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Limited Ability of Humoral Immune Responses in Control of Viremia during Infection with SIVsmmD215 Strain

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Abstract

To investigate the impact of humoral immunity on SIV replication, 11 rhesus macaques (RMs) were inoculated with the neutralization-sensitive strain SIVsmmD215. Seven RMs were treated every three weeks, with 50 mg/kg of an anti-CD20 antibody (Rituxan, gift from Genentech) starting from day -7 p.i., as follows: four RMs were treated for two months, and three were treated for five months. The remaining four RMs were used as controls. Three RMs were completely depleted of CD20 cells. Four RMs only partially depleted CD20 cells in the LNs and intestine. The efficacy of tissue CD20 depletion predicted the ablation of antibody production, with SIVsmm seroconversion being delayed in the animals with complete tissue CD20 depletion, and neutralizing antibody production being significantly delayed and at low levels in all CD20-depleted RMs. There was no significant difference in acute or chronic VLs between CD20-depleted RMs and control monkeys, with a tendency for lower set-point VLs in CD20-depleted RMs. At 6 weeks p.i., cellular immune responses were significantly stronger in CD20-depleted RMs than in controls. After two years p.i., there was no significant difference in survival between CD20-depleted and control RMs. We concluded that CD20 depletion plays no significant role in the control of SIV replication or disease progression in SIVsmmD215-infected RMs.

Introduction

Twenty-five years of AIDS research has not resulted in the development of an effective anti-HIV vaccine. One of the major reasons for this failure is that the correlates of immune protection against HIV infection are still incompletely understood in spite of tremendous efforts (1, 2).

Cellular immune responses were reported to play an essential role in controlling HIV replication and disease progression, based on direct observations in patients and studies in rhesus macaques (RMs) (3-12). However, vaccines aimed at developing sustained cellular immune responses for SIV did not prevent infection or disease progression in RMs infected with pathogenic SIVmac strains (13). Moreover, recent failures of vaccine trials in humans suggest that CTL-based approaches should be significantly improved (14-16).

In this context, humoral immune responses during HIV infection are now considered a crucial component of an effective anti-HIV vaccine. Intravenously-administered antibodies can protect macaques against intravenous or mucosal SHIV challenge (17-20). However, in African green monkeys, very high amounts of passively transferred specific immunoglobulin failed to prevent SIVagm infection (21). Topically applied antibodies can also protect macaques against vaginal SHIV challenge (18, 22). Considering their prime role in many successful vaccines in the past, antibody-based vaccines were the first choice in the initial stages of vaccine development (23). However, the resistance of primary HIV isolates to neutralization has been a major hurdle (23). Hope for a solution to this challenge was provided by the observation that

neutralizing antibodies effective against primary patient isolates develop following many infections (24, 25). However, the neutralization activity tends to be rather type-specific and the high sequence variability in Env means that the virus can easily escape (25-27). Nevertheless, a fraction of patients go on to develop broadly HIV-neutralizing antibodies, providing a paradigm for what we would like to achieve with a vaccine (28).

Antibodies are soluble molecules that persist in circulation for long periods of time, therefore, B cell depletion studies assessing the role of humoral immune responses in controlling SIV replication can only be performed prior to SIV infection in order to prevent specific anti-SIV antibody production. As a result, experimental animal studies have provided less convincing results than $CD8^+$ depletion studies in supporting the involvement of humoral immune responses in the control of post-acute and chronic SIV replication (29, 30). Only two such experiments have been reported thus far and their results did not permit a clear conclusion on whether or not antibodies play an essential role for viral control (29, 30). In the first study, a short-term depletion of CD20 cells by infusion of an anti-CD20 commercial monoclonal antibody (Rituxan, Genentech) had no significant impact on the resolution of peak viremia in SIVmac251-infected RMs (30). However, the authors reported a temporal inverse correlation between the emergence of anti-SIV neutralizing antibodies and the control of viral replication during the post-acute phase of infection, suggesting that humoral immune responses may contribute to the control of chronic SIV replication. Note, however, that both humoral and cellular immune responses emerge simultaneously, and therefore this correlation between the control of VLs and neutralizing antibodies may be an epiphenomenon of viral control exerted by cellular immune responses, which were not ablated in these experiments. A

second study, in which Rituxan was administered continuously at a higher dosage until AIDS developed, reported a significantly higher rate of disease progression for RMs with complete CD20 depletion compared to RMs in which the anti-CD20 antibody induced only an incomplete depletion of the B cells (29). Note, however, that in this study RMs were infected with SIVmac239, a virus that is outstandingly resistant to antibody neutralization (29).

Here we report the identification of a neutralization-sensitive SIVsmm strain (D215) directly derived from a naturally-infected sooty mangabey (SM). Using this strain to infect RMs previously treated with a commercially available CD20-depleting monoclonal antibody (Rituxan, Rituximab, gift from Genentech, San Francisco, CA), we have investigated the role of anti-SIVsmm antibodies in the control of acute and post-acute viral replication. We report that, in spite of successful B cell depletion through Rituximab infusion, which resulted in the ablation of anti-SIV antibody responses, no effect was observed on virus replication in the acute and post-acute SIVsmmD215 infection. Therefore, we concluded that humoral immune responses have a limited impact on the control of SIV replication.

Materials and Methods (6616)

Virus characterization and stock preparation. During our previous survey of SIVsmm diversity in sooty mangabeys from US Primate Centers (31, 32), we identified a neutralization-sensitive SIVsmm strain (D215) (see supplementary information). An

SIVsmmD215 stock virus was grown in SM PBMCs and frozen at -80ºC for future studies (32). Plasma samples from the naturally-infected SM D215 were also collected.

Animals. Eleven male Indian RMs (*Macaca mulatta*) housed at the Tulane National Primate Research Center (TNPRC), were included in this study. Animals were housed at the TNPRC, an AAALAC International-accredited facility. Housing and handling of animals were in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. Public Health Service) (33) and the Animal Welfare Act. All protocols and procedures were reviewed and approved by the Tulane University Institutional Animal Care and Use Committee (IACUC). Included RMs were aged 4 to 7 years old at the project start date. All animals were MHC genotyped at the AIDS Vaccine Research Laboratory of the University of Wisconsin (Madison, WI), as previously described (34).

Rituxan treatments and virus inoculation. All eleven RMs were infected with plasma originating from the naturally-infected SM D215 at an infectious dose equivalent to 100,000 copies of SIVsmmD215.

One week prior to infection, seven RMs were treated intravenously with 50 mg/kg of Rituxan (Rituximab, gift from Genentech, Inc., South San Francisco, CA), a human-mouse chimeric, monoclonal anti-CD20 antibody. Additional administrations of Rituximab were given as follows: four RMs (DG04, CA16, CF03 and BT49) received three administrations (50 mg/kg) for up to two months [days 14, 35 and 56 post-infection (p.i.)]; three RMs (EL55, EI09 and EI74) received additional Rituximab infusions every

three weeks up to 5 months p.i. (days 77, 98, 119, 140 and 161 p.i.). Finally, four RMs (DG34, DH44, DD93 AND BV85), served as controls, were inoculated with SIVsmmD215 only and were not treated with Rituximab.

Follow-up was >2 years for control RMs and SIV-infected RMs that received Rituximab infusions for 2 months, and of 450 days for the 3 RMs that received Rituximab for 5 months.

Sampling of blood, LNs and intestine. For both Rituxan-treated and control RMs, 4.9 ml of EDTA-blood was collected twice per week during the first two weeks of infection, then weekly during the first month, bi-monthly for the first three months and then every two months during the follow-up. Plasma was separated by centrifugation (1700 RPM for 10 minutes), and peripheral blood mononuclear cells (PBMCs) were extracted as described (35).

Superficial LN (axillary and inguinal) biopsies were removed from animals using sterile surgical procedures on days -7, 0, 7, 10, 14, 28, 42, 120, 180, 240, 300, and 360 p.i. LN mononuclear cells were isolated as described (35).

Intestinal samples (proximal jejunum) consisting of approximately 10-15, 1-2 mm^2 pieces were obtained by endoscopic guided biopsy on days -7, 0, 10, 14, 21, 28, 42 and 56 p.i. Subsequent intestinal biopsies were collected at the same time points as blood samples, as described. In addition, two to three inch intestinal resections were removed from the animals surgically: before Rituximab treatment and virus inoculation, at the end of acute infection, and during the chronic infection (days -7; 28 and 300 p.i.).

Lymphocytes were separated from pinch biopsies and resections as described (36-39). Cells from blood, LNs and intestine were stained for flow cytometry or frozen at -80°C in freezing media.

Flow cytometry analysis of lymphocyte populations. Immunophenotyping of lymphocytes isolated from blood, LNs, and intestine was performed by using fluorescently-conjugated monoclonal antibodies and a four-color staining technique. The samples were run using a FacsCalibur flow cytometer (Becton Dickinson, San Jose, CA) and the data were analyzed using Cell Quest software (BD). The monoclonal antibodies used were as follows: CD3-fluorescein isothiocyanate (FITC) (clone no. SP34), CD69-FITC (clone no. FN50), CD95-FITC (clone no. DX2), CD8-phycoerythrin (PE) (clone no. SK1), CD20-PE (clone no. L27), CD3-PE (clone no. SP34), CD25-PE (clone no. 2A3), CCR5-PE (clone no. 3A9), CD4-peridinin chlorophyll protein (PerCP) (clone no. L200), CD3-PerCP (SP34-2), HLA-DR-PerCP (clone no. L243), CD28-allophycocyanin (APC) (clone no. CD28.8), CD20-APC (clone no. 2H7) (BD Bioscience). Ki-67-FITC (clone no. B56) and CD79a-APC (clone no. HM47) were used for intracellular staining (BD Biosciences). Whole blood and mononuclear cells from LNs and intestine were stained as previously described (37-39).

Plasma viral loads were determined by branched DNA assay (bDNA; Bayer Diagnostics).

Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded tissues (LNs and intestine from CD20-depleted and control RMs) using an avidin-biotin complex HRP technique (Vectastain Elite ABC kit; Vector Laboratories) and anti-CD79a (Dako Corporation, Carpinteria, Calif), anti-CD20 (Dako Corporation, Carpinteria, CA), and P28 SIV (Mardex Diagnostics, Carlsbad, Calif) as primary antibodies. Sections were visualized with DAB (Dako) and counterstained with hematoxylin.

Production of specific anti-SIV IgG was determined using four serological methods on serum or plasma samples from all animals at different time points p.i. Anti-gp41 and anti-V3 antibody titers were determined by a specific SIVsmm primate immunodeficiency virus enzyme immunoassay (PIV-EIA), as described (40). SIVmac Western blots (WB) (Zeptometrix Corporation, Buffalo, NY), were performed on serial plasma or serum samples to investigate the dynamics of anti-SIVsmm seroconversion. The neutralizing antibody titers were determined on serum samples from different time points of SIVsmmD215 infection, as described (41).

Cellular Immune Responses. Five milliliters of whole blood were collected in sodium heparin CPT vacutainers (BD Bioscience) on days 0, 14, 28, 42, 56, 90, 180, and 360. To separate the PBMCs, the CPT tubes were centrifuged at 1500 rpm for 15 minutes. CTL assays were performed on PBMCs as described previously (42).

Statistical Analysis of Data. To compare values of measured variables at a given time point between Rituximab-treated and control animals we used the non-parametric Mann-Whitney test. To analyze changes over time, such as those for HLA-DR, we used linear mixed-effects models (43). Significance was assessed at the α=0.05 level.

Results

During our survey of SIVsmm diversity in naturally-infected SMs housed in different Primate Centers in the US (44, 45), we identified a strain (SIVsmmD215) which is highly sensitive to neutralization by both autologous and heterologous sera (Supplementary Figure 1). Here, we have used this strain to identify the role of humoral responses in the control of SIVsmm replication in RMs.

Administration of anti-CD20 mAb depletes B cells in blood and induces variable B cell depletion in tissues. For CD20 depletion, seven RMs were repeatedly infused with Rituximab, as described above. At the time of SIV infection, seven days after the first Rituximab administration, peripheral blood levels of $CD20^+$ B cells were undetectable in all but one RM (Figure 1a). In the last RM (EI74), detectable CD20 levels were observed at the time of the Rituximab administration (days 14, 35 and 56 post-SIV infection), suggesting a more rapid rebound of $CD20^+$ cells. Interestingly, the efficacy of $CD20^+$ B cell depletion was independent of the duration of Rituximab administration. Thus, after the interruption of Rituximab treatment, major rebound of $CD20^+$ B cells occurred between days 180-200 p.i. in all animals, irrespective of the

duration of treatment (Figure 1a). Note that at the end of the follow-up, in two animals from the first group (DG04 and CA16) and one from the second group (EI09) peripheral $CD20^+$ cells were still significantly depleted (Figure 1a).

As Rituximab can cross-block anti-CD20 monoclonal antibodies used for the detection of B cells, CD79a was also included in the flow cytometry panel. Staining with CD79a showed slightly different dynamics of peripheral B cells in Rituximab-infused RMs (Figure 1b). Three animals demonstrated undetectable levels of $CD79a^+$ cells up to day 150 post-infection, similar to what was observed using the CD20 marker. However, the other 3 RMs demonstrated incomplete depletion of $CD79a^+$ B cells (Figure 1b). RM EI74 showed the same $CD79a^+$ dynamic pattern as that observed for the CD20 marker, with repeated rebounds prior to Rituximab administration (Figure 1b).

The flow-cytometric analysis of CD20 expression in LNs showed complete depletion of CD20 cells in 3 RMs (CF03, BT49 and EI09) starting from day 0 p.i. on (Figure 1c), whereas in the remaining 3 RMs (DG04, CA16 and EL55) only a partial CD20 depletion was observed (Figure 1c). The same dynamic pattern was observed in the intestine, where depletion was complete in 3 RMs (CF03, BT49 and EI09) and incomplete in 3 RMs (DG04, CA16 and EL55) (Figure 1d). The duration of tissue depletion of CD20 cells in animals receiving short-term treatments was similar to that observed in RMs that received long-term treatments (Figures 1c and 1d), and similar to what was observed in peripheral blood. Finally, note that RM EI74, in which repeated rebounds of peripheral $CD20^+$ and $CD79a^+$ B cells between consecutive Rituximab administrations occurred, showed a major depletion of CD79a B cells in tissues, different from those observed in

RMs with incomplete tissue CD20 depletion. In control RMs, there was no significant variation in the dynamics of CD20 cells during the follow-up (Figures 1a, 1c and 1d).

IHC for CD79a (Figure 2) confirmed the flow cytometry data, showing complete B cell depletion in three RMs at five weeks after the first administration of Rituxan (day 28 post-SIVsmm infection) in the LNs (Figure 2b) and effector (Figure 2h) and inductive (Figure 2n) sites in the intestine, as compared to baseline levels (Figures 2a, 2g and 2m). Furthermore, B cell depletion was incomplete in 3 RMs in the LNs (Figure 2e) and intestinal effector (Figure 2k) and inductive (Figure 2q) sites, as compared to baseline levels (Figures 2d, 2j and 2p). At day 300 post-infection, a significant, but partial restoration of LN and intestinal $CD79a^+$ cells was observed by IHC (Figures 2c, 2f, 2i, 2l, 2o and 2r). $CD20^+$ B cell depletion resulted in a significant reduction in the size of the germinal centers compared to undepleted control RMs (Figure 2).

Impact of B-cell depletion on anti-SIV dynamics. To investigate the impact of Rituximab-induced B cell depletion on humoral immune responses, we compared the dynamics of anti-SIV Ab in both CD20-depleted and undepleted RMs by using four different serological assays. We first investigated the dynamics of anti-SIVsmm gp41 seroconversion by ELISA. As shown in Figure 3a, delays in anti-gp41 seroconversion were observed in RMs (CF03, BT49 and EI09) that displayed complete tissue CD20 depletion. For RMs showing an incomplete tissue CD20 depletion (DG04, CA16 and EL55), as well as for the RM showing a rapid rebound in $CD20^+$ B cells, there was no difference in the seroconversion patterns or antibody titers, when compared to undepleted RMs (Figure 3a). We then investigated the dynamics of anti-V3 antibodies in both groups, using an SIVagm.sab-specific V3 peptide (40) and showed no significant

difference in the seroconversion pattern of anti-V3 titers between CD20-depleted and control RMs, irrespective of the efficiency of CD20 depletion (data not shown). Note that the anti-V3 seroconversion in RMs occurs later than anti-gp41, similar to what was previously reported in HIV-1-infected patients (46), which might explain the similar dynamics of anti-V3 seroconversions between RMs with complete and incomplete B cell depletion.

Testing serial sera on WB confirmed that there was no differences in seroconversion patterns between those animals in which only partial depletion of tissue CD20 cells occurred (Figure 3b) and control, undepleted animals (Figure 3b). Conversely, in those RMs in which tissue CD20 depletion was complete, significant delays in seroconversion patterns were observed by western blot testing of serial samples (Figure 3b).

Finally, testing the dynamics of SIV neutralizing antibodies demonstrated significant delays and lower titer of neutralizing antibodies in all the CD20 depleted RMs, irrespective of the efficacy of CD20 depletion (Figure 3c).

Impact of B cell depletion on viral trapping. To further confirm the efficacy of Rituximab treatment in ablating anti-SIV humoral responses, we investigated the impact of CD20 depletion on SIVsmm trapping in the germinal centers of the LNs and intestinal Peyer's Patches. Viral trapping occurs as a result of interactions on the surface of follicular dendritic cells between viral antigens, immunoglobulins, and complement particles (47, 48). LNs and intestinal tissues were stained for SIV. No evidence of viral trapping in the germinal centers was observed in any of the Rituximab-infused RMs, with the virus being dispersed throughout the tissue rather than congregating at the

germinal centers (Figure 4a and 4c), as opposed to significant trapping observed in control RMs (Figure 4b and 4d) These results offer an additional, albeit indirect, indication that Rituximab successfully ablated anti-SIV humoral immune responses at inductive tissue sites.

CD20 depletion has no impact on the control of SIVsmmD215 replication. No significant difference in the VLs between those RMs with undetectable levels of B cells in the blood and tissues and those animals with incomplete depletion in these compartments was observed (Figure 5). Moreover, there was no statistically significant difference in the SIVsmm RNA copies/ml between Rituximab-infused RMs and control RMs at either the viral peak ($p=0.11$) or viral set point ($p=0.11$ for the average VL between days 90 and 240) (Figure 5), Although these p-values could indicate a trend towards lower VLs in the Rituximab-treated group compared to the control RMs. Note that 6 out of 7 Rituximab-infused RMs showed undetectable VLs during the follow-up, as opposed to detectable VLs in all control RMs (Figure 5). The control of virus replication was not due to any particular MHC profiles, as all but one RM in this study were negative for the alleles associated with better control of SIV infection (data not shown). The exception was one RM in the Rituximab-treated group (CF03) which was positive for the Mamu-B*17 MHC allele, a marker that has been associated with "elite controller" status of SIV infection (49). However, CF03 replicated the virus at the highest levels in comparison to all other Rituxan treated animals (Figure 5).

Our results, showing no significant difference in post-acute viral replication between CD20-depleted and undepleted RMs infected with a neutralization-sensitive SIVsmm

strain strongly suggest that humoral immune responses are not sufficient in controlling SIV replication.

Comparative changes immune cells from different compartments in CD20$^+$ B cell depleted and undepleted monkeys. We investigated the effect of Rituximab on other lymphocyte subsets in the blood, LNs and intestine using flow cytometry. Comparing the animals treated with Rituximab to the control RMs showed no significant difference in CD4$^+$ T cell depletion in blood (Figure 6a and 6b) ($p>0.4$ at 6 weeks and 8 months), LNs (Figure 6c) ($p=0.11$), and intestine (Figure 6d) ($p=0.06$), indicating no significant impact on the SIV pathogenicity in Rituximab-infused RMs. Note, however, that on the late time points, there was a tendency for lower CD4$^+$ T cell counts in control animals when compared to Rituximab-infused RMs, in agreement with higher levels of viral replication in controls than in CD20$^+$ B cell-depleted RMs. Phenotyping the CD4$^+$ T cell subsets showed no significant difference in the dynamics of naïve, central memory and effector memory CD4$^+$ T cells in the blood (Figure 7 a, c and e) and intestine (Figure 7 b, d and f) between Rituximab-infused RMs with complete or incomplete CD20 depletion or between Rituximab-infused and control RMs (Figure 7).

Different from previous results reported during infection with highly pathogenic SIVmac strains (50, 51), RMs infected with SIVsmmD215 did not experience significant increases in the levels of CD4$^+$ T cell activation, as detected by the use of –DR phenotypic marker. A minimal, transient increase in -DR expression on CD4$^+$ T cells was observed during acute SIVsmmD215 infection in periphery (Figure 8a) and no change was observed in the LNs (Figure 8c). Even in the intestine, where the activation levels of CD4$^+$ T cells are higher than in periphery, there was no major increase in CD4$^+$ T cell immune activation following SIVsmmD215 infection, as detected by the use of –DR (Figure 8e). More significant increases in the levels of –DR expression were observed on CD8$^+$ T cells from peripheral blood (Figure 8b), LNs (Figure 8d) and intestine (Figure 8f). Note that, during the initial stages of SIVsmmD215 infection, no difference was observed in the levels of immune activation in CD20$^+$ B cell-depleted RMs and control RMs ($p>0.9$ and $p=0.2$, for CD4$^+$ and CD8$^+$ T cells in blood, respectively), or between monkeys with complete or incomplete CD20$^+$ B cell depletion (Figure 8). During the follow-up, higher levels of immune activation of CD4$^+$ and CD8$^+$ T cells were observed in control RMs, probably as a consequence of higher viral replication (Figure 8). Analysis of another T cell activation marker (CD69) showed a similar pattern of changes in blood, LNs and intestine (data not shown). The levels of CD4$^+$ and CD8$^+$ T cell proliferation, as detected by using Ki-67, were not significantly different between the different groups of RMs and not significantly different during the initial stages of the follow-up from baseline levels (data not shown). These results show that, unlike anti-CD8 antibodies used for CD8$^+$ T cell depletion *in vivo*, which induce significant increases in activation and proliferation of CD4$^+$ T cells, Rituximab infusion had no significant effect on proliferation and activation of CD4$^+$ and CD8$^+$ T cells in SIVsmmD215-infected RMs.

Cellular immune responses. ELISPOT. Ask miti if she can do some ICS. Two figures. (1) Cellular immune responses (statistical analyses). To determine the

effect that depletion of CD20 cells has on cellular immune responses, we tested PBMCs from Rituximab-treated and control animals in an ELISPOT assay specific for IFN-γ release. Cellular immune responses were higher in Rituximab-treated animals than in control animals (Figure 9). The magnitude of the response was statistically different comparing the Rituximab-treated animals to the control group at day 42 post-infection and almost reached statistical significance at day 14 post-infection. The breadth of the response to viral antigens was not statistically significant between either the Rituximab-treated group or the control animals at any time point. Therefore, the higher cellular responses could be an indicator of a compensatory mechanism in the absence of humoral responses. This result might also explain the trend towards lower viral loads in the Rituximab-treated group.

Discussion

In this study, by using an anti-CD20 depleting antibody to achieve prolonged, persistent B cell depletion and by performing SIVsmm infection of RMs, we investigated the impact of humoral immune responses on SIVsmm replication and disease progression. We report that anti-SIV antibodies have no significant impact on viral replication or disease progression during infection of RMs with the neutralization-sensitive SIVsmmD215 strain.

Although studies have shown that antibodies can prevent infection (18-20), thus raising some optimism with regard to the perspective of developing antibody-based vaccines that can prevent HIV spread, it was also reported that it is unlikely that antibodies can play a major role in controlling virus replication. This failure is due to a delay in antibody

production in the face of a very rapidly evolving virus, likely changed by the time when new generations of antibodies emerge. If this trait is corroborated with the heavy glycosylation of the HIV/SIV envelope which prevents antibodies from reaching their viral targets, one may understand that the odds of antibodies playing a major role in controlling viral replication in an infected host are very low.

However, previous and ongoing CD20 B cell depletion studies in SIV-infected RMs have reported a possible role for antibodies in controlling SIVmac251 replication or impacting the duration for disease progression during SIVsmm239 infection (29, 30). However, the major limitations of these studies that employed highly pathogenic SIV strains are that in most cases SIVmac infection is very unstable in RMs due to rapid progression to simian AIDS. Moreover, SIVmac strains are either partially resistant (SIVmac251) or highly resistant (SIVmac239) to neutralization (2) and therefore, humoral immune responses may have only a limited impact on the outcome of SIVmac infections. In our study, we circumvented these potential problems by performing $CD20^+$ B cell depletions in RMs infected with a different strain, SIVmacD215, that we recently identified in a naturally-infected SM during our surveys of SIVsmm diversity in Primate Centers (44). There are multiple advantages of using the primary SIVsmmD215 strain over the highly pathogenic SIVmac strains to assess the impact of humoral immune responses on SIV pathogenicity: (i) SIVsmmD215 strain is highly susceptible to neutralization by homologous and heterologous sera; (ii) SIVsmmD215 better mimics in RMs the pathogenesis of HIV-1 in humans, with lower set-points, progressive depletion of $CD4^+$ T cells and longer disease progression than highly pathogenic SIVmac strains. Thus, chronic SIVsmmD215 replication in RMs ranged from 10^2 to 10^4 SIVsmm215 RNA

copies/ml of plasma, significantly lower (3-4 logs) than those observed during SIVmac infections (52, 53) and was steady for long periods of time. Hence, changes in viral replication resulting from CD20 depletion could be easily observed during SIVsmmD215 infection, in contrast to highly pathogenic SIVmac infections that display a very limited control of viral replication and only for a short time interval. Moreover, the impact of SIVsmmD215 infection on $CD4^+$ T cells could be evaluated over a long period of time, as opposed to pathogenic infections where catastrophic $CD4^+$ T cell depletion occurs (52). Finally, SIVsmmD215-infected RMs showed significantly slower disease progression compared to SIVmac-infected RMs. Thus, differences in disease progression between CD20-depleted RMs and controls could be readily observed.

Chronic administration of anti-CD20 mAb infusion in RMs resulted in complete depletion in 3/7 monkeys and incomplete depletion in 3/7, whereas in the remaining RM the depletion, although complete, was shorter than the time interval between two administrations (Figure 1). As a consequence, anti-SIV IgG antibody production, as defined by 4 serological methods, varied among RMs included in these experiments: it was completely abolished in those RMs showing complete CD20 depletion, while detectable titers of anti-SIV IgG were observed in the remaining ones. Although the levels of CD20 cells were significantly lower in RMs with incomplete CD20 depletion compared to controls, there was no significant difference in seroconversion timing or antibody titers between animals in the two groups, suggesting that the low levels of residual tissue B cells are sufficient to produce an anti-SIV humoral immune response that is not quantitatively different from those observed in undepleted animals. Note that the incomplete CD20 depletion could only be defined based on the levels observed in

tissues, as CD20 levels in peripheral blood were undetectable in all Rituximab-infused animals.

There were no significant differences in the dynamics of VLs between Rituximab-infused RMs with complete or incomplete CD20 depletion. Moreover, although there was no significant difference in VLs between Rituximab-infused and control RMs, it can be observed from Figure 5 that there was a tendency for lower VLs in the group of Rituximab-infused RMs, as compared to undepleted RMs in the control group. This better control of viral replication in Rituximab-infused animals may be related to stronger cellular immune responses and resulted in a more limited depletion of both total $CD4^+$ T cell population and different (naïve, central memory or effector memory) cellular subsets in Rituximab-infused RMs as compared to the control group.

Our results showing no negative impact on viral replication following $CD20^+$ B cell depletion through Rituximab infusions are different from those reported during experimental CD8 depletion with anti-CD8 monoclonal antibodies that resulted in significant increases of viral replication during both acute and chronic SIV infection (54-57). These differences support the concept that cellular immune responses are essential in controlling viral replication, whereas humoral immune responses have only a limited impact on viral dynamics. However, an alternative explanation to these differences may rely on the observation that the monoclonal antibodies used for $CD8^+$ cell depletions induce significant levels of immune cell activation (Picker et al, unpublished observation), while, as reported in our study, Rituximab infusion did not result in detectable increases of T cell immune activation or proliferation.

Finally, no difference in RM survival was observed between Rituximab-infused and control monkeys in this study, nor between treated RMs showing complete or incomplete CD20$^+$ B cell depletion. At 2 years post-infection survival in the control group was 75%, whereas in the CD20-depleted group survival was 85%. Note that this follow-up is significantly longer than any other survival reported thus far for CD20-depleted animals in previous studies (29, 30). As most of the studies were carried out with highly pathogenic SIVmac strains, the shorter duration of disease progression during such highly pathogenic infections did not allow such a long term assessment of the impact of CD20 B cell depletion on survival.

Based on our results, we concluded that humoral immune responses have only a limited impact on the control of viral replication or disease progression during the chronic infection with the neutralization-sensitive SIVsmmD215 strain. However it has to be stressed that this observation does not preclude a role for antibodies in preventing SIV/HIV transmission and therefore the possibility of developing an effective anti-HIV vaccine based on stimulating specific humoral immune responses.

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Figure captions

Figure 1. Effect of rituximab infusion on concentrations of CD20+ B cells in blood (a-percentags and b-absolute counts), lymph nodes (c) and intestine (d). Black symbols and lines denote the control monkeys (DG34, DH44, DD93 and BV85). Red symbols and lines denote monkeys in which rituximab administration induced complete CD20 depletion in both peripheral blood and tissues (CF03, BT49 and EI09). Green symbols and lines indicate monkeys in which rituximab induced complete peripheral but incomplete tissue CD20+ B cell depletion (DG04, CA16 and EL55). The violet dots and line indicate a RM in which $CD20^{+}$ B cell depletion was achieved after every rituximab administration but CD20 cells rebound prior to the following treatment. Day 0 is the day of SIV inoculation. Rituximab was infused every 21 days beginning 1 week prior to SIV inoculation. RMs DG04, CA16, CF03 and BT49 received 4 rituximab administrations. RMs EI74, EI09 and EL55 received rituximab for up to 160 days p.i.

Figure 2. Effect of rituximab infusion on the frequencies of CD79a+ B cells in tissues. Panels a-f demonstrate the rituximab effect on LN CD79a+ B cells in RM EI09 (a-c) that showed complete B cell depletion in tissues *versus* RM EL55 (d-f) that showed complete B cell depletion in tissues. LNs were collected before (day -7 p.i.) (a and d) at 28 days p.i. (b and e) and at day 300 p.i. (c and f). Panels g-r demonstrate the rituximab effect on intestinal $CD79a^{+}$ B cells from both lamina propria (g-l) and Peyer's patches (m-r).

Figure 3. Impact of B cell depletion following rituximab administration on anti-SIVsmm humoral immune responses. (a) ELISA testing of anti-SIVsmm gp41 antibodies showed significant delay in the seroconversion patterns in RMs showing complete tissue CD20+ B cell depletion (red symbols and lines) and no significant difference between control RMs (black symbols and lines) and Rituximab-treated RMs that showed only incomplete tissue CD20+ B cell depletion (green symbols and lines). (b) Western blot testing on serial samples confirmed the trend observed by ELISA, with no significant difference in the seroconversion patterns between control RMs (illustrated by RMs DG34 and DH44) and Rituximab-infused RMs with incomplete tissue depletion (illustrated by RMs DG04 and CA16). Significant delays in the WB seroconversion were observed in RMs with complete CD20+ B cell depletion after rituximab infusion (illustrated by RMs CF03 and BT49). (c) Neutralizing antibody testing showed delayed seroconversion and lower titers in RMs infused with Rituximab. RMs showing complete tissue CD20 B cell depletion showed the lowest Nab titers. Day 0 corresponds to SIV inoculation. Rituximab was infused every 21 days beginning 1 week prior to SIV inoculation. RMs DG04, CA16, CF03 and BT49 received 4 rituximab administrations. RMs EI74, EI09 and EL55 received rituximab for up to 160 days p.i.

Figure 4. Lack of viral trapping in LNs (a) and Peyer's patches (c) of Rituximab-infused RMs showing complete CD20+ B cell depletion, as compared to control RMs (b and d). The effect on viral trapping indirectly confirms the ablation of anti-SIV humoral immune responses, as the trapping results from complement fixation of AG-Ab

immune complexes captured by the DCs. Both LN sampling and intestinal resections were performed at day 28 p.i. Magnification: Germinal centers are marked by circles.

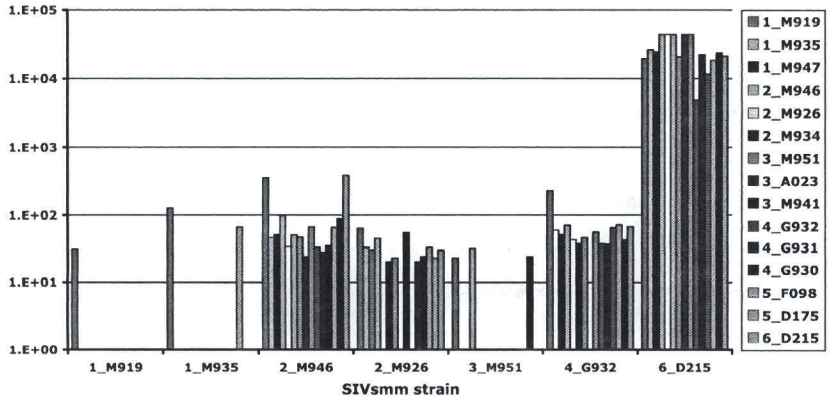
Figure 5. Dynamics of SIVsmm D215 plasma vRNA loads in rituximab-infused RMs and control monkeys. Black symbols and lines denote the control monkeys (DG34, DH44, DD93 and BV85). Red symbols and lines denote monkeys in which rituximab administration induced complete CD20 depletion in both peripheral blood and tissues (CF03, BT49 and EI09). Green symbols and lines indicate monkeys in which rituximab induced complete peripheral but incomplete tissue $CD20^+$ B cell depletion (DG04, CA16 and EL55). The violet dots and line indicate a Rh in which CD20+ B cell depletion was achieved after every rituximab administration but CD20 cells rebounded prior to the following treatment. Day 0 is the day of SIV inoculation. Rituximab was infused every 21 days beginning 1 week prior to SIV inoculation. RMs DG04, CA16, CF03 and BT49 received 4 rituximab administrations. RMs EI74, EI09 and EL55 received rituximab for up to 160 days p.i.

Figure 6. Changes in $CD4^+$ T cells in blood (a-percentages and b-absolute counts), lymph nodes (c) and intestine (d) in rituximab-infused RMs with complete CD20 depletion (ν), incomplete tissue CD20 depletion (τ) and in control RMs (λ). Plots represent the average expression for the animals in each study group. Vertical lines represent the SEM.

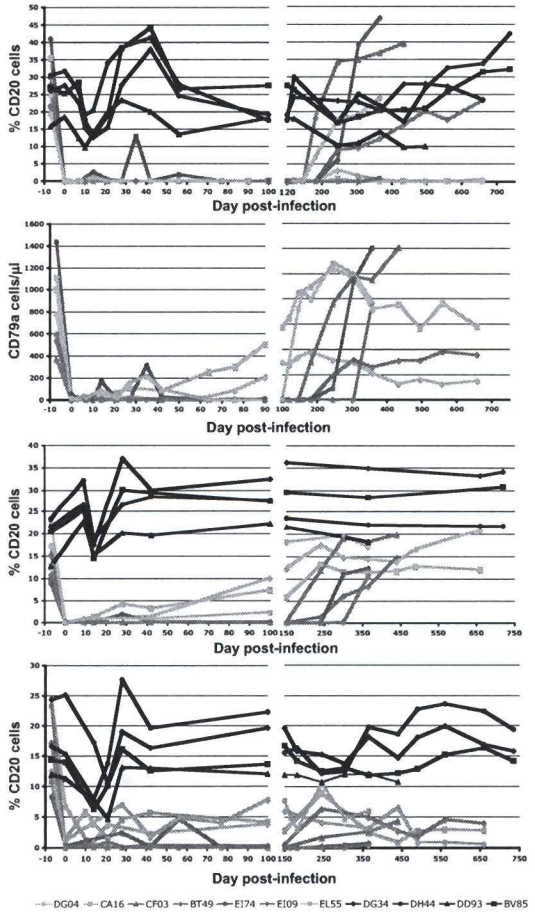
Figure 7. Changes in $CD4^+$ T cells subsets in blood (a, c, e) and intestine (b, d, f) in rituximab-infused RMs with complete CD20 depletion (ν), incomplete tissue CD20 depletion (τ) and in control RMs (λ). Dynamics of naïve ($CD3^+$ $CD4^+$ $CD28^+$ $CD95^{neg}$) (a and b), central memory ($CD3^+$ $CD4^+$ $CD28^+$ $CD95^+$) (c and d) and effector memory ($CD3^+$ $CD4^+$ $CD28^{neg}$ $CD95^+$) (e and f) is presented. Plots represent the average expression for the animals in each study group. Vertical lines represent the SEM.

Figure 8. Dynamics of $CD4^+$ and $CD8^+$ T cell immune activation (as defined by changes in the expression of –DR markers) in blood (a and b), lymph nodes (c and d) and intestine (e and f) in rituximab-infused RMs with complete CD20 depletion (ν), incomplete tissue CD20 depletion (τ) and in control RMs (λ). Plots represent the average expression for the animals in each study group. Vertical lines represent the SEM.

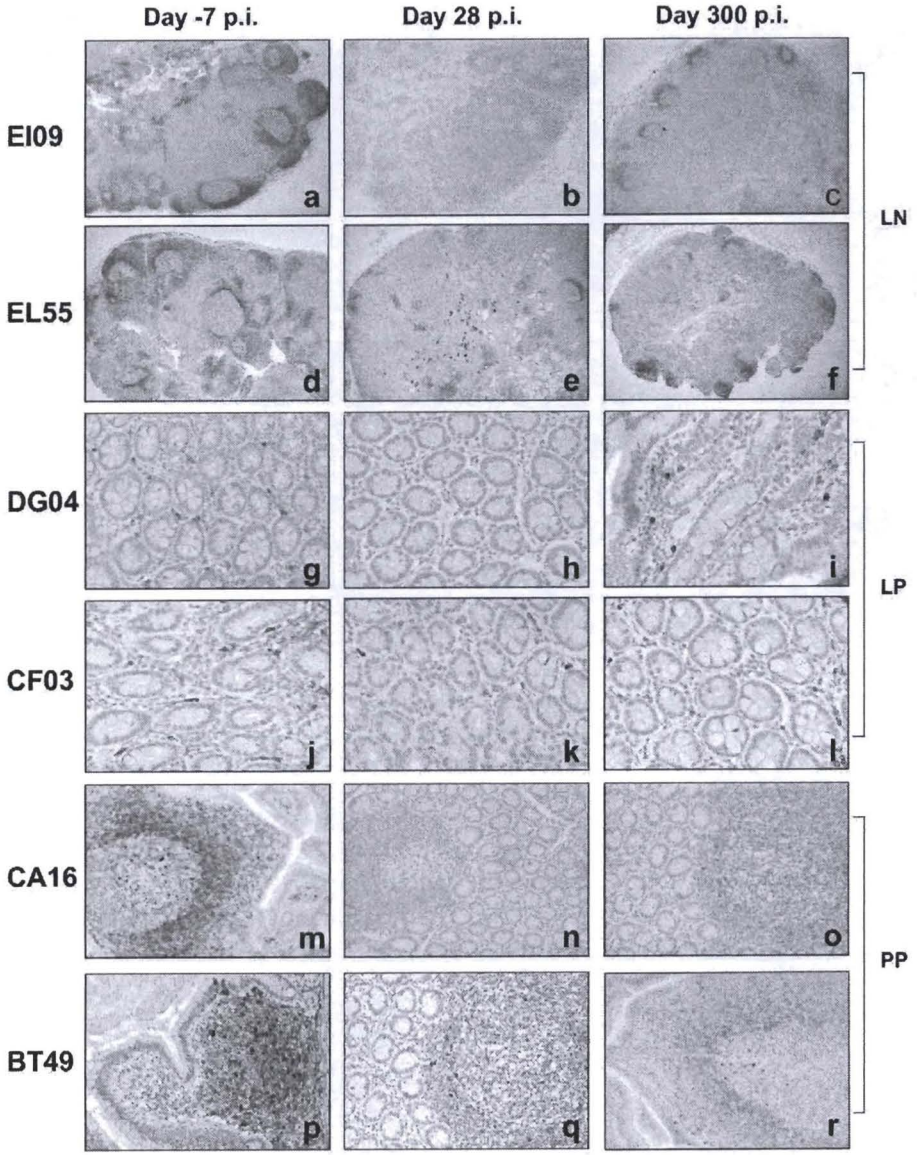
Figure 9. SIV-specific cellular immune responses, as assessed by Elispot assay with peptide pools mapping the entire SIVsmm proteome. Cellular immune responses (in boxes) are presented in relation to SIVsmm RNA plasma VLs (black lines).



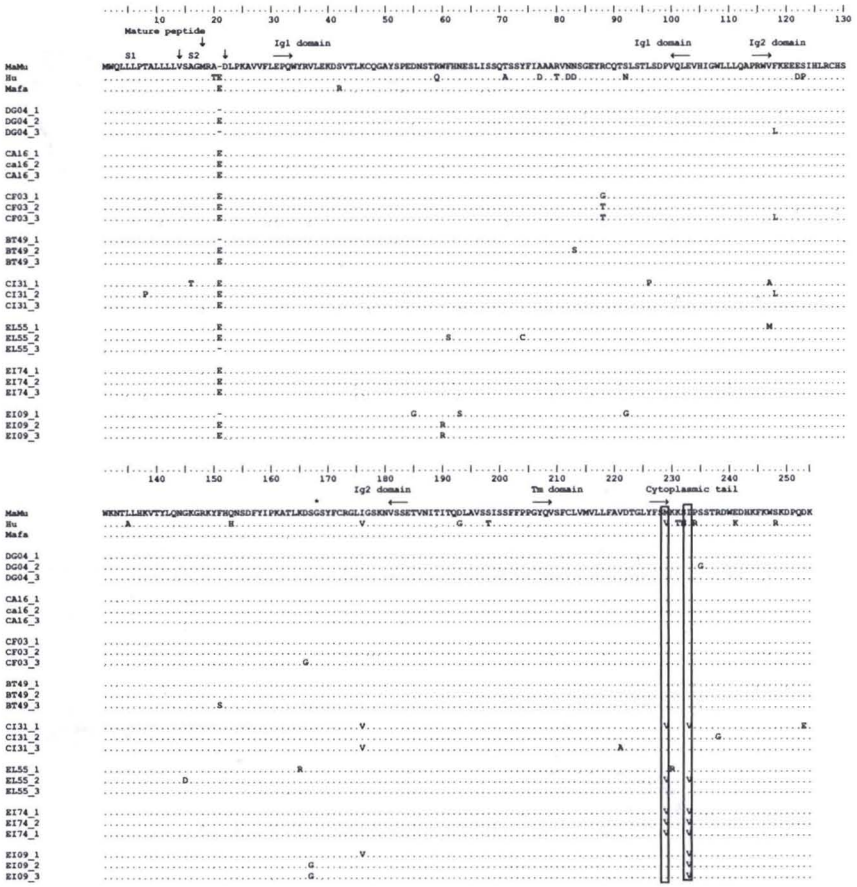
Gaufin et al., Figure 1



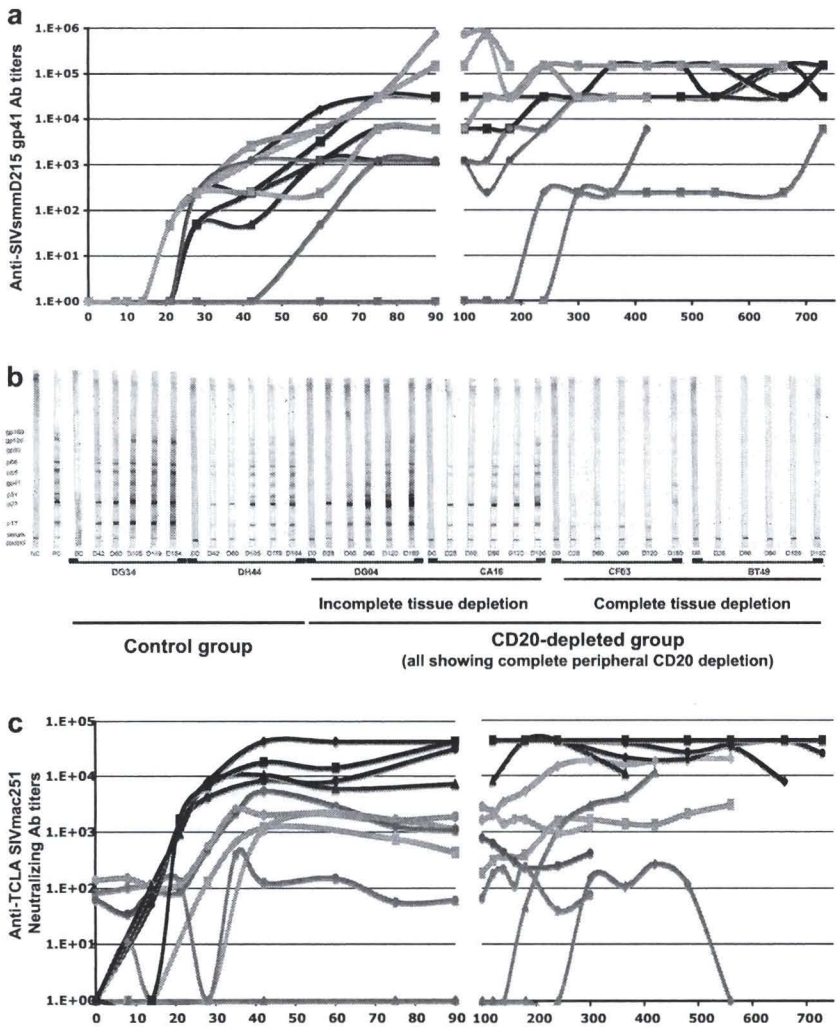
Gaufin et al., Figure 2



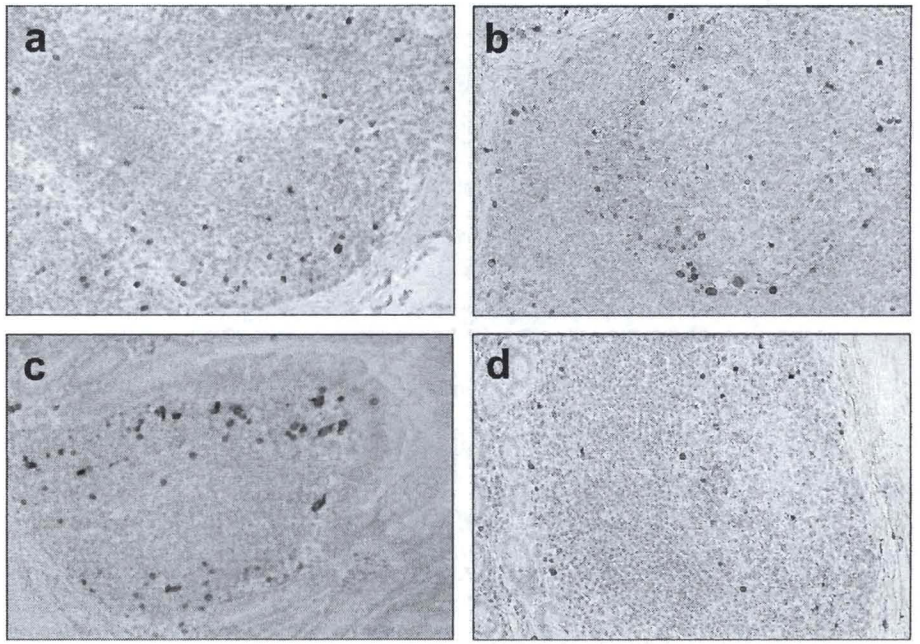
Gaufin et al., Figure 3



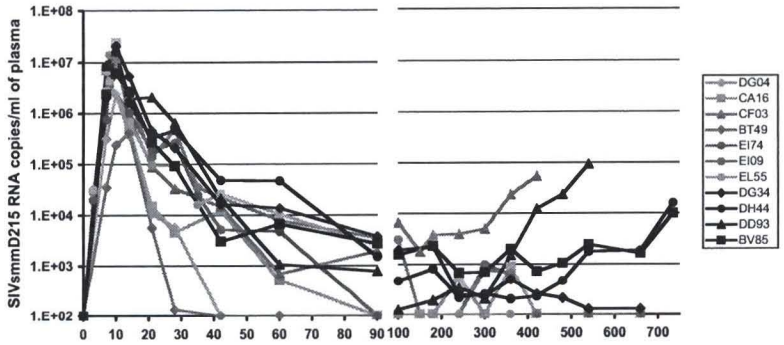
Gaufin et al., Figure 4



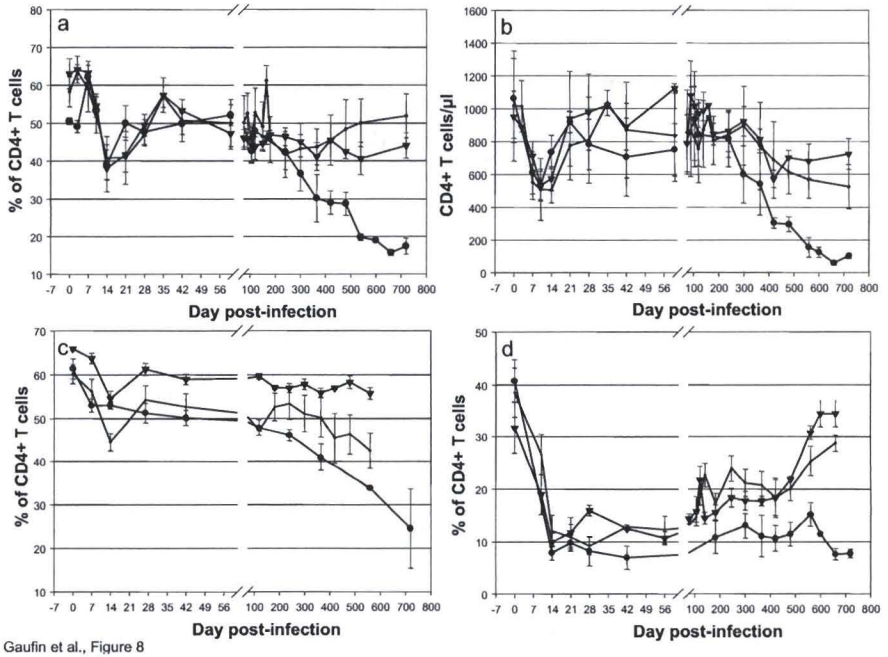
Gaufin et al., Figure 5



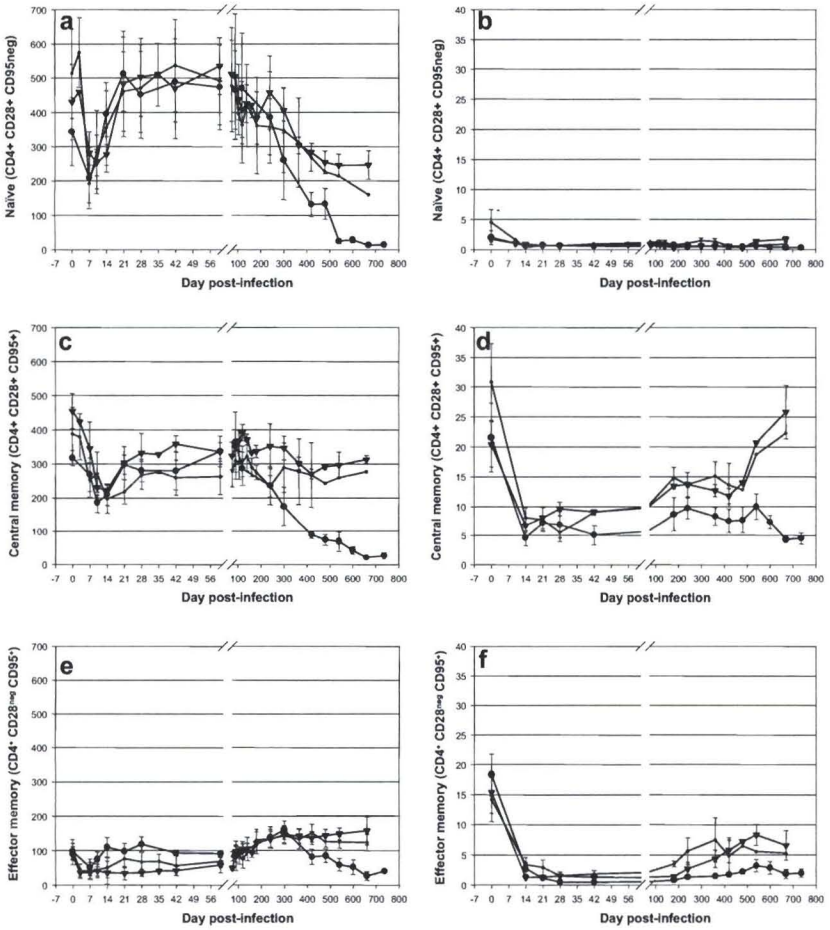
Gaufin et al., Figure 6



Gaufin et al., Figure 7



Gaufin et al., Figure 8



Gaufin et al., Figure 9