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Effect of B-Cell Depletion on Viral Replication and Clinical Outcome of Simian Immunodeficiency Virus Infection in a Natural Host▽†

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Simian immunodeficiency virus (SIV)-infected African nonhuman primates do not progress to AIDS in spite of high and persistent viral loads (VLs). Some authors consider the high viral replication observed in chronic natural SIV infections to be due to lower anti-SIV antibody titers than those in rhesus macaques, suggesting a role of antibodies in controlling viral replication. We therefore investigated the impact of antibody responses on the outcome of acute and chronic SIVagm replication in African green monkeys (AGMs). Nine AGMs were infected with SIVagm.sab. Four AGMs were infused with 50 mg/kg of body weight anti-CD20 (rituximab; a gift from Genentech) every 21 days, starting from day −7 postinfection up to 184 days. The remaining AGMs were used as controls and received SIVagm only. Rituximab-treated AGMs were successfully depleted of CD20 cells in peripheral blood, lymph nodes (LNs), and intestine, as shown by the dynamics of $CD20^+$ and $CD79a^+$ cells. There was no significant difference in VLs between CD20-depleted AGMs and control monkeys: peak VLs ranged from 10^7 to 10^8 copies/ml; set-point values were 10^4 to 10^5 SIV RNA copies/ml. Levels of acute mucosal $CD4^+$ T-cell depletion were similar for treated and nontreated animals. SIVagm seroconversion was delayed for the CD20-depleted AGMs compared to results for the controls. There was a significant difference in both the timing and magnitude of neutralizing antibody responses for CD20-depleted AGMs compared to results for controls. CD20 depletion significantly altered the histological structure of the germinal centers in the LNs and Peyer's patches. Our results, although obtained with a limited number of animals, suggest that humoral immune responses play only a minor role in the control of SIV viral replication during acute and chronic SIV infection in natural hosts.

In marked contrast to pathogenic human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections of humans and macaques, which are characterized by the constant progression to AIDS in a variable time frame (26), African monkey species naturally infected with SIV are generally spared from any signs of disease (reviewed in references 53 and 71).

There are currently three animal models of SIV infection in natural hosts: SIVagm infection of African green monkeys (AGMs), SIVsmm infection of sooty mangabeys, and SIVmnd-1 and SIVmnd-2 infection of mandrills (53, 71). SIV infection in natural hosts is characterized by the following: (i) active viral replication, with set-point viral loads (VLs) similar to or even higher than those found in pathogenic infections (44–46, 49, 50, 52, 61–63); (ii) transient depletion of peripheral $CD4^+$ T cells during primary infection, which rebound to pre-infection levels during chronic infection (12, 30, 44–46, 49, 62); (iii) significant $CD4^+$ T-cell depletion in the intestine, which can be partially restored during chronic infection in spite of significant viral replication (21, 48); (iv) low levels of $CD4^+$ $CCR5^+$ cells in blood and tissues (47); (v) transient and moderate increases in immune activation and T-cell proliferation during acute infection, with a return to baseline levels during the chronic phase (44–46, 49, 50, 52, 61–63), as a result of an anti-inflammatory milieu which is rapidly established after infection (14, 30); and (vi) no significant increase in $CD4^+$ T-cell apoptosis during either acute or chronic infection (37, 48), thus avoiding enteropathy and microbial translocation, which control excessive immune activation and prevent disease progression by allowing $CD4^+$ T-cell recovery in the presence of high VLs (21, 48). Hence, the current view is that the main reason behind the lack of disease progression in natural African hosts lies in a better adaptation of the host in response to the highly replicating virus. A better understanding of the mechanisms underlying the lack of disease in natural hosts for SIV infection may provide important clues for understanding the pathogenesis of HIV infection (53, 71).

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To date, it is still unknown whether or not immune responses are responsible for the lack of disease progression in natural hosts, since data are scarce. Studies of cellular immune responses are significantly more limited than is the case with pathogenic infection, and although not always in agreement (3, 13, 28, 29, 73, 76), their convergence point is that cellular immune responses are not essentially superior to those observed in pathogenic infections (3, 13, 28, 29, 73, 76). This observation is not surprising in the context of the high viral replication in natural hosts. Data are even scarcer on the role of humoral immune responses in the control of disease progression in natural hosts. However, several studies reported that anti-SIV antibody titers are lower in SIV infections of natural hosts, with a lack of anti-Gag responses being characteristic of natural SIV infections in African nonhuman primates (1, 6, 24, 25, 42, 43, 71). Because the viral replication in SIVagm-infected AGMs is of the same magnitude or higher than that in pathogenic infections of rhesus macaques (RMs), it has been hypothesized that these high VLs may be a consequence of the lower antibody titers. Moreover, a recent study has also shown that B cells in lymph nodes (LNs) of AGMs are activated at an earlier time point than is the case for SIVmac251-infected RMs, which implies that humoral immune responses may be important in controlling SIV replication in the natural hosts (9). Conversely, it has been shown that passively transferring immunoglobulins from animals naturally infected with SIVagm prior to infection with a low dose of SIVagm did not prevent infection in AGMs (42, 60), which is in striking contrast to results in studies of pathogenic infections, which convincingly demonstrated with animal models that intravenously administered or topically applied antibodies can protect macaques against intravenous or mucosal simian-human immunodeficiency virus challenge (34–36, 54, 72).

Previous $CD20^+$ B-cell-depletion studies during pathogenic RM infections have indicated that humoral immune responses may be important for controlling both the postpeak VL and disease progression (38, 57). However, these studies used strains that are highly resistant to neutralization (SIVmac251 and SIVmac239), making it difficult to assess the role that antibodies have in controlling SIV replication and disease progression. Moreover, our recent results suggested a limited impact of humoral immune responses in controlling replication of a neutralization-sensitive SIVsmm strain in rhesus macaques (18).

To investigate the effect that $CD20^+$ B cells and antibodies have on SIV replication in natural hosts, we have depleted $CD20^+$ B cells in vivo in AGMs infected with SIVagm.sab92018. We assessed the impact of humoral immune responses on the control of viral replication and other immunological parameters, and we report that ablating humoral immune responses in SIVagm-infected AGMs does not significantly alter the course of virus replication or disease progression.

MATERIALS AND METHODS

Animals. Nine Caribbean AGMs (*Chlorocebus sabaeus*) were included in this study. They were housed at the Tulane National Primate Research Center (TNPRC), which is an Association for Assessment and Accreditation of Laboratory Animal Care international facility. All animals were adults ranging from 4.5 to 10 years. The animals included in the rituximab-treated group were selected with lower weights, and they were younger than the ones in the control group (average age, 4.8 years versus 7.3 years, respectively). The animals were fed and housed according to regulations set forth by the *Guide for the Care and Use of Laboratory Animals* (41) and the Animal Welfare Act. The animal experiments in this study were approved by the Tulane University Institutional Animal Care and Use Committee.

Rituximab treatments and virus inoculation. All nine AGMs were inoculated intravenously with plasma corresponding to 300 50% tissue culture infection doses of SIVagm.sab92018 (46). Seven days prior to the infection, four AGMs were treated intravenously with 50 mg/kg of body weight of rituximab (a gift from Genentech, Inc., south San Francisco, CA), a human-mouse chimeric, monoclonal anti-CD20 antibody. Rituximab was then administered every 21 days for up to 184 days postinfection (p.i.). The five AGMs not treated with rituximab served as controls and were inoculated with virus only.

Blood, LNs, and intestine sampling. Blood was collected from all the animals at three time points preinfection (days −35, −14, and −7 p.i.) and then at the time of SIVagm.sab inoculation, twice per week for the first 2 weeks p.i., weekly for the next 4 weeks, and then every 3 weeks for up to 6 months p.i.

LN biopsy specimens were sampled using sterile surgical procedures on days −14, −7, 10, 21, 42, 98, 132, and 184 p.i., as described previously (46, 48). Intestinal endoscopies (proximal jejunum) consisting of approximately 10 to 15 1- to 2-mm^2 pieces were obtained by endoscopy-guided biopsy on days −14, 0, 8/10, 21, 35, 42, 56, 77, 98, 119, 142, 163, and 184 p.i., as described previously (46, 48). Intestinal resections (5 to 10 cm) were performed at days −7 and 28 p.i., as described previously (46, 48, 51). Additional intestine pieces were obtained at necropsy. The control animals followed a similar sampling schedule.

Isolation of lymphocytes. Mononuclear cells were separated from the blood through Ficoll density gradient centrifugation. Lymphocytes from the intestine and LNs were isolated and stained for flow cytometry as described previously (46, 48, 51). Briefly, lymphocytes were isolated from intestinal biopsy specimens using EDTA followed by collagenase digestion and Percoll density gradient centrifugation (46, 48, 51). Lymphocytes were isolated from the axillary LNs by gently mincing and pressing tissues through nylon mesh screens.

Flow cytometry. Mononuclear cells derived from peripheral blood, intestinal biopsy specimens, and LNs were stained for flow-cytometric analysis using four-color staining combinations with monoclonal antibodies (MAbs) as follows: CD3-fluorescein isothiocyanate (FITC), CD20-phycoerythrin (PE), CD8-peridinin chlorophyll A protein (PerCP), and CD4-allophycocyanin (APC); Ki-67–FITC, CD20-PE, CD3-PerCP, and CD79a-APC; CD3-FITC, CD8β–PE (Beckman Coulter), HLA-DR–PerCP, and CD4 APC; and Ki-67-FITC, CD8-PE, CD3-PerCP, and CD4-APC. All MAbs are from BD Bioscience Pharmingen, San Diego, CA, unless otherwise indicated. Cells were incubated with an excess amount of monoclonal antibodies at 4°C for 30 min, followed by a phosphate-buffered saline wash (400 × *g*, 7 min) and fixation in 2% paraformaldehyde. Whole blood was stained using a whole blood lysis technique, as described previously (46, 48, 51). Samples were stained for Ki-67, as per the manufacturer's instructions. Stained cells were analyzed with a FACSCalibur flow cytometer (BD Immunocytometry Systems) and analyzed with CellQuest software (BD). $CD4^+$ and $CD8^+$ T-cell percentages were obtained by gating first on lymphocytes and then on $CD3^+$ T cells. Memory, activation, and proliferation markers were determined by gating on lymphocytes, next on $CD3^+$ T cells, and finally on $CD4^+$ $CD3^+$ or $CD8^+$ $CD3^+$ T cells.

Viral load quantification. Plasma VLs were quantified by real-time PCR, as described previously (46, 49). SIVagm.sab RNA loads were also quantified in mononuclear cells isolated from blood, LNs, and intestinal biopsy specimens using the same real-time PCR assay (46, 49). For tissue quantification, viral RNA was extracted from 5×10^5 to 10^6 cells from peripheral blood mononuclear cells (PBMCs), LNs, and intestine with RNeasy (Qiagen), and VLs were quantified as described elsewhere (48, 51). Simultaneous quantification of RNase P (RNase P detection kit; Applied Biosystems, CA), a single-copy gene with two copies per diploid cell, was done to normalize sample variability and allow accurate quantification of cell equivalents (48). The assay sensitivity was 10 RNA copies/10^5 cells and 100 RNA copies per 1 ml of plasma.

Analysis of anti-SIVagm.sab immunoglobulin G responses. An in-house SIVagm.sab-specific primate immunodeficiency virus enzyme immunoassay was used for the titration of anti-gp41 and anti-V3 antibody titers, as described previously (64), on serial plasma or serum samples to investigate the dynamics of anti-SIVagm.sab seroconversion. SIVagm.sab neutralization was measured using a new neutralization assay. An SIVagm.sab-specific molecularly cloned Env-pseudotyped virus containing full-length gp160 of SIVagm.sab92018 (clone 28) was prepared as described previously (40). Neutralization titers were then measured as 50% reductions in luciferase reporter gene expression in TZM-bl cells, as reported previously (40).

Immunohistochemistry (IHC) analysis was carried out on LNs and intestinal samples. Tissues were formalin fixed, paraffin embedded (LNs and intestine from CD20-depleted and control RMs), and probed using an avidin-biotin complex

horseradish peroxidase technique (Vectastain Elite ABC kit; Vector Laboratories) and anti-CD79a (Dako Corporation, Carpinteria, CA) and anti-CD20 (Dako Corporation, Carpinteria, CA) as primary antibodies. Sections were visualized with 3,3′-diamidino-benzidene (Dako) and counterstained with hematoxylin.

Cytokine determination. Cytokine testing in plasma was done using a sandwich immunoassay-based protein array system, the Human Cytokine 25-Plex system (Biosource International, Camarillo, CA), as instructed by the manufacturer, and results were read by the Bio-Plex array reader (Bio-Rad Laboratories, Hercules, CA), which uses Luminex fluorescent-bead-based technology (Luminex Corporation, Austin, TX).

FCRγIIIA polymorphisms. Gene sequences of FCRγIIIA were investigated to confirm reports on both rituximab-administered humans and macaques which have indicated that there is an association between FCRγIIIA polymorphisms and the effectiveness of rituximab in inducing CD20 depletion (2, 5, 38).

DNA was extracted from PBMCs using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) and subjected to different PCRs, using primers and conditions as described previously (56). Exons 1 and 2 were each amplified as single PCR amplicons; exons 3 and 4 were each amplified in two overlapping fragments; the coding region and partial 3′ untranslated region of exon 5 were amplified in a single amplicon using seminested primers, as described previously (56). In addition, to characterize the coding regions, RNA was extracted from RM PBMCs using the QIAamp RNA blood mini kit (Qiagen). PCR amplification was carried out using the One Step PCR kit (Qiagen) using PCR conditions and primers as described previously (69). All reactions were carried out in at least two independent reverse transcription-PCRs to verify product sequences. PCR products were cloned using the TOPO XL PCR cloning kit (Invitrogen). Four clones from each AGM were then sequenced, and sequence analyses were performed using methods described previously (69).

Statistical analysis of data. Data comparisons between AGMs depleted of $CD20^+$ B cells and controls were done using two-tailed nonparametric tests (Mann-Whitney). These tests included analyses of maximum depletion of CD4 and increases in HLA-DR and Ki-67 over the first month, when the effects of infection were most pronounced (see Results). The transient effects of infection on CD20 levels in controls were analyzed by linear mixed-effects models over the appropriate period. Where needed, appropriate transformations were applied so that the assumptions of homoscedasticity and normality of residuals were met. Significance was assessed at the $\alpha = 0.05$ level, and analyses were performed using S-Plus 2000 (MathSoft Inc., Massachusetts). The area under the curve of VL was determined by numerical integration of a spline interpolation of the logarithm of the data to avoid overemphasizing the peak of viral infection (Mathematica 6.0; Wolfram Research Inc., Illinois).

RESULTS

Anti-CD20 MAb treatment successfully depleted B cells in blood and tissues of AGMs. Four AGMs were repeatedly infused with rituximab every 3 weeks starting from day −7 up to day 184 p.i. At the time of SIV infection, 7 days after the first rituximab administration, the percentages and absolute numbers of $CD20^+$ B cells in the peripheral blood of the rituximab-administered animals were at undetectable levels (Fig. 1a). At the end of the follow-up, 6 months into infection, peripheral $CD20^+$ cells were still depleted for all treated AGMs (Fig. 1a).

It has been reported that treatment with the anti-CD20 MAb may block the CD20 marker on B cells (38). Therefore, to ensure that B cells were depleted and not blocked through rituximab infusion, staining with another B-cell marker (CD79a) was performed to assess the effectiveness of rituximab in depleting B cells in AGMs. CD79a is one of the components of the B-cell antigen receptor and is expressed at the pro-B-cell stage (17). Flow cytometry assessment of the $CD20^+$ $CD79a^+$ B-cell population confirmed the significant depletion of B cells following rituximab infusion (Fig. 1b).

In our previous study of $CD20^+$ B-cell depletion in RMs infected with SIVsmmD215, we observed that the ablation of humoral immune responses was predicted by the efficacy of B-cell depletion in tissues (18). We therefore examined the changes in the B-cell populations in tissues in rituximab-infused and control AGMs. The flow-cytometric analysis of CD20 and CD79a expression in LNs showed complete depletion of B cells in rituximab-infused AGMs from day 0 p.i. onward (Fig. 1c and data not shown). The same dynamic pattern was observed in the intestine, where depletion was complete in the four rituximab-infused AGMs (Fig. 1d). CD20 depletion persisted during the follow-up, and at the time of necropsy, 6 months after infection, CD20 cells were still depleted in both lymphoid and mucosal tissues (Fig. 1c and d).

In control AGMs, there was a transient depletion of CD20 cells in the periphery during acute infection ($P = 0.0095$) and then a recovery to approximately baseline levels, whereas in LNs, there was a transient increase over the first month p.i. in the fraction of $CD20^+$ B cells ($P = 0.0003$) (Fig. 1a to d).

Immunohistochemical staining for CD20 confirmed the flow cytometry data. Representative data are shown in Fig. 1e to f. Complete B-cell depletion was observed in the rituximab-infused AGMs at 3 weeks after the first administration of Rituxan (day 28 p.i.) in the intestine (Fig. 1e, bottom panel) and LNs (Fig. 1f, right panels) compared to baseline levels (Fig. 1e, upper panel, and f, left panels). LNs from two of the four rituximab-treated animals showed background staining for CD20 antibody (Fig. 1f, middle right panel), suggesting significant but incomplete tissue depletion of CD20 cells in the LNs. Note, however, that at a higher magnification, this CD20 residual staining pattern was dim, disrupted, and fragmented. The staining was dotted and was located only on one side of the cell or in between the cells (Fig. 1f, bottom panel, right). This pattern is very different from the CD20 staining detected in nondepleted AGMs, which is a strong, well-defined, continuous immunostaining, delineating the entire cellular membrane (Fig. 1f, bottom panel, left). Given the differences in staining patterns and the discordant results between IHC and flow cytometry, this background staining is probably an artifact due to either the presence of fragments of destroyed CD20 cells or antigen retrieval techniques.

Both LNs and lamina propria were also stained for CD79a, which showed results similar to those for the tissues stained for CD20 (data not shown). At the end of the follow-up (day 184 p.i.), B cells were still significantly depleted in blood, LNs, and intestine, as assessed by both flow cytometry (Fig. 1a to d) and IHC (data not shown). $CD20^+$ B-cell depletion resulted in a significant reduction in the size of the germinal centers compared to results for undepleted control AGMs (Fig. 1f).

Unlike reports for previous studies of humans and macaques that the efficacy of CD20 cell depletion after rituximab administration is linked to polymorphisms of the FCRγIIIA receptor, we found no link between a particular polymorphism and the ability to deplete $CD20^+$ B cells (data not shown). Thus, all AGMs showed complete $CD20^+$ B-cell depletion while harboring both V229 and I233 polymorphisms (data not shown), which were associated with complete (V229) or incomplete (I233) CD20 depletion upon rituximab administration in RMs (38). Moreover, none of the AGMs tested here yielded Arg48 and Val158, the polymorphisms reported to be predictive for efficient CD20 depletion in human patients with Waldenström's macroglobulinemia (69). Therefore, we concluded that

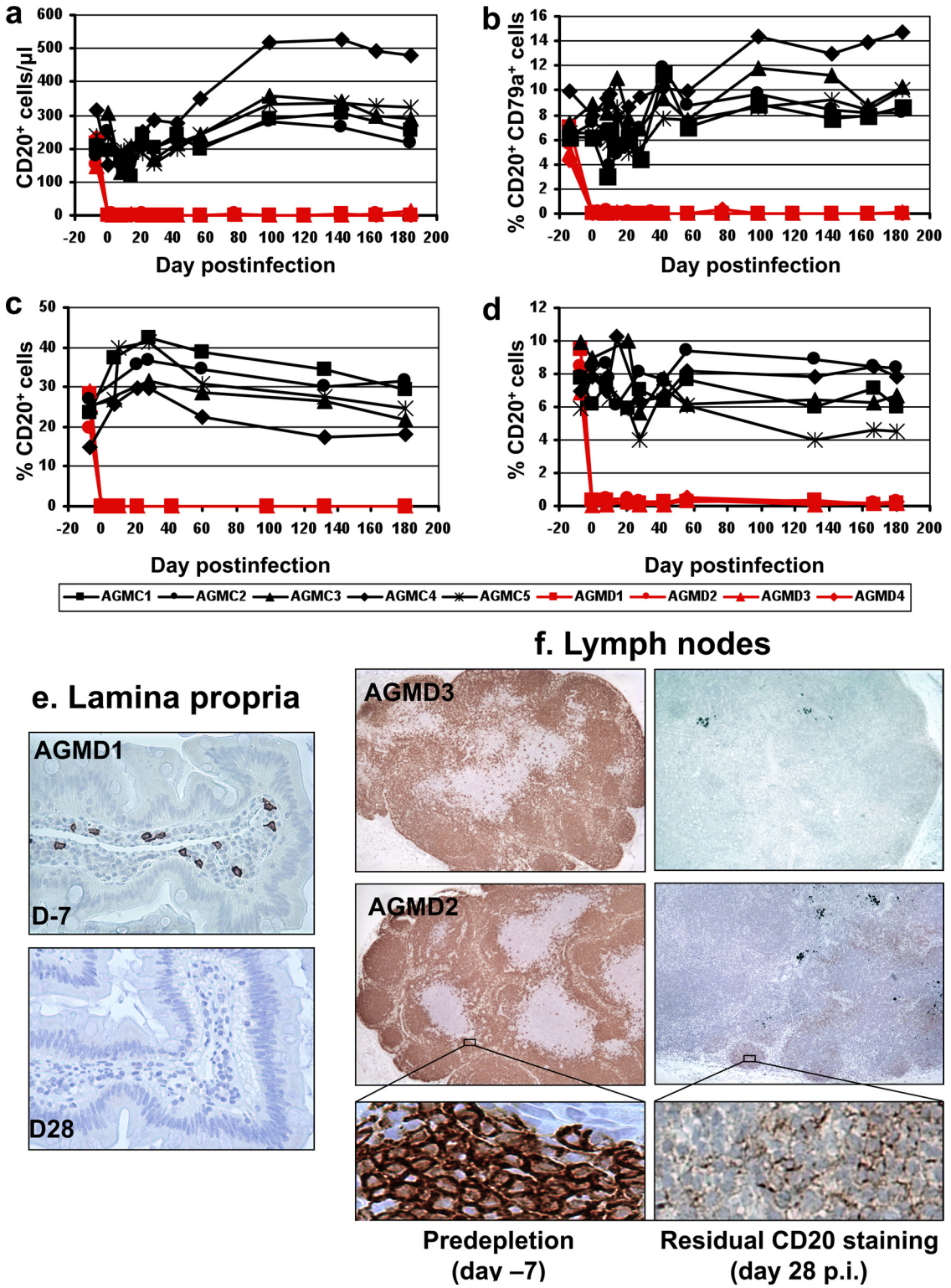

FIG. 1. Effect of rituximab infusion on CD20$^+$ B cells of AGMs. CD20$^+$ B-cell concentrations are shown in blood (a), lymph nodes (c), or intestine (d). Rituximab infusion induced a depletion of B cells and did not mask the CD20 molecule, as shown by the CD79a staining (b). Black symbols and lines denote the control monkeys (AGMC). Red symbols and lines denote AGMs that received rituximab infusions (AGMD). Day 0 is the day of SIV inoculation. Rituximab was infused every 21 days beginning 1 week prior to SIV inoculation. Immunohistochemistry for CD20 in the intestine (e) or lymph nodes (f) confirmed flow cytometry data. Low residual levels of CD20 were detected by IHC in two AGMs (f). The residual CD20 staining is dim, fragmented, and located only on the side of the cell or in between the cells (f, lower right panel), unlike the strong, continuous CD20 immunostaining that delineates the entire membrane in the LNs of nondepleted animals (f, lower left panel).

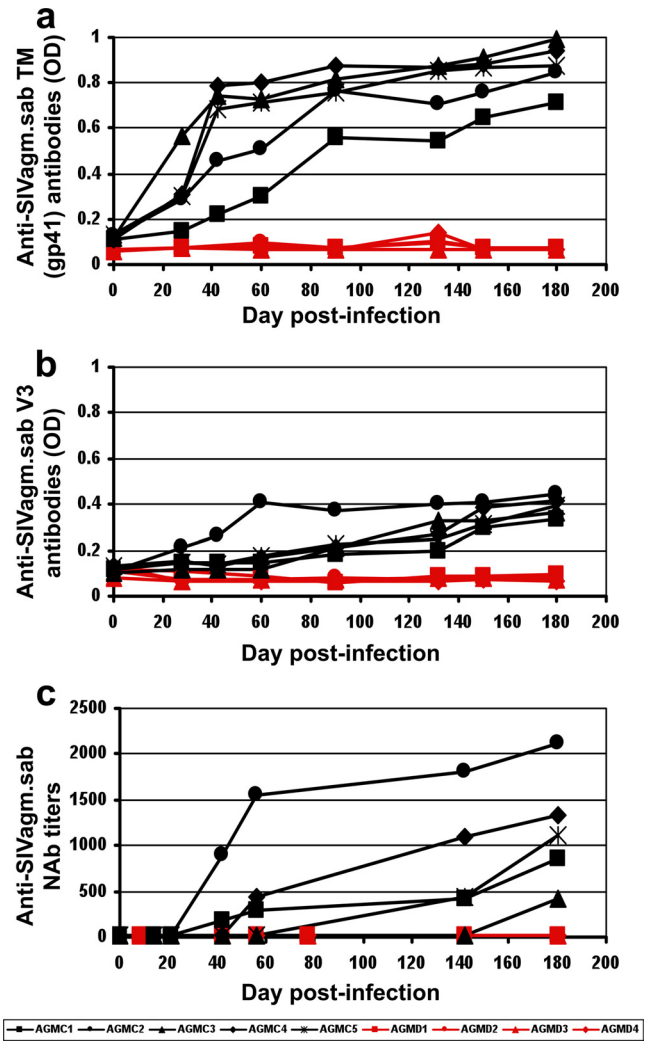

FIG. 2. Impact of B-cell depletion following rituximab administration on anti-SIVagm.sab humoral immune responses. SIVagm.sab-specific peptide enzyme-linked immunosorbent assay testing showed ablation of both anti-SIVagm gp41 (a) and anti-SIVagm V3 (b) antibody production in CD20-depleted AGMs (red symbols and lines) compared to results for controls (black symbols and lines). (c) Neutralizing antibody testing showed ablation of neutralizing antibody production in CD20-depleted AGMs (red symbols and lines) compared to results for controls (black symbols and lines). Day 0 corresponds to SIV inoculation. Rituximab was infused every 21 days beginning 1 week prior to SIV inoculation.

the efficacy of CD20 depletion by rituximab is not predicted by FCRγIIIA polymorphisms in AGMs.

Production of anti-SIVagm antibodies following rituximab administration. To investigate the impact of rituximab-induced B-cell depletion on humoral immune responses, we compared the dynamics of anti-SIV binding antibodies in CD20-depleted and undepleted AGMs by using various serological assays. We first investigated the dynamics of anti-SIVagm gp41 seroconversion by enzyme-linked immunosorbent assay. As shown in Fig. 2a, anti-gp41 antibody production was suppressed in rituximab-infused AGMs compared to results for undepleted AGM controls. We next investigated the dynamics of anti-V3 antibodies, using an SIVagm-specific V3 peptide, which again revealed suppression of anti-V3 seroconversion for rituximab-infused AGMs compared to results for undepleted AGM controls (Fig. 2b). Anti-V3 seroconversion in undepleted AGMs occurred later than anti-gp41 seroconversion, similar to what has been reported previously (64). Therefore, B-cell depletion significantly impacted production of binding antibodies in the rituximab-treated group compared to results for the control animals.

Finally, the dynamics of neutralizing antibodies were assessed in rituximab-infused and control AGMs. This test showed that there was a complete ablation of neutralizing antibodies for the CD20-depleted AGMs (Fig. 2c). In contrast, in the control group, relatively high levels of neutralizing antibodies were observed (Fig. 2c), in agreement with previous reports that identified significant titers of neutralizing antibodies for most SIVagm-infected AGMs (19). The levels of neutralizing antibodies for control AGMs were of an order of magnitude similar to those observed in rhesus macaques infected with a neutralization-sensitive SIVsmm strain (18).

The lack of both nonneutralizing and neutralizing antibody production in all of the rituximab-infused AGMs indicates that rituximab-induced CD20 depletion was effective in suppressing the humoral immune response.

Impact of CD20 depletion on the control of SIVagm.sab92018 replication. Depletion of CD20 cells in AGMs had a limited impact on the SIVagm.sab replication. Plasma VLs at the peak of replication ranged from 1.5×10^7 to 1.2×10^8 copies/ml for control AGMs and 7.1×10^7 to 1.9×10^8 copies/ml for rituximab-infused AGMs ($P = 0.064$) (Fig. 3a; see also Fig. S1a in the supplemental material), whereas VLs at the set point and chronic phase of infection up to 184 days p.i. ranged from 2.3×10^4 to 1.8×10^5 and 9.2×10^4 to 2.9×10^5 copies/ml, respectively ($P = 0.19$) (Fig. 3a; see also Fig. S1b in the supplemental material). We also analyzed the full VL profile; calculating the areas under the viral load curve up to day 184 p.i., we again found no significant differences between the two groups ($P = 0.41$) (see Fig. S1c in the supplemental material). So although rituximab-infused AGMs showed slightly higher VLs, this did not reach significance.

SIVagm replication in tissues was also quantified by real-time PCR at multiple time points postinfection. There were no significant differences in SIVagm.sab RNA loads in PBMCs (Fig. 3b) or LNs (Fig. 3c) between CD20-depleted and control AGMs. In the intestine (Fig. 3d) at 6 months postinfection, the VLs were 2.5×10^3 and 1.9×10^4 copies/10^6 cells for controls and CD20-depleted AGMs, respectively ($P = 0.015$) (see Fig. S1d in the supplemental material).

Therefore, we concluded that the ablation of B cells had a very subtle impact on the control of virus replication in SIVagm-infected AGMs, suggesting that humoral immune responses are not very effective in controlling SIV replication.

Comparative changes in immune cells from different compartments in CD20$^+$ B-cell-depleted and undepleted SIVagm-infected AGMs. We next investigated the effect of rituximab treatment on other lymphocyte subsets in the blood, LNs, and intestine using flow cytometry. There were no significant differences in changes of peripheral blood CD4$^+$ T-cell counts ($P = 0.41$) (Fig. 4a; see also Fig. S1e in the supplemental material) and percentages ($P > 0.5$) (Fig. 4b). Note that the baseline CD4$^+$ T-cell counts were lower for CD20-depleted

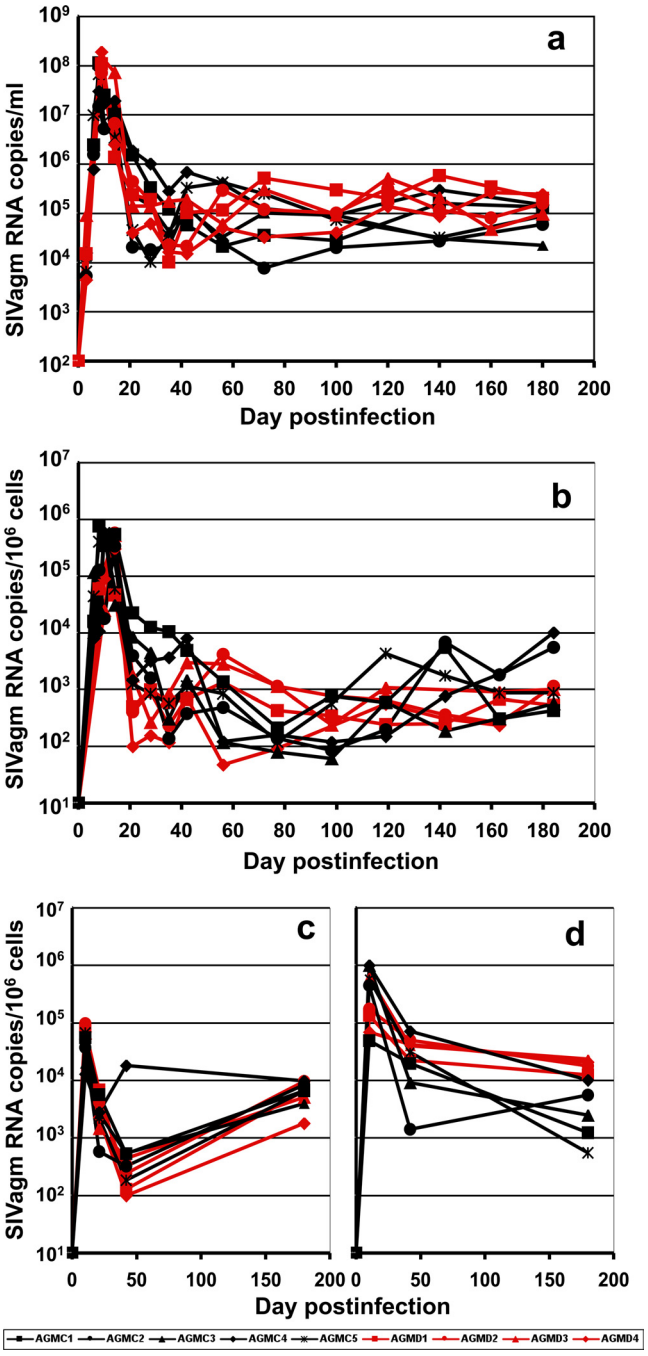

FIG. 3. Impact of B-cell depletion following rituximab administration on SIVagm.sab replication. Ablation of humoral immune responses had no significant impact on SIVagm.sab replication in AGMs, as illustrated by the dynamics of SIVagm.sab VLs in plasma (a), PBMCs (b), lymph nodes (c), or intestine (d). Black symbols and lines denote the control AGMs. Red symbols and lines denote rituximab-infused AGMs. Day 0 is the day of SIV inoculation. Rituximab was infused every 21 days beginning 1 week prior to SIV inoculation.

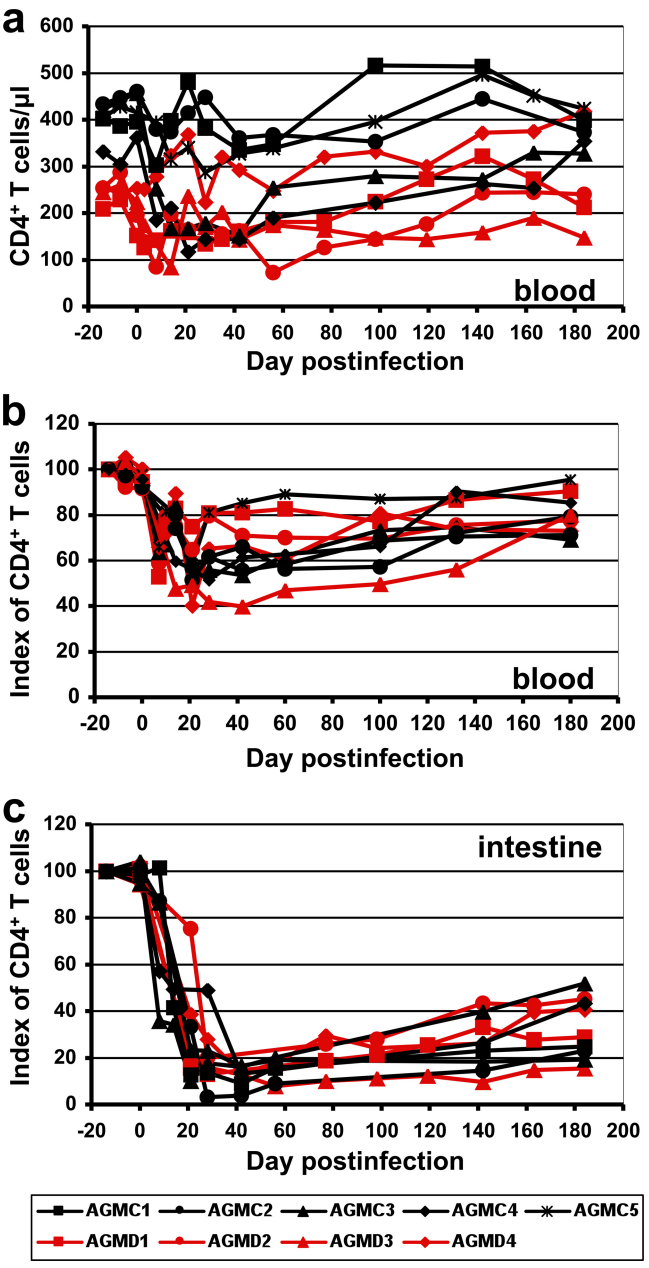

FIG. 4. CD4$^+$ T-cell dynamics in SIVagm.sab-infected CD20-depleted and control AGMs. Changes in peripheral blood CD4$^+$ T-cell counts (a) or percentages (b) or changes of CD4$^+$ T cells in the intestine (c) in rituximab-infused AGMs (red symbols and lines) and in control AGMs (black symbols and lines) are shown.

AGMs than for controls, probably because rituximab-infused animals were older than those in the control group. Therefore, in order to compare changes in CD4$^+$ T-cell counts between the two groups, we have considered percentages of depletion rather than the absolute counts. There were no significant differences in the CD4$^+$ T-cell depletion between the two groups during the acute SIVagm infection (over the first month, $P = 0.41$) or during chronic infection at days 56 or 98 p.i. ($P > 0.42$) (Fig. 4a). As the differences in CD4$^+$ T-cell counts between CD20-depleted AGMs and controls persisted during the follow-up, with the CD4$^+$ T-cell counts being lower for CD20-depleted monkeys at the end of the study (day 184 p.i.), we compared the dynamics of CD4$^+$ T cells as percentages of the lymphocyte population. No significant difference in the percentages of CD4$^+$ T cells was observed during

the follow-up between CD20-depleted AGMs and controls ($P > 0.5$) (Fig. 4b). These results were also supported by the changes in the intestine (Fig. 4c), where similar $CD4^+$ T-cell depletion was observed during acute and chronic infection in CD20-depleted AGMs and controls ($P = 0.56$) (see Fig. S1f in the supplemental material). Moreover, the same degree of $CD4^+$ T-cell recovery was observed in the gut in CD20-depleted AGMs and controls (Fig. 4c). Altogether, these data suggest that rituximab had little or no impact on SIVagm pathogenicity. Phenotyping the $CD4^+$ T-cell subsets did not reveal any differences in the dynamics of naive, central memory, and effector memory $CD4^+$ T cells in the blood and intestine between CD20-depleted and undepleted AGMs (data not shown). Both percentages and absolute counts of $CD8^+$ T cells remained stable during infection in both rituximab-treated and control AGMs (data not shown).

It has been indicated that administration of an anti-CD8 antibody results in increased proliferation and activation of $CD4^+$ T cells (3). To determine if rituximab had an effect on proliferation and activation, we measured the levels of immune activation and cell proliferation of both $CD4^+$ and $CD8^+$ T cells by flow cytometry for HLA-DR and Ki-67. No significant difference in the course of $CD4^+$ T-cell activation over the first month of infection was observed between CD20-depleted AGMs and control monkeys ($P = 0.34$), as detected by the use of the HLA-DR phenotypic marker (Fig. 5a; see also Fig. S1g in the supplemental material), and no change was observed in the LNs (data not shown). Significant increases in the levels of HLA-DR expression were observed on $CD8^+$ T cells from peripheral blood, with the average maximum increase over the first month equal to 123% above baseline ($P = 0.0078$) (Fig. 5b; see also Fig. S1h in the supplemental material), LNs (data not shown), and intestine, with an average maximum increase of 127% ($P = 0.017$) (data not shown). The same pattern of transient increase in immune activation of $CD8^+$ T cells and to a lesser extent of $CD4^+$ T cells was identified by using a second immune activation marker, CD69 (data not shown).

The levels of $CD4^+$ and $CD8^+$ T-cell proliferation, as assessed by using Ki-67, were not significantly different between rituximab-treated and control AGMs during the initial stages of infection (Fig. 5c and d). Thus, both CD20-depleted AGMs and control AGMs demonstrated a significant increase ($P < 0.011$) of Ki-67 expression on both $CD4^+$ and $CD8^+$ T cells at the peak of viral replication (Fig. 5c and d), followed by a return to near-baseline levels during chronic SIVagm.sab infection in both groups (Fig. 5c and d). Moreover, there were no differences in the percentage increase in Ki-67 between the two groups of AGM, either in $CD4^+$ or $CD8^+$ T cells ($P > 0.28$) (see Fig. S1i and j in the supplemental material).

In order to confirm the flow cytometry data, we investigated the dynamics of proinflammatory cytokines in plasma. No significant difference was observed between CD20-depleted AGMs and controls during acute SIVagm infection. Dynamics of selected cytokines are shown in Fig. 5: interleukin 1 (IL-1), a proinflammatory cytokine that targets and costimulates $CD4^+$ T cells (Fig. 5e); IL-2, a cytokine which is involved in T-cell activation (Fig. 5f); IL-12, a cytokine produced by macrophages and B cells, which targets cytotoxic T cells and natural killer cells and is involved in the differentiation of cytotoxic T lymphocytes (Fig. 5g); and alpha interferon (IFN-α), a cytokine produced by dendritic cells, which activates natural killer cells and macrophages, increases antigen presentation to lymphocytes, and induces the resistance of host cells to viral infection (Fig. 5h). Note that unlike findings of a previous study (33), for which no significant increase in IFN-α production as a major mechanism of nonpathogenicity in natural hosts was reported, we observed significant increase in IFN-α expression for both rituximab-infused and control AGMs.

Collectively, these results indicate that unlike the case with antibodies used for $CD8^+$ T-cell depletion in vivo, which induce significant increases in activation and proliferation of $CD4^+$ T cells, administration of rituximab did not result in significant increases in T-cell activation and proliferation.

DISCUSSION

Understanding the correlates of protection in the natural hosts of SIV is important since these animals rarely progress to AIDS. Unlocking the key components in the immune system which function in controlling disease progression in spite of high levels of viral replication will assist in designing new treatment strategies for HIV/AIDS patients (53, 71). The purpose of this study was to examine the ability of humoral immune responses to control SIV replication in the natural hosts of SIV using Caribbean AGMs infected with SIVagm.sab. For invasive studies, such as ablation of immune responses by selective depletion of different immune cell populations during both acute and chronic SIV infection, AGMs are the model of choice, since they are not endangered, unlike sooty mangabeys and mandrills (46).

Our results showed successful depletion of $CD20^+$ B cells in the peripheral blood, LNs, and intestine in all of the rituximab-infused AGMs, as demonstrated by undetectable levels of the $CD20^+$ $CD79a^+$ cell populations in these tissue compartments by flow cytometry. IHC analysis of both the intestinal lamina propria and LN tissues from the CD20-depleted AGMs confirmed the absence of $CD20^+$ cells at these sites. Two rituximab-treated animals showed CD20 background staining in the LNs by IHC, but the differences in staining pattern and the discordant results between IHC and flow cytometry, as well as the lack of anti-SIVagm neutralizing and binding antibody production in these animals, suggest that this background staining was an artifact due to either the presence of fragments of destroyed CD20 cells or antigen retrieval techniques.

In previous studies employing $CD20^+$ B-cell depletion in nonhuman primates and humans (as therapy for autoimmune diseases), complete depletion of CD20 cells occurred in only 50% of cases (2, 7, 22, 38). Here, complete depletion occurred in all cases; however, since our study included a limited number of AGMs, it is not possible to state whether or not AGMs are always susceptible to CD20 depletion by rituximab or if these results are only coincidental. We previously reported that FCRγIIIA receptor polymorphisms cannot be used as predictors of the effectiveness of rituximab to induce depletion of $CD20^+$ B cells in macaques (18). In this study we report that the same observation is true for AGMs: all AGMs included in the CD20 depletion group harbored both FCRγIIIA sequence polymorphisms for the receptor, which were previously reported to be associated with either complete depletion or incomplete depletion, respectively, in RMs (38).

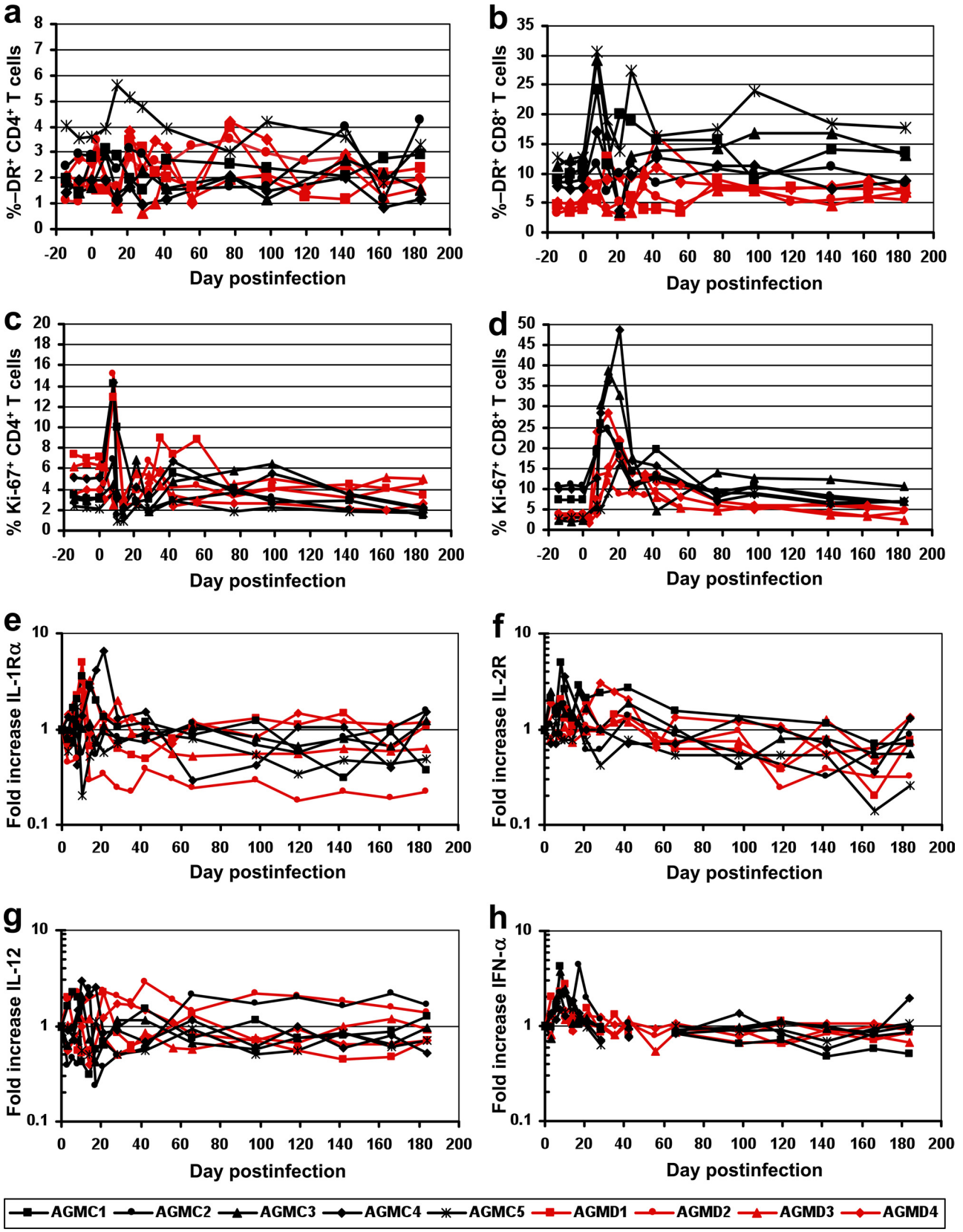

FIG. 5. Kinetic expression of immune activation and T-cell proliferation in SIVagm.sab-infected CD20-depleted and control AGMs. Dynamics of $CD4^+$ and $CD8^+$ T-cell immune activation (as defined by changes in the expression of -DR markers) in blood (a and b). Dynamics of $CD4^+$ and $CD8^+$ T-cell proliferation (as defined by changes in the expression of Ki-67) in blood (c and d). Flow cytometry data were confirmed by the measurement of plasma cytokines: 1L-1rα (e), IL-2R (f), IL-12 (g), or IFN-α (h). Rituximab-infused AGMs are shown as red symbols and lines; control AGMs are shown as black symbols and lines. The dynamics of plasma cytokines (quantified in pg/ml) are shown as *n*-fold increases over baseline levels.

As a result of this effective CD20 depletion through rituximab administration, production of anti-gp41 and anti-V3 antibodies, along with that of neutralizing antibodies, was suppressed in CD20-depleted AGMs. This result indicates that humoral immune responses against SIVagm were ablated in rituximab-treated AGMs. However, there was no statistical difference in SIVagm.sab infection dynamics in plasma, PBMCs, and LNs between CD20-depleted and control AGMs despite the effective ablation of humoral immune responses in rituximab-infused AGMs. In the intestine, the levels of viral replication were comparable between the two groups with the exception of results for day 184 p.i. However, this difference was not significant when historical data on a larger group of AGMs were factored into the calculation. Based on our results, which show that complete depletion of CD20 cells in the blood, LN, and intestine and effective suppression of antibody production in CD20-depleted animals had no or very minimal impact on the dynamics of VLs and on the clinical outcome during acute and chronic AGM infection with SIVagm.sab92018, we concluded that humoral immune responses have a limited role during the course of SIV infection in natural hosts. Note, however, that due to the limited number of animals included, it is still possible that we were not able to detect subtle differences between CD20-depleted and undepleted AGMs. Moreover, differences between treated and control animals could exist outside the immunological parameters we assayed.

In RMs, initial $CD20^+$ B-cell depletion studies reported potential involvement of humoral immune responses in the control of viral replication or duration of disease progression (38, 57). However, both of these studies used SIVmac251 and SIVmac239, two viral strains that are extremely pathogenic and are difficult to neutralize (38, 57). In contrast, we previously reported that depletion of CD20 cells in RMs infected with a neutralizable strain (SIVsmD215) results in no significant difference in the VLs or survival between the rituximab-treated and control RMs (18). Moreover, in our study, there was a trend toward lower VLs for the CD20-depleted RMs than for controls. Therefore, in both pathogenic and persistent, nonprogressive models of SIV infection, we observed that ablation of humoral responses does not have a discernible effect on the control of virus replication.

Immune activation in HIV infection is a major contributor to the pathogenesis of AIDS (4, 20, 23, 65). Polyclonal B-cell activation, observed during HIV type 1 infection, is associated with hypergammaglobulinemia, increased levels of B-cell activation markers, increased autoantibody production, development of B-cell lymphomas, reduced memory B cells, and decreased function of B cells (10, 32, 39, 59). It has been hypothesized that the B-cell dysregulation occurs during the primary SIV infection (66, 67). This is not unexpected, since studies have shown that efficient B-cell function depends upon interaction with $CD4^+$ T cells (27, 39) and also that efficient functioning of $CD4^+$ T cells depends on interactions with B cells and other antigen-presenting cells (32), and thus, the massive depletion of $CD4^+$ T cells during acute SIV infection can contribute to the dysregulation of B cells. On the other hand, in this study, experimental depletion of $CD20^+$ B cells did not alter the dynamics of $CD4^+$ T-cell depletion and recovery.

Because T cells receive costimulatory signals and antigens that are presented by B cells, it would be expected that the depletion of B cells would have an impact on the T-cell population (31). In our experiment, the levels of $CD4^+$ or $CD8^+$ T cells were not modified between depleted and undepleted AGMs. In humans, the use of rituximab for treating complications of systemic lupus erythematosus resulted in a decrease in the immune activation markers and an increase in both the numbers and function of T regulatory cells, which are modulators of immune activation (58, 68, 70). This result was in contrast to that for systemic lupus erythematosus patients that have not received rituximab, who displayed higher levels of immune activation markers and a decrease in T regulatory cells. Furthermore, when B cells are restored in these patients, they continue to show a decrease in costimulatory markers, suggesting that the level of stimulation between T cells and B cells remains depressed, thus prolonging the effects of depletion (31). In comparison, here we report similar levels of immune activation between the rituximab-treated and control AGMs, with slightly lower levels of HLA-DR^+ $CD8^+$ T cells observed in the rituximab-treated animals.

In light of the HIV vaccine failures, it could be stated that developing a protective vaccine against HIV will require a fresh approach focusing on induction of effective humoral immune responses in addition to effective cellular immune responses (74). To date, there has been only one phase 3 trial aimed at producing anti-HIV antibodies. This vaccine, which used two different recombinant gp120 antigens from HIV type 1, was not successful in protecting against HIV infection and did not result in lower viral replication (15). Furthermore, it has been repeatedly reported that although neutralizing antibodies effective against primary patient isolates develop following many infections (16, 75), the initial neutralization antibody response tends to be highly specific for the early autologous virus. Moreover, the high sequence variability in Env means that the virus can easily escape (11, 16, 55). 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 (8). Our research showing that antibodies make little contribution to controlling viral replication in both pathogenic SIV infection (18) and persistent, nonprogressive SIV infections indicates that antibody-based vaccines should aim at preventing SIV infection rather than at controlling viral replication.

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