

Gag p27-Specific B- and T-Cell Responses in Simian Immunodeficiency Virus SIVagm-Infected African Green Monkeys

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Note

**Gag p27-Specific B and T Cell Responses
in SIVagm-Infected African Green Monkeys**

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1 **Abstract**

2 Nonpathogenic SIVagm infection of African green monkeys (AGMs) is characterized by the
3 absence of a robust antibody response against Gag p27. To determine if this is accompanied by a
4 selective loss of T cell responses to Gag p27, we studied CD4⁺ and CD8⁺ T cell responses against
5 Gag p27 and other SIVagm antigens in the peripheral blood and lymph nodes of acutely- and
6 chronically-infected AGMs. Our data show that AGMs can mount a T cell response against Gag
7 p27, indicating that the absence of anti-p27 antibodies is not due to the absence of Gag p27-
8 specific T cells.

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1 Simian immunodeficiency virus (SIV) infection in African green monkeys (AGM) is
2 nonpathogenic, even though it is characterized by plasma viral load (PVL) levels similar to those
3 found during acute and chronic pathogenic infection of humans with HIV-1 and macaques with
4 SIVmac (14). This feature is shared with other African non-human primates (NHP), such as sooty
5 mangabeys and mandrills (19, 20). SIV-infected AGMs also display high viral loads in the
6 gastrointestinal mucosa (11), a transient decline of circulating CD4⁺ T cells during acute infection
7 (13), and longer-lasting CD4⁺ T cell depletion in the intestinal lamina propria (10). Concomitant
8 with the peak viral load during acute infection, SIVagm-infected AGMs display transient
9 increases of CD4⁺ and CD8⁺ T cells expressing activation and proliferation markers, such as
10 MHC-II DR and Ki-67 (4, 13), and anti-SIVagm antibodies (Ab) are induced with kinetics
11 similar to those found in SIVmac infection (5). Interestingly, however, the Ab response against
12 Gag p27 is weak, if present at all (1, 2, 12, 15, 17, 18). This observation is surprising since, in the
13 context of HIV-1 and SIVmac infections, Ab responses to Gag p27 are usually quite strong.
14 Weak or low reactivity to Gag p27 has also been observed in some other natural SIV infections
15 (7, 8, 20), but not in all (21). We wondered whether such a selective lack of Ab reactivity in the
16 SIV-infected AGM might be related to a lack of Gag p27-specific T cells. With this hypothesis in
17 mind, we first confirmed and extended the studies on humoral responses against Gag p27 by
18 characterizing the antigen-specific IgG responses and mid-point titers against total SIVagm
19 antigens (SIVagm virions) and recombinant Gag p27 (SIVagm) in naturally and experimentally
20 SIVagm-infected AGMs. Second, we searched for the presence of Gag p27-specific T cell
21 responses in SIVagm infection, by analyzing the CD4⁺ and CD8⁺ T cell responses specific for
22 Gag p27 and other SIVagm proteins in blood and lymph nodes (LN) of acutely- and chronically-
23 infected animals.

1 Humoral responses against SIV were analyzed in 50 wild-born AGMs (*Chlorocebus sabaeus*)
2 and 17 Rhesus macaques (RM). The animals were housed at the Pasteur Institute in Dakar,
3 Senegal, and the California National Primate Research Center, Davis, CA, respectively,
4 according to institutional and national guidelines. RM were either non-infected (N=5) or
5 intravenously (I.V.) infected with SIVmac251 (N=12). AGMs were either non-infected (N=23),
6 naturally infected (N=17) or I.V. infected with wild type SIVagm.sab92018 (N=10) (5, 9). IgG
7 titers against SIVagm.Sab92018 virions or recombinant Gag p27 protein (rP27) were determined
8 by ELISA using monkey anti-IgG as secondary antibodies (Fig. 1A and B). Virions had been
9 purified by ultracentrifugation on a iodixanol cushion from cell-free supernatants of
10 SIVagm.Sab92018-infected SupT1 cells. The His-tagged rP27 was constructed using DNA from
11 gut cells of an SIVagm.sab92018-infected AGM (96011) (11). A Gag p27 PCR product was
12 subcloned into pET-14b, and the recombinant protein was produced in BL21DE3pLysS *E. Coli*
13 and purified on Ni-TA columns. SIV-infected macaques showed high IgG titers cross-reacting
14 with both SIVagm virions (Fig. 1A and B, left) and rP27 (Fig. 1A and B, right). By contrast, only
15 2 out of 27 SIV-infected AGMs showed detectable IgG responses against rP27 (Fig. 1A and B,
16 right), while 21 out of 27 displayed significant responses against SIVagm virions (Fig. 1A and B,
17 left). Two AGMs out of 23 from the negative control group showed weak responses at the limit
18 of detection against SIVagm and two against rP27, suggesting either natural response against
19 SIVagm proteins, cross-reactivity with unknown pathogens, maternal antibodies or recent SIV-
20 infection. Of note, the titers against whole SIV in the infected monkeys were higher in macaques
21 than AGMs, may be due to lack of anti-p27 Ab in most AGMs.

22 The study of IgGs by Western blot using denatured SIVagm.sab92018 virions showed no or weak
23 anti-Gag responses in SIV-infected AGMs, yet the anti-Env responses were often strong (Fig. 1C).
24 By contrast, SIV-infected macaques showed a dominant IgG cross-reactive response against the

1 SIVagm Gag p27 protein. Even if a response was detected more frequently than with the ELISA
2 assays in AGMs, their magnitude was different and considerable weaker than in macaques.
3 To compare B and T cell responses over time, 5 STLV-seronegative AGMs were infected with
4 SIVagm.sab92018 and the animals followed longitudinally during the acute and post-acute
5 phase of infection until day 90 post-infection (p.i). Sequential blood samples were collected and
6 biopsies of axillary and inguinal LNs were performed at day -5 and at three times p.i. (days 14,
7 43 and 62). PVL was measured by real time PCR (5). Since we searched for Gag p27-specific
8 responses, we also quantified Gag p27 antigen in the plasma (SIV p27 antigen assay, Coulter,
9 Miami, FL). Viral RNA and p27 antigenemia peaks were observed between days 7 and 14 p.i.
10 (Fig. 2A and 2B, respectively). The Gag p27 levels were variable among the animals but in a
11 range similar to those reported previously in AGMs and macaques (3, 5). As has also been
12 observed in SIVmac infection (except for rapid progressors), plasma Gag p27 levels fell below
13 the detection level in the post-acute phase (i.e., after day 28 p.i.) (Fig. 2B and *data not shown*).
14 There were significant increases of circulating CD8⁺DR⁺ T cells at days 7 and 14 p.i., and of
15 CD8⁺Ki-67⁺ T cells at days 14 and 28 p.i. (Fig. 2C and 2D, left panels). After day 28 p.i., the
16 percentages were no more statistically different from baseline levels. In LN cells (LNCs), the
17 percentage of CD8⁺Ki-67⁺ T cells rose from 3.1±1.1% before infection to 6.1±0.3% at day 62
18 p.i., but the difference was not statistically significant (Fig. 2D, right panel). The levels of blood
19 CD4⁺DR⁺, CD8⁺DR⁺, and CD8⁺Ki-67⁺ T cells, and of LNC CD8⁺ Ki-67⁺ T cells, were positively
20 correlated with viremia (p=0.002 for DR⁺ cells and p<0.02 for Ki-67⁺ cells). Altogether, these
21 results confirm previous data showing early, transient T cell activation in the peripheral blood of
22 SIVagm-infected AGMs (13).

23 We next looked for the presence of Ab responses against rP27 in these animals. No Ab were
24 detected before infection. After infection, all five AGMs developed anti-SIVagm IgGs within 4 to

1 9 weeks p.i., AGM2001 showing the fastest response (Fig.3A). While the humoral responses
2 against whole virions were significant (Fig.3B, red line), the anti-rP27 responses were below the
3 threshold for positivity (Fig.3B, green line), with the exception of one animal (AGM 02001). The
4 anti-rp27 response in this animal was only transient since it was no more detectable at week 75
5 p.i., in contrast to the anti-SIV Ab that were sustained (Fig. 3B and *not shown*).

6 We next searched for T cell responses against Gag p27 as compared to other SIVagm antigens in
7 these animals. Gag p27 epitopes were presented in two ways: in the context of rP27 and as
8 synthetic peptides. The peptide pools (comprised of overlapping 15-mers) spanned the following
9 SIVagm proteins: Gag p27, Gag without (w/o) p27, Env and Tat. The amino acid sequences of
10 the Gag and Env peptides corresponded to the autologous wild type SIVagm.sab92018 sequence,
11 and those of the Tat peptides to an SIVagm.sab consensus sequence. The latter was determined
12 using Tat sequences of other SIVagm viruses from Senegal that are available in the databases
13 (SIVagm.sab1c, SIVagm.sabD42 and SIVagm.sabD30). We measured T cell responses by
14 investigating the antigen-induced proliferation. T cells from blood (PBMC) and LNs were
15 analyzed. All assays were performed with fresh cells that were stimulated with 10 µg/ml of Gag
16 rP27 and 5 µg/ml of peptides over a period of 4 days. Dead cells were gated out using 7-amino-
17 actinomycin D (7-AAD) and dividing (CFSE^{low}) cells were analyzed after stimulation with media
18 alone, SIV antigens or concanavalin A as a positive control. We detected significant Gag p27-
19 specific proliferative responses for CD8⁺ T cells in PBMC and for CD4⁺ and CD8⁺ T cells in
20 LNCs (Fig. 3C). The animal with the detectable anti-p27 Ab (AGM 2001) did not show stronger
21 p27-specific T cell responses than the other animals. Thus, all SIV-infected AGMs were able to
22 mount a proliferative T cell response against p27, while anti-p27 IgG were lacking in four of

1 them. However, the SIVagm-specific T cell responses were detected at only a few time points
2 post-infection.

3 We then analyzed the T cell responses in the chronic phase of naturally and SIVagm.sab92018-
4 experimentally infected AGMs. PVL, peripheral blood cell counts (CD4⁺ and CD8⁺ T cells,
5 CD20⁺ B cells), and immune activation (Ki-67⁺CD8⁺ T cells) were similar in naturally-infected
6 and in experimentally-infected AGMs (Fig. 4A). As expected, cell counts and immune activation
7 levels were also not different from SIV negative AGMs (Fig. 4A). Again, we measured SIV-
8 specific responses first by a proliferation assay (Fig. 4B). One out of five animals tested had a
9 proliferative SIV-specific CD4⁺ T cell response (against Gag w/o p27, Gag p27, Gag rP27, Env
10 GP120 and Tat) and two had a CD8⁺ T cell response (against Gag p27 in both and against Env
11 GP120 and Tat in one). Two (one naturally-infected, one experimentally-infected with
12 SIVagm.sab92018) did not show any detectable antigen-specific proliferative CD4⁺ or CD8⁺ T
13 cell response.

14 These results were extended to an analysis of SIV-specific T cell cytokine responses, e.g., the
15 production of IFN- γ and TNF- α in nine chronically-infected as compared to ten non-infected
16 AGMs (Fig. 4C and D). Fresh cells were stimulated for eight hours with the antigens described
17 above. SIV-specific cytokine responses were detected in CD8⁺, but not in CD4⁺ T cells. Seven
18 animals out of nine showed a response against at least one antigen. The two animals showing no
19 response were among the four naturally infected animals tested. We therefore cannot exclude that
20 the absence of response in these two animals is due to the presence of highly divergent viruses.
21 However, a precise epitope mapping in SIVagm sequences would be necessary to confirm this. In
22 those animals showing a SIVagm-specific cytokine T cell response, the latter were directed
23 against Gag p27 (4 out of 9 animals), other Gag proteins than p27 (2 out of 9 animals), and Env
24 GP120 (4 out of 9 animals). In the experimentally infected animals, we might have

1 underestimated the responses against Tat as compared to Gag and Env antigens, since the Tat
2 peptides corresponded to an SIVagm.sab consensus sequence and not to the autologous virus
3 (SIVagm.Sab92018). There was no correlation between the magnitude or breadth of SIV-specific
4 T cell responses and immune activation or VL.

5 Altogether, our study demonstrates that AGMs can mount T cell proliferative and cytokine
6 responses against Gag p27. The T cell response was variable among the animals. In general, it
7 appeared moderate, comparable to chronically SIV-infected RM (9). Of note, T cell responses
8 were not consistently detected at all time points and not in all animals. We cannot exclude the
9 possibility that we underestimated the magnitude of the cytokine responses. For instance, we did
10 not co-stimulate the cells during the assays. However, cytokine responses were also variable in
11 vervet AGMs with a trend for lower levels as compared to RMs even by using more sensitive
12 assays (23). In sooty mangabeys, the responses were also reported to be not stronger than in RM.
13 This is in line with the lack of efficient control of viral replication in natural hosts (6, 22).

14 In our study we show that IgG responses against Gag p27 are either lacking, weak or transient,
15 while Ab against other SIVagm proteins are present. The mechanisms underlying this selective
16 lack of Gag p27 Ab responses are unclear. It could be related to a moderate and/or dysfunctional
17 CD4⁺ T cell responses and/or due to an unknown suppressive regulatory mechanism. SIV-
18 specific T cell cytokine responses were indeed principally found at the CD8⁺ T cell level. This
19 was also reported in SIVsm-infected sooty mangabeys (SM) (6, 22). Here, we also searched for
20 SIVagm Gag p27-specific proliferative responses. Interestingly, they were detected for CD4⁺ T
21 cells indicating the presence of p27-specific CD4⁺ memory cells in AGMs. Moreover, AGMs can
22 potentially mount a strong and sustained anti-Gag p27 humoral response, when appropriately
23 immunized (*D. Favre et al., manuscript in preparation*). This suggests that there is no central B
24 cell tolerance against p27 Gag protein in AGMs, nor an inherent inability for CD4⁺ T cells to

1 provide helper B cell functions. The transient nature of anti-p27 Ab in one animal would be in
2 favor of regulatory mechanisms, but that needs to be confirmed. Another explanation could be
3 that AGMs are able to mount Ab responses against some p27 epitopes but not to those exposed
4 by the native protein, which would explain why we and others detect more frequently humoral
5 responses in WB than in Elisa assays (16).

6 In conclusion, we characterized the IgG responses against SIVagm and confirmed a lower
7 humoral response against p27 than in RM. Moreover, our study reveals that cytokine and
8 proliferative T cell responses against SIVagm Gag p27 are detectable in AGMs. Thus, the
9 reduced ability of the AGM to produce antibodies against Gag p27 post-infection is not related to
10 a lack of Gag p27-specific T cells.

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1 **FIGURE LEGENDS**

2

3 **Figure 1: Cross-sectional analysis of IgG antibody responses against SIV_{agm} or Gag p27 in**
4 **SIV-infected AGMs and RMs.**

5 (A and B) Cross-sectional analysis by ELISA. IgG antibodies against SIV_{agm}.sab₉₂₀₁₈ virions or
6 recombinant p27-Gag antigens were determined in SIV negative and chronically SIV_{mac251}-
7 infected RMs and in SIV negative and chronically SIV_{agm}-infected AGMs, that were either
8 naturally or experimentally-infected with SIV_{agm}.sab₉₂₀₁₈. Antibody titers were calculated for
9 each animal by limited dilution of plasma on coated ELISA plates with 5µg/ml of (p27
10 equivalent) virions (left) or 1µg/ml of the monomeric recombinant protein (rP27) (right). IgG
11 detection by ELISA displayed high background for rP27, especially at the highest plasma
12 concentration (e.g. 1/100 and 1/400 plasma dilution) in SIV-negative RM and AGMs. To
13 discriminate between positive responses and background, calculated dose-response curves were
14 compared to theoretical sigmoid-dose response curves corresponding to the 95% confidence
15 interval of SIV-negative animals. By convention, responses were considered as background when
16 sigmoid dose-response curves were graphically within the 95% confidence interval of SIV
17 negative animals, and when the calculated -LogEC₅₀ was lower than the upper theoretical
18 sigmoid dose-response curve from SIV negative animals (corresponding to a threshold of -
19 logEC₅₀ = 2.8). (A) Results (O.D. 450) are represented for both virions (left) and rP27 (right)
20 over plasma dilution (log₁₀) on a per animal basis (data points) and for each group (lines). Lines
21 represent the sigmoid dose-response curves for each group (Prism 4, Graphpad) (B) Mid-point
22 IgG titers were determined for each animal from individual sigmoid dose-response curves, and
23 presented as the log₁₀ value from the reciprocal of the effective concentration that corresponds to
24 50% response between minimum and maximum O.D. 450 (-log EC₅₀). Horizontal bars represent

1 the median mid-point titer per each group. Man-Whitney non-parametric tests were applied for
 2 statistical analysis (n.s., non significant with p values > 0.1) (C) Cross-sectional analysis of Ab
 3 against SIVagm proteins by Western blot using denatured SIVagm.sab₉₂₀₁₈. For the positive
 4 controls on the left, we used sera from an SIVmac₂₅₁-infected macaque and a SIVagm.sab₉₂₀₁₈
 5 infected AGM. Development times and reagents were identical for all western blots. Mo: months
 6 of infection, y= years of infection, C-: negative control, and C+ : positive control.

7
 8 **Figure 2. Plasma viremia and T cell activation in blood and lymph nodes of five**
 9 **longitudinally-followed SIVagm.sab₉₂₀₁₈-infected African green monkeys.** (A) SIVagm.sab
 10 RNA copy numbers in plasma; (B) Plasma Gag p27 concentrations; (C) Percentages of MHC-II
 11 DR positive CD4⁺ (●) and CD8⁺ (○) T cells within, respectively, total CD4⁺ and CD8⁺ T cells
 12 from PBMC and LNCs; (D) Percentages of Ki-67 positive CD4⁺ (●) and CD8⁺ (○) T cells
 13 within, respectively, total CD4⁺ and CD8⁺ T cells from PBMC and LNCs. Results are shown as
 14 the mean \pm SEM. Asterisks indicate statistically significant differences as compared to levels
 15 before infection ($p < 0.05$).

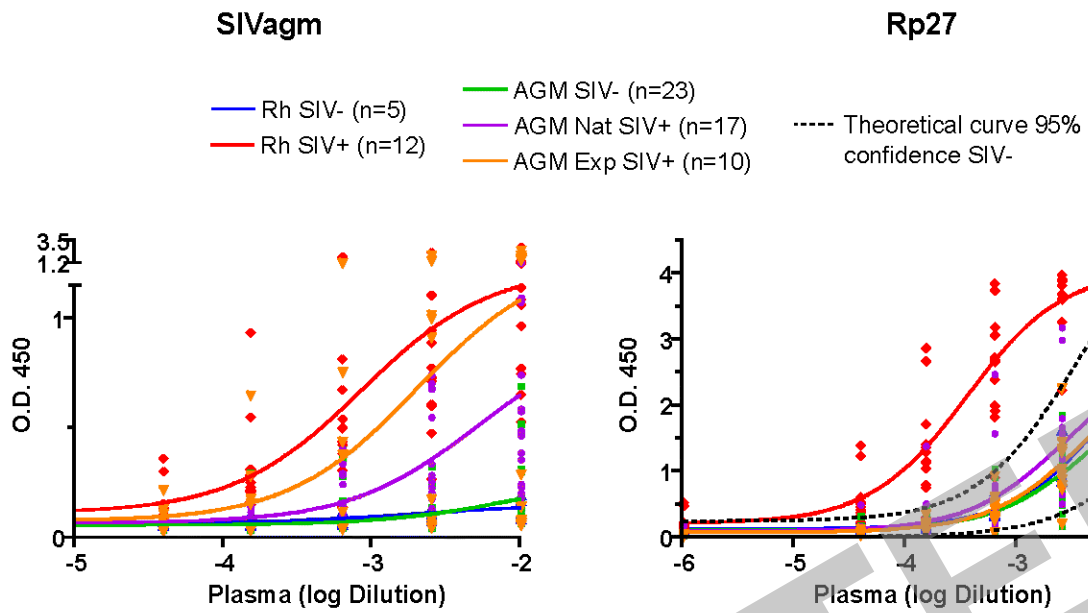
16
 17 **Figure 3. Longitudinal analysis of IgG titers and T cell proliferative responses against**
 18 **SIVagm and Gag p27 in five SIVagm.sab₉₂₀₁₈-experimentally infected AGMs.** (A+B) Ab
 19 responses were analyzed by ELISA. (A) IgG dose-response curves against SIVagm (upper) and
 20 rP27 (lower) are shown over time (week -1 to week +24 p.i.). (B) Mid-point titers were calculated
 21 as described in Figure 1A. Continuous lines correspond to median titers from all 5 animals (red,
 22 anti-SIVagm IgGs; green, anti-p27 IgGs). (C) Proliferative responses of CD4⁺ and CD8⁺ T cells
 23 were assessed by flow cytometry, using CFSE staining. CD4⁺ and CD8⁺ T cell responses in
 24 PBMCs (left) and LNCs (right) after stimulation with peptide pools (Gag w/o p27, P27, and Tat)

1 and Gag rP27 are shown for each animal. All data are reported after background subtraction.
2 Results are presented in columns as mean \pm SEM. Asterisks indicate statistically significant
3 differences compared to individual values before infection ($p < 0.05$).

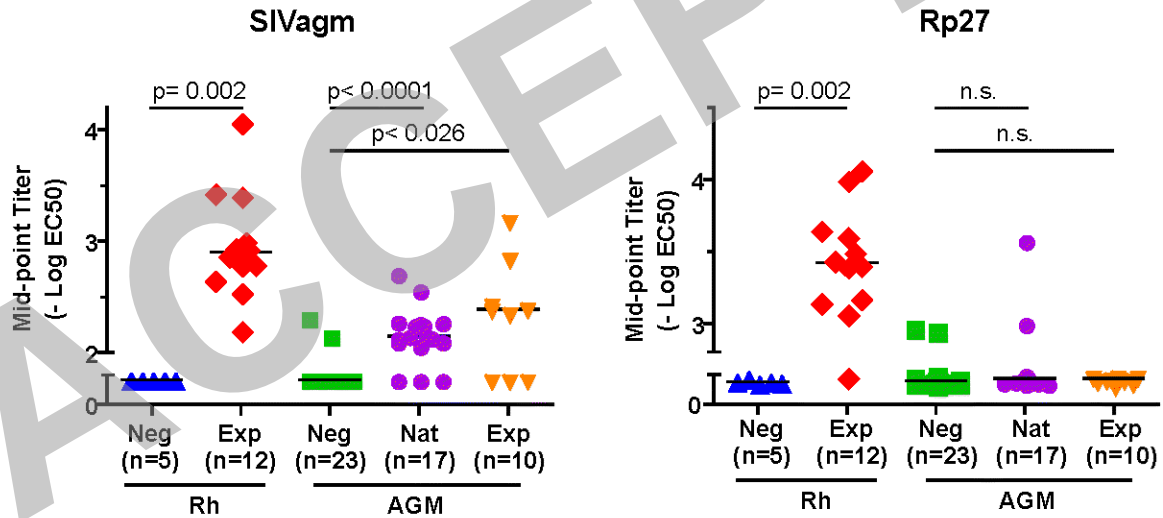
4
5 **Figure 4. Immune parameters and SIVagm-specific proliferative and cytokine T cell**
6 **responses in chronically-infected AGMs. (A)** Cell counts ($CD4^+$ and $CD8^+$ T cells, B cells) ,
7 and immune activation levels (% of $Ki-67^+$ in $CD8^+$ T cells) in SIVagm-naturally infected AGMs
8 ($n=4$) and SIVagm.sab₉₂₀₁₈-experimentally infected AGMs ($n=6$) compared to uninfected AGMs
9 ($n=10$). PVL if known is indicated. Green, blue and orange symbols correspond, respectively, to
10 non-infected, naturally infected and experimentally infected AGMs. **(B)** Proliferative response to
11 SIVagm antigens in chronically-infected AGMs ($n=5$) compared to uninfected AGMs ($n=3$).
12 PBMCs were stimulated with the same antigens as described in Fig. 3. **(C)** Analysis of cytokine
13 responses ($IFN-\gamma$ and $TNF-\alpha$) by SIVagm-specific T cells. ConA was used as a positive control.
14 Representative results from a single animal are shown here. **(D)** Cumulative values of SIVagm-
15 specific $TNF-\alpha$ and $IFN-\gamma$ responses in chronically-infected animals. The responses to SIVagm
16 antigens were analyzed in peripheral blood specimens of 4 naturally- and 5 experimentally-
17 infected AGMs, as well as 10 uninfected AGMs. The data are reported after background
18 subtraction corresponding to the subtraction of the frequency of positive events from the
19 unstimulated samples to the frequency of positive events from the antigen-specific stimulation.
20 Proliferative T cell responses and cytokine T cell responses in SIV-infected AGMs were defined
21 as positive when higher than the mean + 3St.Dev of the responses from uninfected animals.

22

A



B



C

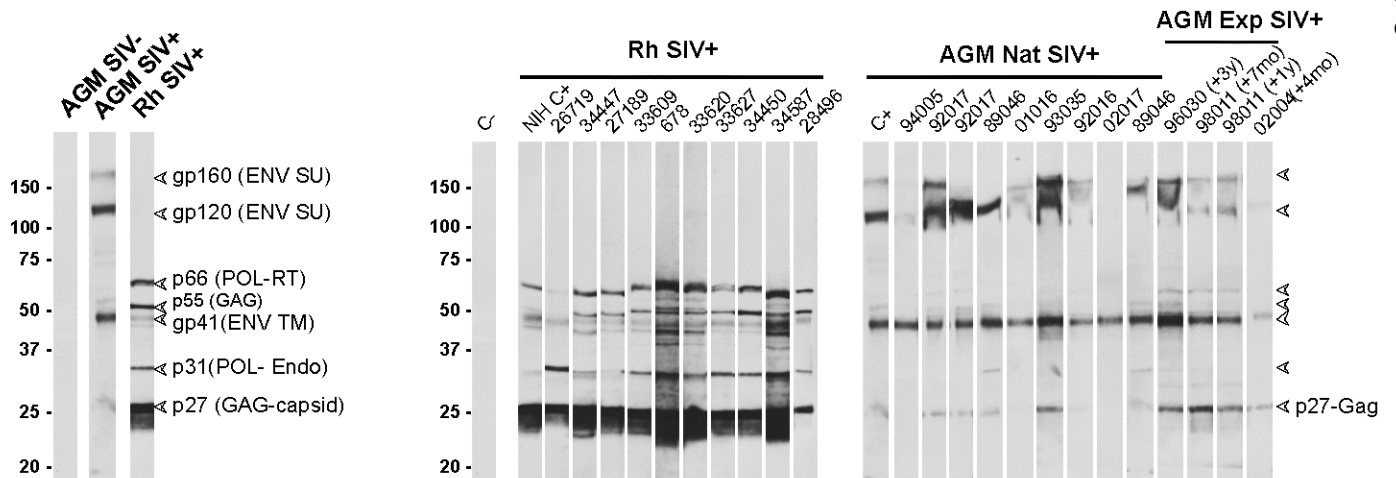


Figure 1

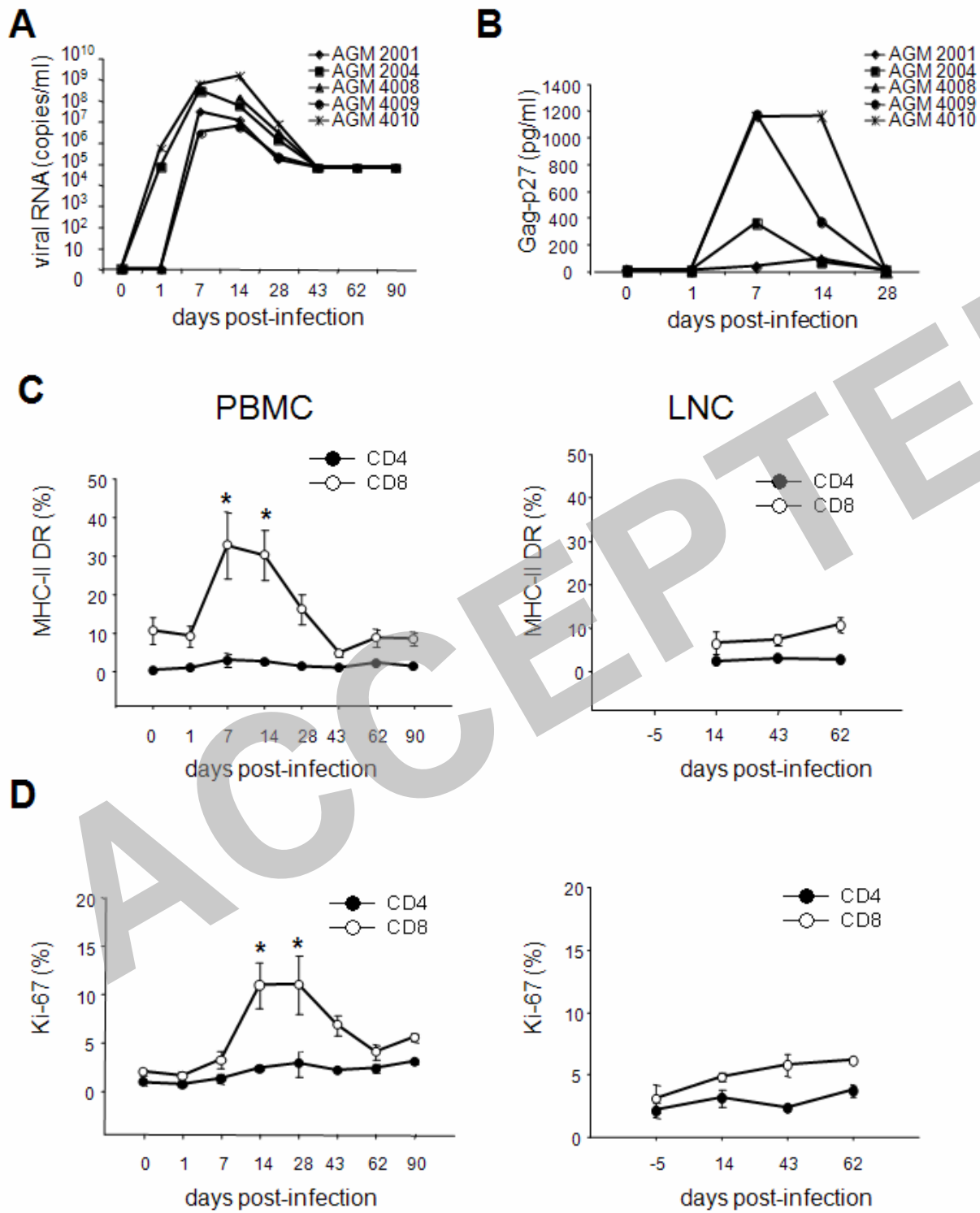
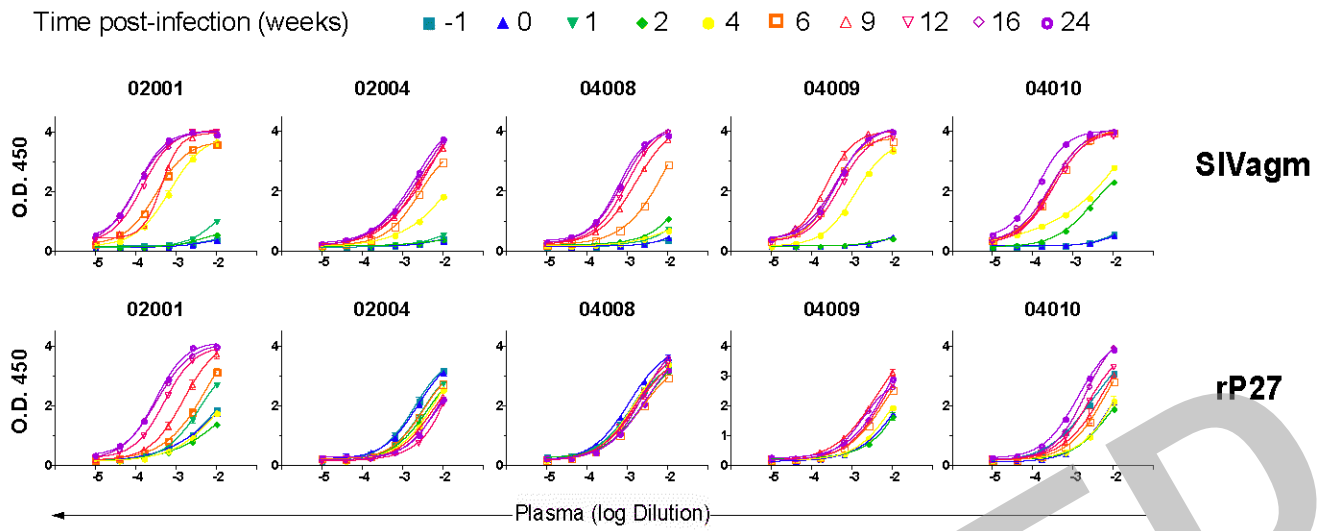
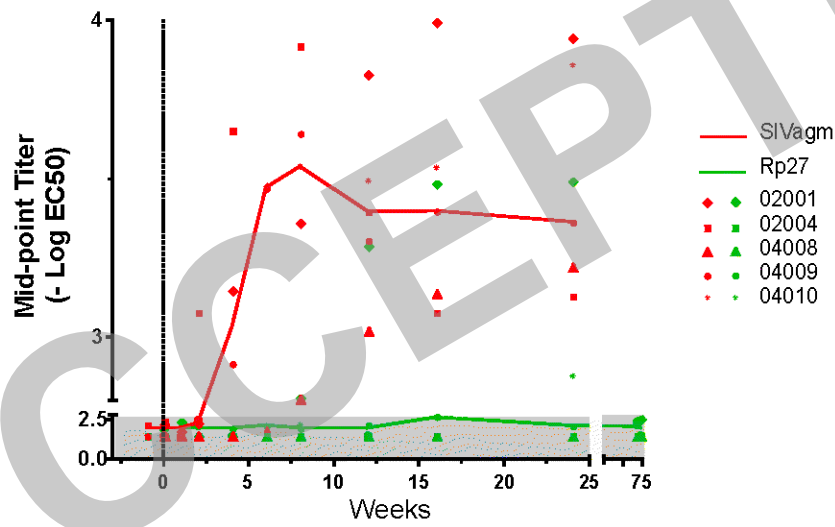


Figure 2

A



B



C

PBMCs

LNCs

CD4

% of CFSElow

% of CFSElow

CD8

Time post-infection (weeks)

Time post-infection (weeks)

Antigens:

□ Gag w/o p27

□ P27

□ rP27

□ Tat

AGMs ID:

◆ 02001

■ 02004

▲ 04008

● 04009

● 04010

