounted for six cases of Nef-G63E selection (Figure 3A), and the remaining one animal possessed Mamu-A1*032:02 predicted to bind to Nef63–70 GW8 (NetMHCpan). Ten of nineteen NAb non-inducers also had either of these alleles (Figure 1-figure supplement 1), indicating that NAb induction is not associated with these MHC-I genotypes ($P = 0.25$ by Fisher’s exact test, data not shown) but with the Nef-G63E mutation itself (Figure 2C). Nef-G63E was confirmed to be an escape mutation from Nef62–70-specific CD8+ T-cell responses (Figure 3B). NAb non-inducers possessing these alleles elicited little or no Nef62–70-specific CD8+ T-cell responses (Figure 3-figure supplement 1A), suggesting that in vivo selection and fixation of this Nef-G63E SIV was indeed Nef62–70-specific CD8+ T cell-dependent. Replication kinetics of SIV carrying the Nef-G63E mutation was comparable with wild-type (WT) on a cynomolgus macaque HSC-F CD4+ T-cell line (Akari et al., 1996), stating that the mutation does not critically impair viral replication (Figure 3-figure supplement 1B), and plasma viral loads were comparable.
between Nef-G63E mutant-selecting NAB inducers versus non-inducers (Figure 3-figure supplement 1C). These somewhat contrasted conditional reversion of a more replication-impairing nef mutation related with CD3 downregulation loss in SIV<sub>mac239</sub> infection (Joas et al., 2020). These results indicate NAb induction following <i>in vivo</i> selection and fixation of the CD8<sup>+</sup> T-cell escape nef mutation, Nef-G63E.

G63E mutation reduces aberrant Nef interaction-mediated drive of PI3K/mTORC2

We next focused on the functional phenotype of this highlighted Nef-G63E mutant SIV in infected cells. An essence of host perturbation by Nef is its wide-spectrum molecular downregulation, ultimately increasing viral replication (Schindler et al., 2004; Zhang et al., 2009). To address possible amelioration, we compared downregulation of major targets CD3, CD4, MHC-I, CXCR4 and BST-2 (Jia et al., 2009) in infected HSC-F cells. Nef-G63E mutation did not confer notable change compared with wild-type (WT) [<i>P</i> = not significant (n.s.) for all molecules, data not shown], implicating other non-canonical changes (Figure 4A). Literature suggested ferritin negative influence on B cells proportionate to HIV-infected macrophage-derived production (Swingler et al., 2008). Here, plasma ferritin showed no differences (Figure 4-figure supplement 1A) and viral loads were comparable between Nef-G63E-selecting NAb inducers and non-inducers (Figure 4-figure supplement 1B and 1C), arguing against gross involvement of ferritin as well as other viral replication-changing Nef phenotypes at least in this model.

Thus, we took interest in immunosignaling-modulating properties of Nef-G63E SIV. Analysis of Akt, the predominant immune-intrinsic negative brake of B-cell maturation and AFC/antibody responses <i>in vivo</i> (Omori et al., 2006; Limon et al., 2014; Fruman et al., 1999; Ray et al., 2015; Sander et al., 2015), revealed that its serine 473 phosphorylation, non-canonically known for Nef-mediated upregulation (Kumar et al., 2016), was significantly lower on day 3 after Nef-G63E SIV infection at low multiplicity of infection (MOI) compared with WT (Figure 4B), stronger than histogram deviation levels in PI3K gain-of-function mice with full disease phenotype (Avery et al., 2018). The threonine 308-phosphorylated Akt (pAkt
Thr308) level was more unaffected (Figure 4-figure supplement 1B).

We further assessed properties of serine 473-phosphorylated Akt (pAkt Ser473) inhibition by Nef-G63E SIV, and found that external PI3K stimuli (serum starvation) (Kennedy et al., 1997) accelerated phenotype appearance from day 3 to day 1 post-infection (Figure 4C, left). A similar trend was obtained by short-term PI3K stimulation with interferon-γ (Nguyen et al., 2001), interleukin-2 (Marzec et al., 2008) and SIV Env (François & Klotman, 2003) (Figure 4-figure supplement 1C). Env-stimulating high-MOI infection also accelerated phenotype appearance, with enhanced 50% reduction (Figure 4C, right). These showed that the Akt-inhibitory G63E mutant Nef phenotype is PI3K stimuli-dependent. Transcriptome analysis signatred decrease in PI3K-pAkt-FoxO1-related genes in mutant SIV infection (Figure 4-figure supplement 1D). As Akt is also a survival mediator this implicates that Nef-G63E mutation may be Th-cytopathic, decreasing Th dysfunctional expansion in LCMV (Recher et al., 2004) and HIV/SIV (Gray et al., 2011; Lindqvist et al., 2012; Petrovas et al., 2012) infections. Here, reduced peripheral CXCR3+CXCR5+PD-1+ memory follicular Th (Tfh) (Locci et al., 2013) was in keeping (Figure 4-figure supplement 1E and 1F).

We further explored molecular traits of this decreased Akt hyperactivation. We reasoned that comparing endogenous Nef binding patterns would be adequate and analyzed SIV-infected HSC-F cells with a flow cytometry-based proximity ligation assay (PLA) (Leuchowius et al., 2009; Avin et al., 2017), by calculating a mean fluorescence intensity (MFI) deviation-based binding index for each. We found that this Nef-G63E mutation causes significant decrease in Nef binding to PI3K p85/p110α and downstream mTORC2 components mTOR (Sarbassov et al., 2005) and GβL/mLST8 (Kim et al., 2003) in the CD4+ SIV Gag p27 infected population (Figure 4D). Collectively, the Nef-G63E mutation results in decreased aberrant Nef bridging and signal transduction of PI3K/mTORC2, explaining decreased substrate Akt Ser473 phosphorylation by mTORC2. Thus, Nef-G63E SIV is a mutant virus decreased in aberrant interaction/drive of B-cell-inhibitory PI3K/mTORC2 signaling. This molecular signature was reciprocal to a recently found human
inborn error of immunity (IEI), activated PI3 kinase delta syndrome (APDS) (Angulo et al., 2013; Lucas et al., 2014; Avery et al., 2018).

Next, we analyzed in vivo targeting of virus-specific B cells by Nef in lymph nodes to explore the potential B-cell-intrinsic influence of the G63E-Nef phenotype. Previously suggested influence of soluble Nef itself (Qiao et al., 2006) and/or related host factors (Swingler et al., 2008) may derive generalized influence on B cells; however, SIV antigen-specific binding antibody responses were rather decreased in NAb inducers (Figure 1-figure supplement 1), viremia was comparable (Figure 3-figure supplement 1C) and ferritin levels were not altered (Figure 4-figure supplement 1A), arguing against these. Therefore, we surmised that some targeted Nef intrusion against Env-specific B cells may be occurring and that the decreased aberrant Nef-PI3K/mTORC2 drive may result in their enhanced lymph node maturation.

While reports (Xu et al., 2009) histologically proposed Nef B-cell transfer, quantitative traits, e.g., invasion frequency and influence on virus-specific B cells, have remained unvisited. Intracellular Nef staining is dim (particularly for B cell-acquired Nef), difficult to examine by conventional flow cytometry. Defining staining cutline requires single-cell images, overcoming confounding of high Nef false-staining via pre-permeation rupture and post-permeation confounding as judged by biologically discontinuous staining and/or batch-inflated signals. Collectively, at the expense of spatial information, we judged that sophisticating imaging cytometry would best visualize Nef-mediated B-cell perturbation in vivo. We analyzed lymph node B cells with Image Stream X MKII, with high-power (> 10-fold) antigen detection [e.g., molecules of equivalent soluble fluorochrome (MESF) 5 vs. MESF 80 in an average flow cytometer for FITC detection] ideal for detection/single-cell verification.

We designed a triple noise cancellation strategy to overcome the above. Firstly, amine reactive dye staining (Perfetto et al., 2006) gated out B cells being Nef-positive due to pre-experimental membrane damage (Figure 5-figure supplement 1A, left). Next, we
deployed secondary quantitative parameters of Nef signals deriving from each pixel of the images. Definition of a gray-level cooccurrence matrix (GLCM) (Haralick et al., 1973) numerating adjacent signal deviation as their frequencies on a transpose matrix enables calculation of a variety of feature values summarizing traits of the whole image. A biological assumption of Nef signal continuity in a true staining suggested that the sum of weighted square variance of GLCM, i.e., the \( \text{sum of} \left[ \text{square of} \ (\text{value} \ - \ \text{average signal strength}) \times \right. \ \text{frequency of each value occurrence} \) would separate natural versus artificial Nef signals. This value, Haralick variance, was computed for multiple directions and averaged, deriving Haralick variance mean (Figure 5-figure supplement 1A, X axis), proportionate with staining image unnaturalness. Finally, Nef signal intensity threshold (Figure 5-figure supplement 1A, Y axis) gated out overtly-stained cells void for true-false verification. These excluded B cells showing non-specific, strong-signal binary-clustered staining pixels for Nef deriving a large summated variance (X axis), and/or cells batch-stained for Nef (Y axis), likely originating from post-experimental membrane damage.

With this approach we acquired images of a Live/Dead–Nef signal Haralick variance mean–Nef signal intensity threshold–Nef\(^{\text{int}}\)CD19\(^{+}\) B-cells, with fine-textured gradation of Nef\(^{\text{int}}\)–staining with continuity from membrane-proximal regions without sporadic staining speckles (Figure 5A and Figure 5-figure supplement 1A). Images resembled Nef-transferred B cells generated \textit{in vitro} by a SIV-infected CD4\(^{+}\) T-cell coculture (Figure 5-figure supplement 1B). A linear discriminant analysis-based machine learning module, scoring segregation of typical void/valid images, showed this gating to provide the highest two-dimensional separation (Figure 5-figure supplement 1A). Combined with biotinylated Env/streptavidin staining, this reproducibly obtained images of Env-specific (Ch 12) CD19\(^{+}\) (Ch 11) B cells without membrane ruptures (Ch 08) and showing fine-textured transferred Nef (Ch 02) (Figure 5B). Nef invasion upregulated pAkt Ser473 (Ch 03) to a range resembling \textit{in vitro} analysis (Figure 5C), stating that Nef-driven aberrant PI3K/mTORC2 signaling does occur in Nef-invaded B cells \textit{in vivo} and the current phenotypic change likely affects B-cell dynamics. Strikingly, lymph node Env-specific B cells showed significantly higher Nef-positive
frequencies as compared with batch non-Env-specific B cells (Figure 5D). This indicated that infected cell-generated Nef preferentially targets adjacent Env-specific B cells, putatively through infected CD4+ cell-to-B-cell contact/transfer (Xu et al., 2009; Hashimoto et al., 2016) and that a Nef phenotypic change likely dictates Env-specific B-cell maturation.

**Enhanced Env-specific B-cell responses after PI3K-diminuting mutant selection**

Finally, peripheral SIV Env-specific IgG+ B-cell responses comprising plasmablasts (PBs) and memory B cells (B_mem) were analyzed to assess *in vivo* B-cell quality in NAb inducers with Nef-G63E mutant selection. After excluding lineage-specific cells [T/NK/pro-B cells/monocytes/myeloid dendritic cells (DCs)/plasmacytoid DCs], we defined IgG+ PBs as showing: high replication (Ki-67+), post-activation (HLA-DR+), transcriptional switching for terminal differentiation (IRF4hi) and downregulated antigen surface binding (surface [s]Envlo-cytoplasmic [Cy]Env+) (Nutt et al., 2015; Silveira et al, 2015). Env-specific PBs were defined as CD3−CD8−CD10−CD14−CD11c−CD123−CD19−CyIgG+Ki-67−IRF4hiHLA-DR+ (total PB) sEnvloCyEnv+ (Env-specific) (Figure 6-figure supplement 1). NAb inducers showed significantly higher Env-specific IgG+ PB responses around week 30 p.i., after Nef-G63E selection, compared to those in NAb non-inducers (Figures 6A and 6B). At 1 year, this difference became enhanced; Env-specific PBs were reduced in non-inducers, while this reduction was ameliorated in the NAb inducers with Nef-G63E.

Concomitant determination of Env-specific IgD−CD27+IgG+ B_mem responses allowed pair-wise analysis of Env-specific IgG+ B_mem/PB as a projection of germinal center (GC) output as an indicator of overall B-cell response quality (Zotos et al., 2010) (Figure 6C, left). In this two-dimensional vector charting of Env-specific B_mem/PB responses, vector protrusion towards upper right reasonably represents higher gross GC output of antibody-forming cells (AFCs) (B_mem/PBs). In NAb non-inducers, all vectors converged on an empirically defined polygonal attractor area $D_n$ (gray area surrounded with dotted lines in Figure 6C, right) at year 1 p.i. and beyond, describing that these NAb non-inducers failed to sustain Env-specific GC output. In contrast, the vectors were consistently tracked outside $D_n$ in the NAb inducers.
with Nef G63E (Figure 6C). At the moment of NAb induction, they converged on another upper-right GC output attractor area $D_i$ (red area surrounded with dotted lines in Figure 6C, right), mutually exclusive with $D_n$ ($P < 0.0001$ by Fisher’s exact test on NAb non-inducer/inducer vector distribution frequency within $D_n$). These suggest more robust virus-specific IgG+ B-cell responses following Nef-G63E CD8+ T-cell escape mutant selection, predisposing to NAb induction. This enhanced cognate B-cell signature was also an inverted pattern of impaired terminally-differentiated B-cell responses in APDS (Al Qureshah et al., 2021).

Taken together, in the current model, Nef62-70-specific CD8+ T-cell responses in persistently SIVmac239-infected macaques selected for an escape mutant, Nef-G63E SIV, which decreased aberrant Nef binding and drive of B-cell-inhibitory PI3K/mTORC2. B-cell Nef invasion in vivo occurred more preferentially in Env-specific B cells, suggesting diminution in tonic Nef-mediated B-cell dysregulation after mutant selection. These predisposed to enhanced Env-specific B-cell responses and subsequent SIVmac239-specific NAb induction, altogether, in a manner reciprocal to human APDS-mediated immune dysregulation.
Discussion

In the present study, we found NAb induction against an NAb-resistant SIV after selection of a CD8+ T-cell escape nef mutant virus. The Nef-G63E mutation, positioned in cognate B cells, results in reduction of binding/drive of PI3K/mTORC2, being concordant with in vivo enhancement of Env-specific IgG+ B cells up to NAb induction following mutant selection. Importantly, this manifested through a pAkt deviation level more vivid than wild-type versus germline PIK3CD gain-of-function mutation heterozygote mice showing full APDS phenotype, stating that the current Nef-G63E-associated B-cell/NAb phenotype likely occurs in a “reciprocal APDS-like” manner. This proposition was enhanced based on extension of immune cell-intrinsic Nef influence on cognate B cells (Fig. 5), in addition to infected T cells. This work is, to our knowledge, the first to interlink a PI3K/mTORC2-modulating viral signature and enhanced B-cell/NAb responses in a viral infection model.

A link between viral T-cell escape and consequent immune modulation has been previously partially explored, including enhanced mutant HIV-1 epitope peptide binding to inhibitory MHC-I receptor on DCs impairing T cells (Lichterfeld et al., 2007) and decreased CTL-mediated lymph node immunopathology rather driving LCMV-specific antibody production (Battegay et al., 1993). Our results now evidence a new pattern of NAb responses bivalently shaped through viral interactions with both humoral and cellular immunity in AIDS virus infection. SIVmac239 infection of macaques possessing MHC-I alleles associated with Nef-G63E mutation can be one unique model to analyze virus-host interaction for B cell maturation leading to NAb induction. A nef polymorphism was extracted, partially appending to a virological impact of Nef (Kirchhoff et al., 1995; Gauduin et al., 2006; Stolp et al., 2012) in perturbing immunity whereas the current phenotype differed from such replication-related properties. The nef region encoding Nef-G63E is not conserved in HIV-1 (Schindler et al., 2004) and beyond this model study it remains to be addressed whether mutant HIV-1 with a similar phenotype can be obtained.

The major in vivo readout of this study is autologous neutralization of a highly resistant
SIV, differing from HIV-specific broadly neutralizing antibodies (bNAbs). Importantly, however, obtained signatures of enhanced IgG⁺ Env-specific AFC B cells (class switch/terminal differentiation) and virus neutralization (hypermutation) directly evidence enhanced activity of activation-induced cytidine deaminase (AID), the canonical positive driver of B-cell fate, showing a strong conceptual continuity with bNAb regulation by AID.

Our results extracted an in vivo link between a decreased aberrant Nef-PI3K/mTORC2 interaction and major enhancement in B-cell responses. The relationship is indeed reciprocal to immunogenetic mechanisms of human PI3K gain-of-function mutations resulting in APDS with multiply impaired anti-viral B-cell responses. While the exact hierarchy of binding between Nef and PI3K/mTORC2 components (including PI3K isoforms) and its perturbation by Nef-G63E mutation remains much to be investigated, PI3Kp85 did reasonably show the strongest Nef-binding index in PLA assay (Figure 4D) as Nef and PI3Kp85 possess SH3-binding PxxP and SH3 domains, respectively (Rickles et al., 1994). Binding decrease in canonical partner PI3Kp85 may domino-trigger decrease with others, resulting in quadruple PI3K/mTORC2 binding decrease and fragile downstream Akt signaling.

In conclusion, we exemplify in a non-human primate AIDS model that NAb induction against a highly resistant SIV occurs after selection of a CD8⁺ T-cell escape variant reduced in excess PI3K/mTORC2 drive. These results provide an understanding of how immunodeficiency virus-specific NAb responses can be shaped by both wings of adaptive immune pressure and depicts that immune cell-intrinsic PI3K/mTORC2 manipulation may be pivotal for harnessing antiviral NAb responses. Furthermore, search of other analogies between viral immune dysregulations and human IEIs may lead to identification of novel targets to modulate and harness immune responses in translational settings.

Limitations of study

A limitation of this study is the use of retrospective samples, posing constraints for detecting exact temporal changes in tonic viral B-cell perturbation. Related with this, sampling of lymph node cells in animals belonging to the subgroup of interest at the optimal moment was
unpredictable. Nevertheless, usage of such specific animal models does help to depict a
temporal cascade of *in vivo* events leading to NAb induction, assisting human immunology.
As future extension of this study extracting the NAb-involved molecular axis,
manipulation/reconstitution experiments shall be designed.
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Author Contributions

H.Y. conceived/designed/performed study and wrote manuscript; T.M. provided animal cohort, co-edited manuscript and co-directed study.

Declaration of Interests

The authors declare no competing interests.
Figure Legends

Figure 1. NAb induction against NAb-resistant SIV<sub>mac239</sub>.

(A) Study design.

(B) Plasma viral loads (SIV <i>gag</i> RNA copies/ml plasma) in NAb non-inducers (left) and inducers (right). Viral loads have been previously partially reported (Yamamoto et al., 2007; Nomura et al., 2012; Takahashi et al., 2013; Nakane et al., 2013; Iwamoto et al., 2014).

(C) Plasma SIV<sub>mac239</sub> 100% neutralizing end point titers by 10 TCID<sub>50</sub> killing assay on MT4-R5 cells. Points on the dotted line show marginally NAb-positive results (<1:2). In some animals, titers were comparable with our reported results using MT4 cells (Kawada et al., 2007).

Figure 2. Selection of a viral <i>nef</i> mutation (Nef-G63E) before NAb induction.

(A) Viral <i>nef</i> mutations in NAb inducers. A linear schema indicating Nef functional domains is aligned above. Mutations at timepoints with NAb<sup>-</sup> (indicated by white wedge; mostly before 6 months p.i.), NAb<sup>±/−</sup> (green wedge) and NAb<sup>+</sup>) (purple wedge; mostly after 1 year) are shown in individual animals. Black and dark gray represent dominant and subdominant mutations (or deletion in R-360) by direct sequencing, respectively. Red and pink indicate dominant and subdominant G63E detection, respectively.

(B) Schema of SIV<sub>mac239</sub> Nef structure and Nef-G63E mutation orientation.

(C) Comparison of frequencies of macaques having Nef-G63E in plasma viruses between NAb non-inducers and inducers. Compared by Fisher’s exact test.

(D) Temporal relationship of Nef G63E frequencies in plasma virus and NAb induction. Black boxes (left Y axis) show log<sub>2</sub> NAb titers; red triangles and green diamonds (right Y axis) show percentage of G63E and G63R mutations detected by subcloning (15 clones/point on average), respectively.

Figure 3. Nef-G63E is a CD8<sup>+</sup> T-cell escape mutation.

(A) Nef<sub>62–70</sub> QW9-specific CD8<sup>+</sup> T-cell frequencies and related MHC-I alleles in seven NAb
inducers selecting Nef-G63E. Mamu-B*039:01 and Mamu-B*004:01 are known to restrict Nef$_{62-70}$ QW9 epitope (Evans et al., 1999; Sette et al., 2012) and binding of Nef$_{63-70}$ peptide to Mamu-A1*032:02 was predicted. (B) CD8$^+$ T-cell responses specific to wild-type Nef$_{62-70}$ or mutant Nef$_{62-70}$G63E or Nef$_{62-70}$G63R peptides. See also Figure S3.

Figure 4. Nef-G63E mutation reduces PI3K/mTORC2 binding and pAkt drive.

(A) Representative surface expression level histograms of CD3, CD4, MHC class I, CXCR4 and BST-2 in CD4$^+$Nef$^+$ subpopulations after wild-type (WT) or Nef-G63E mutant SIV infection at multiplicity of infection (MOI) 0.1 on HSC-F cells.

(B) Left: representative histograms of relative pAkt serine (Ser) 473 levels in p27$^+$ subpopulations after WT or Nef-G63E mutant SIV infection at MOI 0.1 on HSC-F cells. Numbers show pAkt Ser473 mean fluorescence intensities (MFIs) for each. Right: Deviation of pAkt Ser473 MFIs in p27$^+$ HSC-F cells compared with mean MFI of uninfected cells. Compared by unpaired t test.

(C) Left: Relative pAkt Ser473 levels (normalized to mean MFI of uninfected controls) in Nef$^+$ HSC-F cells assessed for serum starvation (MOI 0.2, 1 day p.i.). Adjusted $P$ values show results of comparison via Sidak's post-hoc test of 2-way ANOVA (C, left). Right: Deviation of pAkt Ser473 MFIs in Nef$^+$ HSC-F cells assessed for high-MOI infection (MOI 5, 1-day p.i.). Compared by unpaired t test.

(D) Proximity ligation assay (PLA) of Nef binding with mTOR, G$\beta$/mLST8, PI3Kp85 and PI3Kp110$\alpha$. MFI-based binding index was calculated as (anti-Nef/anti-partner) - (isotype/anti-partner) - (anti-Nef/isotype) + (isotype/isotype). Histograms for samples and isotype/isotype are representatively shown. Differences in MFI binding indexes can be enhanced compared with comparison of raw MFIs. Compared by paired t tests. Data represent one of two [(A); (B); (C, right)] independent experiments in quadruplicate, four independent experiments performed in triplicate [(C, left)] or four independent single-well comparison experiments pooled for statistical analysis (D). Bars: mean ± SD (B, C right),
mean ± SEM (C left).

**Figure 5. Preferential targeting of lymph node Env-specific B cells by Nef in vivo.**

(A) Representative gating of triple noise-cancellation in vivo Nef staining in B cells analyzed by ImageStreamX MKII. Pre-experimental damaged cells are first excluded with Live/Dead from focused/centroid/singlet image-acquired CD19+ B cells (first lane right/R4 gated on “Focused”). Following Nef+ gating (second lane left/R5 gated on R4), a second step of Nef noise cancellation (second lane middle/R7 on R5) comprises double-negative removal of post-experimental stochastic irregular staining building a disparate intracellular staining gradient (X axis, Nef signal pixel Haralick variance mean) and post-experimental batch overt cellular staining (Y axis, Nef signal pixel intensity threshold). This outputs a B-cell population with a fine-textured pericellular Nef\textsuperscript{st-lo} staining, biologically concordant with Nef membrane-anchoring. Probing of anti-Env BCR (αEnv) by recombinant SIV Env (second lane, right) is combined, resulting in a 2-D panel of intracellular Nef versus αEnv for noise-cancelled Nef+ B cells (R7) plus Nef− B cells (R8) (third lane left/“R7+R8”). pAkt Ser473 expression (third lane right) and cellular morphology (B) was further analyzed. DN, double-negative.

(B) Typical images of Nef-transferred Env-specific B cells defined αEnv+-intracellular Nef+–Nef Haralick variance mean–Nef Intensity threshold–Live/Dead–CD19+ cells [“Nef+aEnv+” population of lower left panel in (A), gated on “R7, R8”]. Note the pericellular pAkt Ser473 upregulation in these cells (Ch 03/yellow). Data on inguinal lymph node lymphocytes of macaque R10-007 at week 62 post-SIV\textsubscript{mac239} infection are shown in (A) and (B).

(C) Comparison of pAkt Ser473 median fluorescence (medFI) intensity levels in Nef− B cells (R8) versus noise-cancelled Nef+ B cells (R7). Analyzed by paired t test.

(D) Comparison of Nef-positive cell frequencies in non-Env-specific (left) versus Env-specific (right) B cells in lymph nodes of persistently SIV-infected macaques (n = 6). Analyzed by paired t test.
Figure 6. Enhanced SIV Env-specific B-cell output up to NAb induction following Nef-G63E selection.

(A) Changes in SIV Env gp140-specific plasmablast (PB) frequencies in viremic NAb non-inducers (left, n = 12) and Nef-G63E-selecting NAb inducers (right, n = 6). Available samples of twelve viremic NAb non-inducers (including ten with ≥ 1-year survival) and six NAb inducers were tracked. In the right panel, the frequencies in NAb non-inducers are shown in background (gray) for comparison.

(B) Comparison of Env gp140-specific PB frequencies between NAb non-inducers and inducers by Mann Whitney U tests. In the right, the frequencies at NAb induction were compared with those at year 1 in NAb non-inducers with ≥ 1-year survival (n = 10). Bars: mean ± SD.

(C) Left: vector chart of Env gp140-specific memory B cell (B_{mem}) and PB levels. Legends for each animal correspond to the ones in (B). Right: NAb non-inducer vectors empirically define a polygonal GC output attractor area $D_n$ (gray area surrounded with dotted lines) on which they converge by and beyond year 1 p.i. The NAb inducer vectors (shown up to the time of NAb induction) remained outside of $D_n$. At the moment of NAb induction they converged on a second GC output attractor area $D_i$ (red area surrounded with dotted lines), mutually exclusive with $D_n$ ($P < 0.0001$ by Fisher’s exact test on NAb inducer vector convergence frequency within $D_n$). Legends for year 1 p.i. in the NAb non-inducers and moment of NAb induction in the NAb inducers are specified.
Materials and Methods

Material availability
This study did not generate new unique reagents.

Data and code availability
Data were analyzed using existing computational packages. The accession number for the transcriptome data [wild-type SIVmac239-infected HSC-F cell line ($n = 3$), Nef-G63E SIVmac239-infected HSC-F cell line ($n = 3$) and uninfected HSC-F cell line ($n = 3$)] has been deposited under the accession number GenBank: GSE65806.

Rhesus macaques retrospectively utilized for samples
Seventy Burmese rhesus macaques (*macaca mulatta*) (57 males and 13 females) were retrospectively analyzed in this study. Experiments were previously carried out (Matano et al., 2004; Yamamoto et al., 2007; Iseda et al., 2016; Nomura et al., 2012; Ishii et al., 2012; Takahashi et al., 2013; Nakane et al., 2013; Shi et al., 2013; Iwamoto et al., 2014; Terahara et al., 2014) in the Tsukuba Primate Research Center, National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN) with the help of the Corporation for Production and Research of Laboratory Primates and the Institute for Frontier Life and Medical Sciences, Kyoto University (IFLMS-KU) after approval by the Committee on the Ethics of Animal Experiments of NIBIOHN and IFLMS-KU under the guidelines for animal experiments at NIBIOHN, IFLMS-KU and National Institute of Infectious Diseases in accordance with the Guidelines for Proper Conduct of Animal Experiments established by Science Council of Japan (http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf). The experiments were in accordance with the "Weatherall report for the use of non-human primates in research" recommendations (https://royalsociety.org/topics-policy/publications/2006/weatherall-report/).

Animals were housed in adjoining individual primate cages allowing them to make sight and sound contact with one another for social interactions, where the temperature was kept at
25°C with light for 12 hours per day. Animals were fed with apples and commercial monkey
diet (Type CMK-2, Clea Japan, Inc.). Blood collection and virus challenge were performed
under ketamine anesthesia.

Cells and viruses
MT4-R5 cells were maintained in RPMI1640 (Invitrogen) supplemented with 10% fetal bovine
serum (FBS) (Clontech) and antibiotics. HSC-F cells (cynomolgus CD4⁺ T-cell line) and
HSR5.4 cells (rhesus macaque CD4⁺ T-cell line) were maintained in RPMI1640 (Invitrogen)
supplemented with 10% fetal bovine serum (FBS) (Clontech), human interleukin-2 (IL-2) (10
IU/ml, Roche), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Invitrogen) and
2-mercaptoethanol (Gibco). SIVmac239 molecular clone derivative was generated by
mutagenesis PCR (Agilent Technologies) using the primers listed. For virus preparation,
COS-1 cells were transfected with pBRmac239 proviral DNA using FuGENE 6 (Promega). At 48
h post-transfection, culture supernatants were harvested, centrifuged and filtered through a
0.45-μm pore-size filter (Merck Millipore). To titrate infectivity, prepared viruses were serially
diluted and infected on HSC-F cells in 96-well plates (Falcon) in quadruplicate. At 10 days
post-infection, the endpoint was determined using SIV p27 antigen enzyme-linked
immunosorbert assay (ELISA) kit (ABL), and virus infectivity was calculated as the 50
percent tissue culture infective dose (TCID₅₀) according to the Reed-Muench method.

Identification of SIVmac239-NAb inducers
Burmese rhesus macaques previously challenged with the highly pathogenic molecular clone
virus SIVmac239 (n = 70) were retrospectively examined approximately up to 2 years for their
plasma NAb profiles. Animals were challenged intravenously with 1,000 TCID₅₀ of SIVmac239-
In the current study, virological and immunological profiles were compared between the newly
identified NAb inducers (n = 9) and representative NAb non-inducers (n = 19). These NAb
non-inducers and eight NAb inducers except R06-032 were previously partially reported for
their plasma viral loads (Yamamoto et al., 2007; Iseda et al., 2016; Nomura et al., 2012;
Takahashi et al., 2013; Nakane et al., 2013; Iwamoto et al., 2014) (Figure 1B). MHC-I haplotypes and alleles were determined by reference strand-mediated conformation analysis, PCR-SSP (PCR amplification utilizing sequence-specific priming) and cloning as described (Naruse et al., 2010). MHC-I binding prediction was made on April 27, 2013 using the IEDB analysis resource NetMHCpan tool (Hoof et al., 2009). Alleles of interest in the study have been previously identified in macaques (Nomura et al., 2012; Evans et al., 1999; Sette et al., 2012). Macaques R06-032, R03-015, R01-010 and R05-010 received a prime-boost vaccination (Matano et al., 2004) composed of a DNA prime/intranasal Sendai virus vector expressing SIV<sub>mac239</sub> Gag (SeV-Gag). R03-015, R06-019, R06-038 and R10-001 received 300 mg of non-specific rhesus IgG at day 7 post-SIV<sub>mac239</sub> challenge as an experimental control in our previous reports (Yamamoto et al., 2007; Iseda et al., 2016; Nakane et al., 2013).

**Plasma viral load quantitation**

Plasma viral RNA samples were extracted with High Pure Viral RNA kit (Roche Diagnostics). Serial fivefold sample dilutions were amplified in quadruplicate by reverse transcription and nested PCR using SIV<sub>mac239</sub> gag-specific primers to determine end point via the Reed-Muench method as described previously (Matano et al., 2004; Iseda et al., 2016). The lower limit of detection is approximately 400 viral RNA copies/ml plasma.

**SIV<sub>mac239</sub>-specific neutralization assay**

NAbs were titrated as described (Yamamoto et al., 2007; Iseda et al., 2016). Serial twofold dilutions of heat-inactivated plasma, or polyclonal IgG affinity-purified with Protein G Sepharose 4 Fast Flow (GE Healthcare) from heat-inactivated and filtered plasma were mixed with 10 TCID<sub>50</sub> of SIV<sub>mac239</sub> at a 1:1 ratio (5 μl:5 μl) in quadruplicate. After 45-min incubation at room temperature, the 10 μl mixtures were added into 5 x 10<sup>4</sup> MT4-R5 cells/well in 96-well plates. Progeny virus production in day 12 culture supernatants was examined by SIV p27 ELISA (ABL) to determine 100% neutralizing endpoint. The lower limit of titration is
Neutralization in three out of four wells at a dilution of 1:2 is defined as marginally NAb-positive (< 1:2). Results were comparable when the same assay was performed with macaque HSC-F cells (Akari et al., 1996) as targets. NAb inducers R01-012, R02-004, R01-010, R02-007 and NAb non-inducer R01-011 were previously partially reported for their NAb titers measured with the same method using MT4 cells as targets (Kawada et al., 2007), which derived comparable results. For assessment of neutralizing activity in IgG, SIV\textsubscript{mac239}-specific IgGs purified from pools of plasma with SIV\textsubscript{mac239}-specific NAb titers were obtained from each animal as described (Yamamoto et al., 2007). After complement heat-inactivation at 56°C, 30 min and 0.45 μm filtration, IgGs were purified by Protein G Sepharose 4 Fast Flow (GE Healthcare) and concentrated by Amicon Ultra 4, MW 50,000 (Millipore) to 30 mg/ml and similarly examined for their 10 TCID\textsubscript{50} SIV\textsubscript{mac239} killing titers on MT4-R5 cells.

**SIV Env-specific IgG ELISA and immunoblotting**

Plasma Env-specific IgG titers were measured as described (Nakane et al., 2013). SIV\textsubscript{mac251} Env gp120 (ImmunoDiagnostics) were coated on 96-well assay plates (BD) at 1000 ng/ml (100 μl/well). Wells were prewashed with phosphate-buffered saline (PBS), blocked with 0.5% bovine serum albumin (BSA)/PBS overnight and plasma samples were incubated at a 1:20 dilution (5 μl:95 μl) for 2 hours. Wells were washed with PBS and SIV Env-bound antibodies were detected with a horseradish peroxidase (HRP)-conjugated goat anti-monkey IgG (H+L) (Bethyl Laboratory) and SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL). Absorbance at 450 nm was measured. Samples from week 0 pre-challenge and month 3, month 6 and year 1 post-challenge were assessed in duplicate. Week 0 average values were subtracted from corresponding later time point values for calibration.

For immunoblotting, SIV virion-specific IgGs in plasma were detected with a SIV\textsubscript{mac239}-cross-reactive western blotting system (ZeptoMetrix). In the NAb non-inducers, samples from those showing rapid progression (euthanized due to AIDS progression within
approximately 1 year) with low plasma anti-SIV reactivity (Nakane et al., 2013) were not included.

Sequencing
Sequencing was performed as described (Matano et al., 2004; Iseda et al., 2016). Viral cDNA fragments spanning from nt (nucleotide) 4829 to nt 7000, nt 6843 to nt 8831 and nt 8677 to nt 10196 in SIV_{mac239} (GenBank accession number MM33262) covering SIV env and nef were amplified from plasma viral RNA by nested RT-PCR using Prime-Script one-step RT-PCR kit v2 (TaKaRa) and KOD-Plus v2 (Toyobo). PCR products were either directly sequenced or subcloned with a TOP10-transforming TOPO blunt-end cloning system (Invitrogen). Sequencing was performed using dye terminator chemistry with an ABI 3730 DNA sequencer (Applied Biosystems). On average, 15 clones were obtained per sample and 20 clones were assessed when nef mutations of interest in early time points were subdominant.

SIV_{mac239}-specific CD8^{+} T-cell responses
Virus-specific CD8^{+} T-cell frequencies were measured as described (Matano et al., 2004; Iseda et al., 2016). Peripheral blood mononuclear cells (PBMCs) were cocultured for 6 hours with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCLs) pulsed with Nef peptides (Sigma-Aldrich Japan) at 1 \mu M concentration or as indicated otherwise under GolgiStop (monensin, BD) presence. Intracellular gamma interferon (IFN-\gamma) staining was performed using Cytofix/Cytoperm kit (BD) and the following conjugated anti-human monoclonal antibodies (mAbs): anti-CD4-FITC (M-T477, BD Pharmingen), anti-CD8-PerCP (SK1, BD Biosciences), anti-CD3-APC (SP34-2, BD Pharmingen) and anti-IFN-\gamma-PE (4S.B3, BioLegend). Specific CD8^{+} T-cell frequencies were determined by subtracting nonspecific IFN-\gamma^{+} CD8^{+} T-cell frequencies from those after peptide-specific stimulation; frequencies beneath 100 cells/million PBMCs were considered negative. Cells acquired by FACS Canto II (BD) were analyzed by FACS Diva (BD) and FlowJo (Treestar). Approximately 1 \times 10^{5}
PBMCs were gated per test.

**Nef-mediated signaling perturbation analysis**

Virus supernatants obtained from COS-1 cells after transfection with wild-type or mutant SIV<sub>mac239</sub> molecular clones were used for infection of CD4<sup>+</sup> T-cell lines, cynomolgus macaque-derived HSC-F and rhesus macaque-derived HSR5.4 (Akari et al., 1996). QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) was used to construct a mutant SIV<sub>mac239</sub> molecular clones possessing a nef mutation Nef G63E (G-to-A mutation at nt 9520) from the wild-type SIV<sub>mac239</sub> molecular clone (Kestler et al., 1991) (nt number from GenBank accession number M33262). Cells (1 x 10<sup>5</sup> cells/well in U-bottomed 96-well culture plates [BD]) were infected with wild-type or mutant SIV<sub>mac239</sub> cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) for indicated periods at MOI 0.1 (intracellular signaling analysis) or 0.001 (supernatant analysis). Culture supernatants were subjected to measurement of SIV capsid p27 concentrations by ELISA. Harvested cells were fixed and permeabilized with Cytofix/Cytoperm kit, washed twice and subjected to immunostaining. The following antibodies were used; anti-SIV<sub>mac251</sub> Gag p27 mAb (ABL) manually conjugated with Alexa 647 (Life Technologies), anti-SIV<sub>mac251</sub> Nef mAb (clone 17, epitope peptide corresponding to SIV<sub>mac239</sub> Nef 71-80 not including the residue G63: Thermo Scientific/Pierce) manually conjugated with Alexa 488 or Alexa 647, anti-human CD3-APC-Cy7 (SP34-2, BD Pharmingen), anti-human CD4-PerCP (L200, BD Pharmingen), anti-human HLA-ABC-FITC (G46-2.6, BD Pharmingen), anti-human CXCR4-APC (12G5, BioLegend), BST2-Alexa 647 (RS38E, BioLegend), Alexa 488-conjugated anti-phospho-Akt (Ser473) (D9E, CST) and Alexa 488-conjugated anti-phospho-Akt (Thr308) (C31E5E, CST).

Cells acquired by FACS Canto II were analyzed by FACS Diva and FlowJo. Approximately 5 x 10<sup>4</sup> cells were gated for each test.

**Ferritin ELISA**

Plasma ferritin levels in NAb inducers and control animals were analyzed by monkey ferritin
sandwich ELISA kit (LS Bio) according to the manufacturer’s instructions.

PI3K stimulation assay

5 x 10⁴ HSC-F cells were infected with wild-type or Nef G63E mutant SIVmac239 at MOI 0.2 and cultured for 1 day in medium supplemented with 10% (normal), 1% (1/10 starvation) or 0.1% (1/100 starvation) FBS. For ligand stimulation (Figure 4-figure supplement 1C), cells at the end of 1-day culture were pulsed for 20 minutes with 40 ng/ml of recombinant human IFN-γ (Gibco/Thermo Fisher), 100 IU/ml of recombinant human IL-2 (Roche Diagnostics) or 10 μg/ml of SIVmac251 Env gp130 (ImmunoDx). Cells were intracellularly stained using Cytofix/Cytoperm kit with anti-SIVmac251 Nef mAb or anti-SIVmac251 p27 mAb manually conjugated to Alexa 647 and PE-conjugated anti-human phospho-Akt (Ser473) (D9E, CST) or Alexa 488-conjugated anti-human phospho-Akt (Ser473). Cells acquired by FACS Canto II were analyzed by FACS Diva and FlowJo. Approximately 7 x 10⁴ HSC-F cells were gated per test.

Transcriptome analysis

Total RNAs were extracted using RNeasy Plus Mini kit (Qiagen) from 2 x 10⁶ HSC-F cells 1 day after infection with wild-type or Nef G63E mutant SIVmac239 at MOI 5. Negative control RNA samples were extracted from 2 x 10⁶ uninfected HSC-F cells after culture with the same condition. Three sets of experiments were performed. Total RNA samples were subjected to a quality control (QC) analysis using an Agilent 2100 Bioanalyzer. The obtained amounts of total RNAs were: 12.37 ± 0.39 (uninfected), 8.80 ± 0.44 (wild-type) and 9.02 ± 0.17 (Nef G63E) μg (P = 0.66 for wild-type versus Nef G63E by unpaired t test). In all samples, two bands of 18S and 28S rRNA were confirmed and the RNA integrity number (RIN) was 10. 500 ng of total RNA samples were processed with GeneChip WT Plus reagent (Affymetrix/Thermo Fisher Scientific) to produce 150 μl of fragmented and labeled cDNA samples. These were incubated with a Human Gene 2.0 ST Array (Affymetrix/Thermo Fisher...
Peripheral CD4+ T-cell surface staining

Cryopreserved/thawed PBMCs were stained for 30 minutes at 4°C with the following reagents or conjugated anti-human mAbs: Live/Dead Aqua (Life Technologies), anti-CD4-PerCP (L200, BD Pharmingen), anti-CD8-APC-Cy7 (RPA-T8, BD Pharmingen), anti-CD3-Alexa 700 (SP34-2, BD Pharmingen), anti-CD95-PE-Cy7 (DX2, eBioscience), anti-CXCR5-PE (87.1, eBioscience), anti-PD-1-Brilliant Violet 421 (EH12.2H7, BioLegend) and anti-CXCR3-Alexa 488 (G025H7, BioLegend). Cells acquired by FACS LSRII Fortessa (BD) were analyzed by FACS Diva and FlowJo. Approximately 1.5 x 10^5 PBMCs were gated per test.

Proximity ligation assay (PLA)

A flow cytometry-based arrangement of proximity ligation assay (PLA) (Avin et al., 2017) was performed to quantitatively assess Nef binding to candidate interacting molecules. 1 x 10^5 HSC-F cells were infected with SIV_{mac239} at MOI 0.05 in U-bottomed plates and permeated with Cytofix/Perm kit (BD Biosciences). After two washes, they were resuspended in 0.5% BSA/PBS for prevention of experimental procedure-related loss and stained with mouse anti-SIV_{mac251} Nef mAb (clone 17, Thermo Scientific/Pierce) or mouse IgG1 isotype control.
mAb (P3.6.2.8.1, Abcam) in combination with either of the following rabbit antibodies: anti-GßL (86B8, CST), anti-mTOR (7C10, CST), polyclonal anti-human PI3K p85 (Merck Millipore), anti-PI3 Kinase p110α (C73F8, CST) or rabbit IgG1 isotype control mAb (DA1E, CST). Antibody-stained cells were subsequently probed with Duolink In Situ PLA Probe anti-mouse PLUS and anti-mouse MINUS probes (Sigma/Merck). Next, they were detected for intermolecular binding using Duolink flow PLA detection kit (Green) (Sigma/Merck) with a reaction time of 100 minutes for post-mouse/rabbit probe linking amplification. Finally, these PLA-subjected cells were additionally stained with anti-SIVmac251 Gag p27 mAb manually conjugated with Alexa 647 and anti-CD4-PerCP for 30 minutes for 20 minutes at 4°C. Cells acquired by FACS Canto II were analyzed by FACS Diva and FlowJo. Approximately 1 x 10^5 cells were gated per test. Binding index (Y axis) was calculated by deriving the deviation from the summation of: {baseline P: (MFI of background reaction with mouse isotype control/rabbit isotype control) + anti-Nef antibody-derived background Q: [(MFI of reaction with mouse anti-Nef/rabbit isotype control) - (MFI of background reaction with mouse isotype control/rabbit isotype control)] + anti-binding partner antibody-derived background R:[(MFI of reaction with mouse isotype control/rabbit anti-binding partner molecule) - (MFI of background reaction with mouse isotype control/rabbit isotype control)]}.

**B-cell Nef invasion in vitro assay**

For Nef invasion inhibition assay, 5 x 10^4 HSC-F cells were infected at MOI 1 for 48 hours. These were then additionally cocultured with 7.5 x 10^4 Ramos B cells for 12 hours. Ramos B cells were double-stained with Alexa 488-conjugated Nef and anti-CD19-PC-5.5 (J3-119, Beckman Coulter), and interacting infected cell-invaded B cell doublet images acquired with ImageStream X MKII (Amnis/Merck Millipore) were analyzed by IDEAS 6.3 (Amnis/Merck Millipore/Luminex).

**Quantitative in vivo imaging flow cytometry**
Cryopreserved/thawed lymph node cells (LNCs) from several persistently SIV-infected macaques used in previous experiments (Nakane et al., 2013; Takahashi et al., 2013; Iwamoto et al., 2014) were seeded in V-bottomed 96-well plates (Nunc) and blocked with 25 μg/ml of anti-human CD4 (clone L200: BD) in 100 μl volume for 15 minutes at 4°C. After three washes, they were stained with 10 μg/ml of recombinant SIVmac239 Env gp140-biotin (Immune Technology) for 30 minutes at 4°C. Cells were then stained with anti-CD19-PC5.5 (J3-119, Beckman Coulter), Live/Dead Aqua (Invitrogen) and streptavidin-APC-Cy7 (BioLegend) for 30 minutes at 4°C. After two washes, cells were processed with Cytofix/Cytoperm kit (BD) for 20 minutes at 4°C, washed twice, and next intracellularly stained with PE-conjugated anti-human phospho-Akt (Ser473) (D9E, CST) and anti-SIVmac251 Nef mAb (clone 17, Thermo Scientific/Pierce) manually conjugated with Alexa 488 or mouse IgG1 isotype control mAb (P3.6.2.8.1, Abcam) for 30 minutes at 4°C. After final two washes, cells were suspended in 0.8% PFA/PBS. All centrifugations for washing (1,200 x g, 2 minutes) were performed at 4°C. Cells were subjected to image acquisition with Image Stream X MKII imaging flow cytometer (Amnis/Merck Millipore/Luminex) and analyzed with IDEAS 6.3 software (Amnis/Merck Millipore/Luminex). Approximately 5 x 10^5 LNCs were acquired for analyses (Figure 5). A custom-implemented linear discriminant analysis-based machine learning module (Luminex) was utilized for verifying candidate Nef staining signal-related secondary parameters (Figure 5 and Figure 5-figure supplement 1A) for their efficacy of target population separation.

Peripheral SIV Env-specific B-cell responses

Peripheral SIV Env gp140-specific memory B cells (B_{mem}) and plasmablasts (PBs) were measured by flow cytometry with procedures modified from previous reports and our experience (Silveira et al., 2015; Hau et al., 2022). First, cryopreserved/thawed PBMCs seeded in V-bottomed 96-well plates (Nunc) were blocked with 25 μg/ml of anti-human CD4 (clone L200: BD) in 100 μl volume for 15 minutes at 4°C. This concentration attains blockade of promiscuous SIV Env binding to CD4 comparable to levels by magnetic depletion of CD3^+.
T cells (data not shown). After three washes, they were next stained with 10 μg/ml of recombinant SIV<sub>mac239</sub> Env gp140-biotin (Immune Technology) for 30 minutes at 4°C. They were subsequently stained with the following anti-human antibodies and fluorochromes for 30 minutes at 4°C with combinations as follows; PB/B<sub>mem</sub>: anti-CD3-APC-Cy7 (SP34-2, BD Pharmingen), anti-CD8-APC-Cy7 (RPA-T8, BD Pharmingen), anti-CD14-APC-Cy7 (M5E2, BioLegend), anti-CD16-APC-Cy7 (3G8, BioLegend), streptavidin-Brilliant Violet 421 (BioLegend), anti-CD19-PC5.5 (J3-119, Beckman Coulter) and Live/Dead Aqua (Invitrogen); PB: anti-CD10-APC-Cy7 (HI10a, BioLegend), anti-CD11c-PE-Cy7 (3.9, BioLegend), anti-CD123-PE-Cy7 (6H6, BioLegend) and anti-HLA-DR-PE-Texas Red (TU36, Invitrogen); B<sub>mem</sub>: anti-CD27-PE/Dazzle 594 (M-T271, BioLegend), anti-CD10-PE-Cy7 (HI10a, BioLegend), anti-IgD-FITC (DaKo), anti-CD38-Alexa 647 (AT1, Santa Cruz Biotechnology), anti-IgG-Alexa 700 (G18-145, BD Pharmingen) and anti-CD138-PE (DL-101, eBioscience).

After two washes, B<sub>mem</sub> samples were suspended in 0.8% PFA/PBS. For PB staining, surface-stained cells were further processed with Cytofix/Cytoperm kit (BD) for 20 minutes at 4°C, washed twice, and next intracellularly stained with 10 μg/ml of recombinant SIV<sub>mac239</sub> Env gp140-biotin (Immune Technology) for 30 minutes at 4°C. After three washes, they were next stained with anti-human IgG-PE (G18-145, BD), anti-human/mouse IRF4-eFluor 660 (3E4, eBioscience), anti-human Ki67-Alexa 700 (B56, BD Pharmingen) and streptavidin-Alexa 488 (BioLegend) for 30 minutes at 4°C. After final two washes, cells were suspended in 0.8% PFA/PBS. Cells acquired with FACS LSRII Fortessa were analyzed with FACS Diva and FlowJo. Approximately 1.5 x 10<sup>5</sup> PBMCs were acquired for B<sub>mem</sub> and 4 x 10<sup>5</sup> PBMCs were acquired for PB analyses (Figure 6). All centrifugations for washing (1,200 x g, 2 minutes) were performed at 4°C. Env-specific PB frequencies were 0 cells/million PBMCs for pre-challenge samples in all animals examined, ruling out background confounding for high-sensitivity quantitation of the population.

**Statistical Analysis**
Analyses were performed via Prism 8 (GraphPad Software). $P < 0.05$ were considered significant in two-tailed unpaired t tests, paired t tests, Mann Whitney U tests, Fisher’s exact tests, Wilcoxon signed-rank tests and two-way ANOVA with Sidak’s post-hoc multiple comparison tests. For analysis of Nef-invaded B-cell pAkt levels, median fluorescence intensities (mFls) were analyzed due to relatively small numbers of Nef$^+$ B cells. Analyses involving pAkt Ser473 levels all derived comparable results between mFl and MFI for wild type versus Nef G63E virus. Machine learning-based rating of Nef signal parameters was verified with IDEAS 6.3 Machine Learning Module (Luminex).
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Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV


Supplemental Information Legends

Fig. 1-Supplement 1. Profile of SIV\textsubscript{mac239}-NAb non-inducer subgroup cohort.
Summary of a SIV\textsubscript{mac239}-NAb non-inducer subgroup cohort (n = 19), composed from previously partially characterized, MHC class I haplotype-balanced naïve animals. Symbols A+, B+, E+, J+ and D+ represent possession of MHC-I haplotypes 90-120-la, 90-120-lb, 90-010-le, 90-088-ij and 90-010-ld, respectively. For the titers, - represents negative; +/ represents neutralization in three out of four wells in a quadruplicate test. Gray shadings indicate euthanasia due to AIDS progression. Mamu-A and Mamu-B alleles of interest in this study are listed. These NAb non-inducers were previously partially reported for their plasma viral loads and MHC-I alleles (Nomura et al., 2012; Takahashi et al., 2013; Nakane et al., 2013; Iwamoto et al., 2014). R01-011 was previously partially reported for NAb titers measured with the same method using MT4 cells as targets (Kawada et al., 2007), which derived comparable results.

Fig. 1-Supplement 2. IgG class-switched NAbs in the inducers.
Titration of SIV\textsubscript{mac239}-neutralizing IgGs in the NAb inducers. SIV\textsubscript{mac239}-specific IgGs, purified from pools of plasma with SIV\textsubscript{mac239}-specific NAb titers, were obtained from each animal as described (Yamamoto et al., 2007) and examined for their neutralizing activity by 10 TCID\textsubscript{50} SIV\textsubscript{mac239} killing on MT4-R5 cells. NAb-negative anti-SIV IgG and control IgG were prepared from a representative animal (R01-011) with NAb-negative plasma and pooled plasma of uninfected rhesus macaques, respectively.

Fig. 1-Supplement 3. Anti-SIV binding IgG profiles in NAb inducers and non-inducers.
(A) Temporal changes in SIV\textsubscript{mac251} Env gp120-specific antibody titers in the NAb non-inducers without rapid progression [NAb-negative non-rapid progressors, NAb(-) non-RPs] (left, n = 11) and NAb inducers [NAb(+)] (right, n = 9). NAb non-inducers showing rapid progression (euthanized with AIDS onset within approximately 1 year) were not included except for...
maque R10-001 (shown in the left). Samples for non-RPs R06-001 and R10-002 were unavailable.

(B) Unpaired t test of log-transformed anti-SIV Env Ab titers in NAb(-) non-RPs and NAb inducers. Bars represent geometric mean absorbance within each group.

(C) Lysed SIV<sub>mac</sub> virion linear antigen-binding at year 1 p.i. in NAb(-) non-RPs and NAb inducers. Plasma anti-SIV<sub>mac251</sub> IgGs were detected using a commercial western blotting system against the parental strain SIV<sub>mac251</sub> (ZeptoMetrix). White arrowheads indicate Env gp120-specific bands. Experiments were performed twice with comparable results.

**Fig. 1-Supplement 4. Env sequence variation pattern is not a major characteristic of NAb induction.**

SIV<sub>mac239</sub> Env sequence variations before and after NAb induction. For each animal, the upper time point shows pre-induction and the lower time point (in purple) shows post-induction (first or second time point with NAb titer > 1:2). Dominant residue changes are shown as follows: gp120 (gray), variable regions V1-V5 (blue), and gp41 (blue gray). Subdominant mutations are uncolored. Unlabeled gray and black shadings show subdominant and dominant deletion of residues. Parental SIV<sub>mac239</sub> sequence is listed for V1-V5. Residues mutated in multiple animals within V1-V5 are shown in red.

**Fig. 3-Supplement 1. Selection of Nef-G63E mutant SIV is linked with Nef<sub>62-70</sub>-specific CD8<sup>+</sup> T-cell response positivity.**

(A) Nef<sub>62-70</sub>-specific CD8<sup>+</sup> T-cell responses around week 20 post-SIV challenge in seven viremic NAb non-inducers possessing either of the MHC-I alleles Mamu-B*039:01, Mamu-B*004:01 or Mamu-A1*032:02.

(B) Temporal supernatant SIV p27 concentrations after wild type (WT) or Nef-G63E mutant SIV infection at multiplicity of infection (MOI) 0.001 on HSC-F cells. Data represent one of two independent experiments performed in triplicate. Bars: mean ± SEM.

(C) Comparison of plasma viral loads in Nef-G63E-selecting NAb non-inducers (n = 7) versus...
Fig. 4-Supplement 1. Decreased Akt hyperactivation properties of Nef-G63E mutant SIV.

(A) Plasma ferritin levels (ng/ml) in examined NAb non-inducers versus Nef-G63E mutant SIV-selecting NAb inducers around year 1 post-infection. Bars represent mean in each group. Compared by unpaired t test.

(B) Relative levels of pAkt Ser473 (left panel) and threonine (Thr) 308 (right panel) in SIV Nef+ HSR5.4 cells (MOI 0.1, 4 days p.i.).

(C) Relative pAkt Ser473 levels in Nef+ HSC-F cells without stimuli and transiently pulsed with IFN-γ, IL-2 or SIVmac Env gp130 after infection (MOI 0.2, 1 day).

(D) Gene expression significantly differing between WT and Nef-G63E SIV infection (MOI 5, 1 day). Black bars show potentially Akt-related genes. Asterisks show dynamics concordant with the Nef-G63E phenotype (PI3K-pAkt Ser473 downstream downregulation or inhibition upregulation).

(E) Representative flow cytometric gating (NAb inducer R01-012, wk 30 p.i.) of peripheral blood CXCR3+CXCR5+PD-1+ memory follicular CD4+ T cells. Cells were gated as CD3+CD8+CD4+CD95+CXCR5+PD-1+CXCR3+ live singlet PBMCs. Available samples of six NAb inducers showing peak Nef-G63E mutant selection around week 30 p.i. and thirteen viremic NAb non-inducers were tracked.

(F) Comparison of peripheral CXCR3+CXCR5+PD-1+ memory Tfh cell frequencies between pre-SIV infection and around week 30 post-infection in NAb non-inducers developing disease before week 60 (left: n = 5) and alive more than 70 weeks (middle: n = 8) and Nef-G63E-detected NAb inducers alive more than 70 weeks (right: n = 6). Analyzed by Wilcoxon signed-rank tests.

MFIs relative to those in uninfected controls (%) are shown. **: P < 0.01, ***: P < 0.001, n.s.: not significant between WT and Nef-G63E mutant by unpaired t tests (B-C). Data represent
one of two (B, C) independent experiments performed in triplicate (C: unstimulated) or quadruplicate (B, C: PI3K-pulsed). Bars: mean ± SEM.

**Fig. 5-Supplement 1. Machine learning-verified morphological gating of Nef-invaded B cells in vivo.**

(A) Representative gating strategy plus schematic of inguinal lymph node Nef+CD19+ B cells of an SIVmac239-infected rhesus macaque analyzed by ImageStreamX MK II imaging flow cytometry (R10-012, week 160 p.i.). First-step incorporation of Live/Dead staining gates out CD19+ B cells staining positive for Nef potentially due to pre-experimental membrane permeation (e.g., image #24896, #248952 and #71859). Second-step dual gating for intracellular Nef signal pixel Haralick variance mean (H Variance mean, X axis), a textural feature calculated from a gray level co-occurrence matrix (GLCM) for each image representing adjacent pixel signal strength heterogeneity and Nef signal intensity threshold (Y axis) gates out CD19+ B cells showing potentially non-specific, strong intracellular binary clustered staining pixels for Nef deriving a large variance (X axis), and/or cells stained overtly strong for Nef and hence becoming void for feasible image verification (high threshold) (Y axis), both likely originating stochastically from post-experimental rupturing procedures including membrane permeation (e.g. image #18541). This two-step noise cancellation results in acquisition of a Live/Dead-Nef signal Haralick variance mean-Nef signal intensity threshold-Nef+CD19+ B-cell population (e.g. image #116318, #116250 and #132927) with a pericellular Nefint-lo staining, biologically concordant with Nef membrane anchoring, resembling Nef+ cells experimentally generated *in vitro* and showed the highest score in IDEAS6.3 machine learning module scoring for segregation of typical void vs. valid images (upper right). This gating was implemented for counting and analysis of Env-specific B-cell Nef invasion *in vivo* (Figure 5).

(B) Detection of contact-dependent Nef invasion from SIV-infected HSC-F CD4+ T cells to cocultured Ramos B cells *in vitro*. Two typical images of an intercellular conduit (SSC, purple) with Nef (green) protrusions from infected HSC-F cells to CD19+ (red) Ramos B cells from...
two independent experiments are shown.

**Fig. 6-Supplement 1. Gating strategy of SIV<sub>mac239</sub> Env gp140-specific B-cell responses.**

Representative gating (R02-004, week 32 p.i.) of SIV Env gp140-specific memory B cells (B<sub>mem</sub>) and plasmablasts (PBs). Two panels for PB gating are shown in lower resolution for visibility. B<sub>mem</sub> staining was performed separately and gatings are merged with PB panels (second row, panels 2–4). For lineage-specific exclusion (first row, panel 4), CD10 was stained independently for B<sub>mem</sub> (see parenthesis).
Infection max 100 wks

SIVmac239 (Highly NAb-resistant)

Rhesus Mq

SIVmac239-NAb retrospective screen (n = 70)

NAb inducer identification (n = 9)

B

Viral load

NAb non-inducers

NAb inducers

Viral load (copies/ml)

Weeks post-SIV challenge

C

SIVmac239-NAbs

SIV 100% neutralizing titers

Weeks post-SIV challenge

R06-032
R01-012
R03-015
R02-004
R01-010
R06-019
R-360
R06-032
R02-007
R05-010
A

N-myristoylation (Q2)  N-anchor 89  Core

env

1

AP-2-binding

G63E

104PxxP107  LTR129

SIVmac239 Nef

R06-032

R01-12

R03-015

R02-004

R01-010

R06-019

R-360

R02-007

R05-010

Status: ▶ NAb (-) ▶ NAb (+/-) ▶ NAb (+)

B

N-anchor (1-89)

N-myristoylation

PxxP motif

Acidic cluster

D89

Core domain loop

C

Nef-G63E selection

% detected in group

P = 0.0009

D

Temporal Nef-G63E mutant selection vs NAb induction

SIV 100% neutralizing titers (log2)

Mutant SIV frequencies (%)

Weeks post-SIV challenge
Specific CD8+ T-cell frequencies (/million PBMCs)

Peptide concentration (nM)

Nef 62-70 QW9: QGQYMNPW70

R01-012 wk30
1000
100
10

R03-015 wk16
1000
100
10

R02-004 wk12
1000
100
10

R06-019 wk8
100
10
1

R-360 wk40
1000
100
10

Nef 62-70
Nef 62-70.G63E
Nef 62-70.G63R

B

R01-010 wk7
1000

100

1,000

R06-032 wk15
1000
100
10

100

1,000

B*039:01
B*004:01
A*032:02

wk11 wk30
wk2 wk30
wk12 wk38
wk12 wk40
wk7 wk31
wk8 wk44
wk16 wk40

B*039:01
B*004:01
A*032:02

R06-032 R01-012 R03-004 R01-010 R06-019 R-360

A

R01-012 wk30
R03-015 wk16
R02-004 wk12

R01-010 wk7
R06-019 wk8
R-360 wk40

Specific CD8 T-cell frequencies (million PBMCs)

Peptide concentration (nM)
A. Surface downregulation

- **CD3**
- **CD4**
- **MHC Class I**
- **CXCR4**
- **BST-2**

Gated on singlet CD4^+\text{Nef}^-

B. MOI 0.1 Day 3

- **pAkt Ser473**

C. Acceleration w/upstream PI3K stimuli Day 3 → Day 1

- **Serum starvation**
- **High MOI**

D. Intracellular PI3K-pathway binding

- **mTORC2**
- **PI3 Kinase**

- **GβL/mLST8**
- **mTOR**
- **PI3Kp85**
- **PI3Kp110α**

Gated on singlet p27^+ HSC-F

- **CD4^+\text{p27}^+\text{singlet HSC-F}**

- **P** values for each condition:
  - pAkt Ser473
  - GβL/mLST8
  - mTOR
  - PI3Kp85
  - PI3Kp110α

Binding Index (ΔMFI)

- **WT**
- **Nef-G63E**
- **WT (isotype)**
- **Nef G63E (isotype)**
**A**

- **B-cell specificity**
- pAkt Ser473 relative mFIs (% of Nef- B cells)

**B**

- **DIC**
- Nef- (Ch01)
- Nef- AF488 (Ch02)
- pAkt473 PE (Ch03)
- Live/Dead- Aqua (Ch08)
- CD19- PC5.5 (Ch11)
- oEnv- PE-Cy7 (Ch12)

**Merged**

- Nef- Live/Dead CD19
- Nef- oEnv
- Nef- pAkt473

**C**

- pAkt S473

**D**

- Nef invasion

---

**Non-Env**

- Nef- (R8)
- Nef+ (R7)

---

Statistical significance:

- $P = 0.0038$
- $P = 0.015$
A) NAb non-inducers

B) wk 30

C) Temporal 2-D plot of Env gp140-specific Bmem vs PB

\[ \text{SIV Env gp140-specific IgG+ plasmablasts/million PBMCs} \]

\[ \text{time post-SIV challenge} \]

\[ \text{wk 0} \quad \text{wk 30} \quad \text{yr 1} \]

\[ P = 0.0131 \quad P = 0.0030 \]

\[ \text{gp140-specific IgG+ plasmablasts/million PBMCs} \]

\[ \text{NAb- Nef-G63E+ NAb+} \]

\[ \text{NAb- Nef-G63E+ NAb+} \]

\[ \text{gp140-specific IgG- memory B cells/million PBMCs} \]

\[ D_1 = \text{GC output attractor 1 (NAb-, at yr 1)} \]

\[ D_3 = \text{GC output attractor 2 (Nef-G63E+, at NAb induction)} \]
### NAb non-inducer cohort (n = 19)

<table>
<thead>
<tr>
<th>MHC-I/ID</th>
<th>SIV&lt;sub&gt;mac239&lt;/sub&gt; neutralization at 1:2 plasma dilution by 10tcid&lt;sub&gt;50&lt;/sub&gt; killing assay (wks p.i.)</th>
<th>pVL at 6 mo</th>
<th>Nef G63E at 1 yr</th>
<th>Detected Mamu alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
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<td>R06-037</td>
<td>wk25 - wk46 +/- wk72 - wk87 - wk95 -</td>
<td>6.4 x 10&lt;sup&gt;2&lt;/sup&gt; - (pVL &lt; 400)</td>
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<tr>
<td>R07-001</td>
<td>wk25 - wk42 - wk88 +/- wk96 -</td>
<td>&lt; 400 - (pVL &lt; 400)</td>
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<tr>
<td>B&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>R06-001</td>
<td>wk25 - wk48 - wk60 - wk80 - wk90 -</td>
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<td>R06-039</td>
<td>wk25 - wk46 - wk56 -</td>
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<td>wk22 - wk40 - wk52 - wk71 - wk85 - wk104 -</td>
<td>1.3 x 10&lt;sup&gt;5&lt;/sup&gt; -</td>
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<td>R05-007</td>
<td>wk26 - wk55 +/- wk71 - wk89 - wk97 -</td>
<td>1.6 x 10&lt;sup&gt;4&lt;/sup&gt; -</td>
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<td>wk25 - wk36 - wk58 +/- wk80 +/- wk95 -</td>
<td>1.1 x 10&lt;sup&gt;5&lt;/sup&gt; -</td>
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<td>J&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>1.5 x 10&lt;sup&gt;5&lt;/sup&gt; - + (wk40)</td>
<td>B&lt;sup&gt;+&lt;/sup&gt;039:01</td>
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<td>1.4 x 10&lt;sup&gt;7&lt;/sup&gt; - - (wk23)</td>
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<td>B&lt;sup&gt;+&lt;/sup&gt;039:01</td>
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<td>D&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>wk25 - wk48 - wk60 - wk80 - wk90 - wk98 -</td>
<td>5.4 x 10&lt;sup&gt;2&lt;/sup&gt; -</td>
<td>A&lt;sup&gt;+&lt;/sup&gt;032:02 , B&lt;sup&gt;+&lt;/sup&gt;004:01</td>
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<td>4.5 x 10&lt;sup&gt;3&lt;/sup&gt; -</td>
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<td>R08-005</td>
<td>wk25 - wk48 - wk68 - wk100 -</td>
<td>9.0 x 10&lt;sup&gt;5&lt;/sup&gt; -</td>
<td>A&lt;sup&gt;+&lt;/sup&gt;032:02 , B&lt;sup&gt;+&lt;/sup&gt;004:01</td>
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<td>Others</td>
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<td>R10-002</td>
<td>wk14 - wk59 - wk104 -</td>
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<td>R10-006</td>
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<td>Group</td>
<td>Rhesus ID</td>
<td>mg/ml</td>
<td>Collected/pooled (wks post-challenge)</td>
<td>10 TCID&lt;sub&gt;50&lt;/sub&gt; SIVmac239 (IgG)</td>
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<td>R06-032</td>
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A

NAb(-) non-RPs

NAb(+)

B

Anti-Env Ab 1 yr

P = 0.044

C

NAb- non-RPs

NAb+

Serum control
A. Nefs2-70 QW9-specific responses in NAb non-inducers

B. Days post-SIV infection on HSC-F cells

C. Plasma viral loads (vRNA copies/ml)

3 mo: $P = 0.2495$

6 mo: $P = 0.5078$

1 year: $P = 0.8928$

1.5 year: $P = 0.6488$
**A**

Plasma ferritin

- **B**

Day 4

- **C**

No stimuli

- **D**

+ Short-term PI3K stimuli (20 min)

- **E**

Circulating memory Tfh

- **F**

NAb- (-60 wk survival) vs NAb+ (>70 wk survival vs wk 30 Nef-G63E+)

- **G**

NC WT Nef-G63E

- **H**

NC WT Nef-G63E

- **I**

NC WT Nef-G63E

---

*PI3K/Akt Ser473/FoxO1-associated Others

***n.s.***
### A

#### Intracellular pixel Nef signal

**Intensity threshold**

Live/Dead CD19- Nef- B cells (void)

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<th>CH02/CH08/CH11</th>
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**Signal intensity threshold (int/hi)**

Live/Dead CD19+ Nef+ B cells

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**Signal intensity threshold (lo)**

Live/Dead-CD19- Nef- B cells (void)

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**Intracellular Nef Haralick variance mean (void)**

Live/Dead-CD19- Nef- B cells

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**Intracellular Nef Haralick variance mean**

Live/Dead CD19+ Nef+ B cells (valid)

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### B

#### In vitro CD19+ Nef+ B cells

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