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Altered Ratio of T Follicular Helper Cells to T Follicular Regulatory Cells Correlates with Autoreactive Antibody Response in Simian Immunodeficiency Virus–Infected Rhesus Macaques

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Abstract

Individuals with chronic HIV-1 infection have an increased prevalence of autoreactive Abs. Many of the isolated HIV broadly neutralizing Abs from these individuals are also autoreactive. However, the underlying mechanism(s) that produce these autoreactive broadly neutralizing Abs remains largely unknown. The highly regulated coordination among B cells, T follicular helper (T_{FH}) cells, and T follicular regulatory (T_{FR}) cells in germinal centers (GCs) of peripheral lymphatic tissues (LTs) is essential for defense against pathogens while also restricting autoreactive responses. We hypothesized that an altered ratio of T_{FH}/T_{FR} cells in the GC contributes to the increased prevalence of autoreactive Abs in chronic HIV infection. We tested this hypothesis using a rhesus macaque (RM) SIV model. We measured the frequency of T_{FH} cells, T_{FR} cells, and GC B cells in LTs and anti-dsDNA and anti-phospholipid Abs from Indian RMs, with and without SIV infection. We found that the frequency of anti-dsDNA and anti-phospholipid Abs was much higher in chronically infected RMs (83.3% [5/6] and 66.7% [4/6]) than in acutely infected RMs (33.3% [2/6] and 18.6% [1/6]) and uninfected RMs (0% [0/6] and 18.6% [1/6]). The increased ratio of T_{FH}/T_{FR} cells in SIV infection correlated with anti-dsDNA and anti-phospholipid autoreactive Ab levels, whereas the frequency of T_{FR} cells alone did not correlate with the levels of autoreactive Abs. Our results provide direct evidence that the ratio of T_{FH}/T_{FR} cells in LTs is critical for regulating autoreactive Ab production in chronic SIV infection and possibly, by extension, in chronic HIV-1 infection.

Human immunodeficiency virus–1 infection of humans leads to immunodeficiency that is characterized by massive $CD4^+$ T cell depletion. Importantly, HIV also causes B lymphocyte dysfunction (1–3) and an increased prevalence of autoreactive Abs (4–7). During chronic infection, HIV neutralizing Abs, including broadly neutralizing Abs (bNAbs), have

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enhanced polyreactive and autoreactive characteristics (8–12). For example, a previous study found that 101 of 134 monoclonal anti-HIV–gp140 neutralizing Abs isolated from HIV-infected individuals were polyreactive and likely to bind self-antigens (9).

To maintain humoral immunologic homeostasis, a highly regulated coordination among B cells, T follicular helper (T_{FH}) cells, and T follicular regulatory (T_{FR}) cells in germinal centers (GCs) of peripheral lymphatic tissues (LTs) is required. These interactions promote the development of protective Abs against pathogens (13–16); however, disruption of homeostatic GC reactions can result in the production of autoreactive Abs or even autoimmune disease (17–19). Regulation of GC reactions, in part, is dependent on the frequency of T_{FH} cells. T_{FH} cells are indispensable for Ab affinity maturation of B cells (15, 16), in which a stochastic process of somatic hypermutation results in a greater risk for development of autoreactive B cells (20, 21). Previous studies have shown that increased frequency of T_{FH} cells in mice was associated with an increased frequency of GC B cells, and the mice were more prone to develop humoral-mediated autoimmunity (18, 22). Furthermore, increased frequency of T_{FH} cells has been implicated in the pathogenesis of autoimmune disease in humans (23, 24).

T_{FR} cells regulate GC reactions through interactions with GC B cells and T_{FH} cells. T_{FR} cells are an effector subset of regulatory T cells (T_{REGs}) that can suppress T_{FH} cell function, limit the frequency of T_{FH} and B cells in GCs (14, 25–28), and prevent autoreactive Ab production (29–31). During chronic HIV infection of humans and SIV infection of rhesus macaques (RMs), T_{FH} cells exhibit increased frequency (32, 33). Recent studies revealed that the frequency of T_{FR} cells in the LTs of SIV-infected RMs declines postinfection (34, 35); however, the role of T_{FH} and T_{FR} cells in autoreactive Ab production and the frequency of GC autoreactive B cells in HIV-infected individuals remain largely unknown.

We hypothesized that an altered ratio of T_{FH}/T_{FR} cells in the GC contributes to the increased prevalence of autoreactive Abs in HIV infection. We tested this hypothesis using an RM SIV model, which is the best available model of HIV infection in humans. We measured autoreactive anti-dsDNA and anti-phospholipid Abs in peripheral blood and quantified the frequency of T_{FH}, T_{FR}, and B cells in the GC of LTs. We found that an increased ratio of T_{FH}/T_{FR} cells in SIV infection correlated strongly with anti-dsDNA and anti-phospholipid Ab levels, whereas the frequency of T_{FR} cells alone did not correlate with autoreactive Ab levels. Our results provide direct evidence that the proper balance and adequate ratio of T_{FH}/T_{FR} cells are crucial in regulating the quality of GC reactions and autoreactive Ab production in SIV infection and possibly, by extension, HIV-1 infection.

Materials and Methods

Virus and animals

This study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln (protocol number 559) and BIOQUAL, Inc. (protocol number 10-0000-01). Adult male Indian RMs (*Macaca mulatta*) were housed and maintained in animal-housing facilities at BIOQUAL, in accordance with the *Guide for the Care and Use of Laboratory Animals*. All animals were free of simian retrovirus type D,

simian T-lymphotropic virus type 1, and herpes B virus. Animals were sedated with ketamine or TELAZOL for all technical procedures and were fully anesthetized for SIV intrarectal inoculation with SIVmac251 (3.4×10^4 $TCID_{50}$). Animals were euthanized by exsanguination, under deep (surgical plane) anesthesia using TELAZOL, which was performed under the direction of the attending veterinarian on a designated date. Macaques were euthanized before SIV infection, at days 28 and 180 d postinfection ($n = 3$ for each time point). We also included four additional lymph node tissue samples from RMs not infected with SIV. Longitudinal peripheral blood samples from three macaques were collected before infection, as well as at 28 and 180 d postinfection (dpi). Three additional peripheral blood specimens were included at 180 dpi.

Detection of total IgG, SIV Env Ab, and anti-phospholipid and anti-dsDNA Abs in plasma by ELISA

Total IgG in plasma was detected using a Monkey IgG ELISA kit (catalog number IGG-3; Life Diagnostics). SIV-specific Env Ab titers were determined using ELISA. SIVmac239 gp130 Env protein [from Dr. K. Uberla (36)] was obtained through the National Institutes of Health AIDS Reagent Program (catalog number 12797). High-binding flat-bottom 96-well plates (catalog number 3361; Corning, Kennebunk, ME) were coated with SIV-mac239 gp130 protein (1 μg/ml) and blocked with PBS containing 5% milk. A two-fold serial dilution of plasma sample (starting at 1:200) was added to each well. Plates were incubated at 37°C for 1 h. After incubation, plates were washed with washing buffer (0.5% Tween-20 in PBS), 0.16 μg/ml goat anti-human IgG-HRP (catalog number 627120; Invitrogen) was added, and plates were stored at 37°C for 1 h. Plates were washed and developed using OPD substrate (catalog number P9187-50SET; Sigma) and stopped with 1 M H_2SO_4. Absorbance was read at 490 nm with an ELx800 Microplate Reader (BioTek, Winooski, VT). The cutoff value was set as the mean OD of control plasma + 3 SD. Binding Ab titers were defined as the end point dilution with an OD value greater than the cutoff + 0.05.

Anti-phospholipid and anti-dsDNA IgG autoreactive Abs were detected in plasma using commercial kits (ORGENTEC Diagnostika, Mainz, Germany), and absorbance was detected at 450 nm (ELx800 Microplate Reader; BioTek) for samples and standards. Using known standard concentrations (provided by the ELISA manufacturer), a linear-regression analysis (Microsoft Excel) was used to calculate IgG concentrations for each sample. Cutoff values were determined based on recommendations from the manufacturer (2.5 times the OD value of the negative control).

Flow cytometry

Detection of T_{FH} and T_{FR} cells—A total of $3–4 \times 10^6$ cryopreserved cells isolated from lymph node tissues was stained for viability using a LIVE/DEAD Fixable Blue Dead Cell Stain Kit, following the manufacturer's instructions (Life Technologies/Thermo Fisher Scientific, Waltham, MA). Cells were then stained with Brilliant Stain (BD Biosciences, Franklin Lakes, NJ) in FACS buffer with titrated amounts of the following surface Abs to detect T follicular cells: mouse anti-human CD3–Alexa Fluor 700 (clone SP34-2, 1:60; BD Biosciences), mouse anti–human CD4–Brilliant Violet 605 (clone OKT4, 1:60; BD Biosciences), mouse anti–human CXCR5-PE (clone MU5UBEE, 1:40; eBioscience, San

Diego, CA), mouse anti–human PD-1–Brilliant Violet 785 (clone EH12.2H7, 1:30), mouse anti–human CD20–Brilliant Violet 421 (clone 2H7, 1:40), mouse anti–human CCR7–Brilliant Violet 711 (clone G043H7, 1:40), mouse anti–human CD95–PE–Cy7 (clone DX2, 1:30), and mouse anti–human ICOS–PerCP–Cy5.5 (clone C398.4A, 1:75) (all from Bio-Legend, San Diego, CA), and mouse anti–human CD25–FITC (clone 4E3, 1:15; Miltenyi Biotec, Bergisch Gladbach, Germany).

Foxp3 staining—After surface staining, cells were fixed and permeabilized for 30 min in 1× Foxp3 Fix/Perm Buffer (eBioscience) and then stained with Foxp3-allophycocyanin (clone PCH101; eBioscience). Cells were washed twice with 1× Perm Buffer (eBioscience). Foxp3 Ab was added to cells and allowed to incubate for 45 min, and cells were washed twice with 1× Perm Buffer and washed once in FACS buffer prior to running on a BD LSR II flow cytometer (BD Biosciences). A minimum of 700,000 live lymphocyte-gated events was detected for each sample. Gating strategy was determined based on fluorescence minus one and appropriate isotype controls.

Detection of GC B cells—Cryopreserved lymphocytes isolated from lymph node tissues were stained with the following Abs: mouse anti-human CD3–Alexa Fluor 700 (clone SP34-2, 1:60; BD Biosciences), mouse anti–human Ki67–Alexa Fluor 488 (clone B56, 1:50; BD Biosciences), mouse anti–human BCL-6–PE–Cy7 (clone K112-91, 1:50; BD Biosciences), and mouse anti–human CD20–allophycocyanin (clone 2H7, 1:40; BioLegend). BCL-6 staining was performed using the Foxp3 staining procedure described above. A minimum of 350,000 lymphocyte-gated events was collected for each sample on a BD FACSAria II Flow Cytometer (BD Biosciences).

Real-time RT-PCR to quantify SIV viral loads in plasma

Real-time RT-PCR assays were performed to determine the levels of SIVmac251 in plasma specimens using a previously reported method (37).

Immunofluorescence staining

To visualize T_{FH} and T_{FR} cells in lymph node tissues, immunofluorescence staining was conducted according to a previously described method (38). Goat polyclonal anti-human PD-1 Abs (catalog number AF1086, 1:100; R&D Systems), rabbit monoclonal anti-human CD4 Abs (clone EPR6855, 1:200; Abcam), and mouse monoclonal anti-human Foxp3 Abs (clone 236A/E, 1:200; Abcam) were incubated with tissues sections. Following primary Ab incubation and washing, donkey anti-goat IgG conjugated with Alexa Fluor 488 (catalog number A-11055, 1:100), donkey anti-rabbit IgG conjugated with Alexa Fluor 594 (catalog number R37119, 1:100), and donkey anti-mouse IgG conjugated with Alexa Fluor 647 (catalog number A-31571, 1:100; all from Thermo Fisher Scientific) were used. Cell nuclei were counterstained with DAPI. A Nikon A1R-TiE live cell imaging confocal system was used to visualize and capture images of stained samples.

Statistics

Statistical analyses of cell subset frequencies detected by flow cytometry were performed using a Mann–Whitney nonparametric *t* test (GraphPad Prism). Correlation analyses were

performed using the Pearson *r* correlation method (GraphPad Prism). The *p* values < 0.05 were considered significant.

Results

Increased autoreactive Abs in chronic SIV infection

In individuals with chronic HIV infection, the prevalence of autoreactive Abs increases significantly (4–7). Previous studies reported that up to 40–50% of HIV-infected individuals produce Abs against the self-antigens cardiolipin and phosphatidylserine (6). An elevated frequency of autoreactive Abs has also been observed in SIV-infected RMs (39). We first sought to determine the frequency of autoreactive Abs in our cohort of SIV-infected RMs. SIV infection was confirmed by detection of SIV viral RNA in plasma using real-time quantitative PCR and in LTs using in situ hybridization (data not shown). Anti-dsDNA and anti-phospholipid autoreactive Abs were measured by ELISA in plasma specimens from RMs that were SIV naive or were in the acute (28 dpi) or chronic (180 dpi) stage of SIV infection.

We found that the levels of anti-dsDNA Abs at the chronic stage of SIV infection were significantly increased compared with uninfected animals ($p = 0.0050$) (Fig. 1A). The level of anti-dsDNA Abs in chronically infected RMs was also higher than in animals in the acute stage of SIV infection ($p = 0.0737$), but the difference was not statistically significant. The percentage of RMs that had anti-dsDNA Abs was much higher in those that were chronically infected (83.3%, 5/6) compared with uninfected RMs (0%, 0/6) and acutely infected RMs (33.3%, 2/6).

A similar trend was observed for anti-phospholipid (a mixture of cardiolipin, phosphatidyl serine, phosphatidyl inositol, phosphatidic acid, and human β-2-glycoprotein I) autoreactive Abs. Anti-phospholipid Ab levels in chronic SIV infection were increased significantly compared with uninfected animals ($p = 0.0425$) (Fig. 1B). The percentage of animals with anti-phospholipids Abs was much higher in association with chronic infection (66.7%, 4/6) than with no infection (18.6%, 1/6) or acute infection (18.6%, 1/6). Our results confirmed the increased prevalence of autoreactive Abs during chronic SIV infection.

To determine whether there is a correlation between the levels of autoreactive Abs and total IgG, we measured total IgG in plasma and performed a correlation analysis of total IgG levels and autoreactive Ab levels. We found an increased trend in total IgG in RMs with chronic SIV infection compared with acute and uninfected RMs; however, this increase was not statistically significant (Supplemental Fig. 1A). No significant correlation between total IgG levels and autoreactive Ab levels was found (Supplemental Fig. 1B, 1C).

Altered frequency of T_{FH} and T_{FR} cells in LTs following SIV infection

Next, we sought to examine the frequency of T_{FH} and T_{FR} cells in LTs during acute and chronic SIV infection using flow cytometry. We gated T_{FH} and T_{FR} subsets according to published methods (34) (Fig. 2A). The percentage of T_{FH} cells ($Foxp3^{-}$ $CXCR5^{+}$ $PD\text{-}1^{hi}$ $ICOS^{+}$ CD4 T cells) within total lymphocytes and CD4 T cells decreased significantly in acute SIV infection compared with SIV-naive animals (Fig. 2B, 2C). During chronic

infection, the T_{FH} cell population in LTs expanded significantly, and their frequency increased significantly within CD4 T cells (Fig. 2C) and total lymphocytes (Fig. 2B), regardless of the decline in CD4 T cells in total lymphocytes (data not shown). During acute SIV infection, the percentage of T_{FR} cells ($Foxp3^+$ $CD25^+$ $CXCR5^+$ $CCR7^-$ CD4 T cells) within total lymphocytes and CD4 T cells was significantly decreased (Fig. 2D). However, T_{FR} cell frequency in CD4 T cells also increased significantly during chronic infection compared with acute SIV infection (Fig. 2E). Furthermore, we determined the location of T_{FH} and T_{FR} cells in lymph node tissues using immunofluorescence staining (Fig. 3). T_{FH} cells, defined as PD-1^+ $CD4^+$ $Foxp3^-$ (Fig. 3F, 3G, yellow arrows), and T_{FR} cells, defined as PD-1^+ $CD4^+$ $Foxp3^+$ (Fig. 3F, 3G, white arrows), were identified in GCs.

Altered frequency of GC B cells in LTs following SIV infection

Next, we evaluated the frequency of GC B cells ($CD3^-$ $CD20^+$ $Ki67^+$ BCL-6^+) in LTs of SIV-infected macaques using flow cytometry, according to previously published methods (32, 40) (Fig. 4A). The frequency of GC B cells did not change significantly during acute infection, but it increased significantly during chronic infection (Fig. 4B). Because T_{FH} cells and T_{FR} cells have been suggested to play a critical role in regulating the survival of GC B cells (13–16), we sought to determine whether there was an alteration in the T_{FH}/T_{FR} cell ratio and what role it played in the expansion of GC B cells during SIV infection. Consistent with the increased frequency of GC B cells, the T_{FH}/T_{FR} cell ratio was also increased significantly during chronic SIV infection (Fig. 4C). We found that the T_{FH}/T_{FR} cell ratio correlated strongly with GC B cells in uninfected and SIV-infected RMs (Fig. 4D); however, T_{FH} cells alone correlated with GC B cells in SIV-infected RMs but not in uninfected RMs (Supplemental Fig. 2A). There was no correlation between T_{FR} cells and GC B cells in SIV-infected or uninfected RMs (Supplemental Fig. 2B).

Autoreactive Ab levels correlated strongly with T_{FH}/T_{FR} cell ratio

To examine the relationship between autoreactive Abs and T_{FH} cells, T_{FR} cells, and GC B cells, we performed a set of correlation analyses in SIV-infected and uninfected RMs. We found that the frequency of GC B cells had a weak correlation with anti-dsDNA autoreactive Abs ($p = 0.0471$) but not with anti-phospholipid autoreactive Abs ($p = 0.0634$) (Fig. 5A). The frequency of T_{FH} cells correlated weakly with anti-dsDNA autoreactive Abs ($p = 0.0373$), but not with anti-phospholipid autoreactive Abs ($p = 0.0832$) (Fig. 5B). The frequency of T_{FR} cells did not correlate with anti-dsDNA or anti-phospholipid autoreactive Abs (Fig. 5C); however, the T_{FH}/T_{FR} cell ratio correlated strongly with anti-dsDNA ($p = 0.0004$) and anti-phospholipid ($p = 0.0018$) autoreactive Abs (Fig. 5D). We also did a correlation analysis that excluded naive animals, and correlations remained significant (data not shown).

We performed correlation analyses to determine the relationship between T_{REGs} and autoreactive Abs. There was no association between T_{REGs} ($CD4^+$ $Foxp3^+$ $CD25^+$ $CXCR5^-$), expressed as a percentage of total lymphocytes or CD4 T cells, and the levels of anti-dsDNA or anti-phospholipid Abs (Supplemental Fig. 3A). In addition, there was no association between $CCR7^+$ T_{REGs} ($CD4^+$ $Foxp3^+$ $CD25^+$ $CXCR5^-$ $CCR7^+$), expressed as a

percentage of total lymphocytes or CD4 T cells, and the level of anti-dsDNA or anti-phospholipid Abs (Supplemental Fig. 3B).

To decipher the relationship between autoreactive Abs and SIV-specific Env Abs, we measured SIV-specific Abs against gp130 in plasma in the six chronically infected animals. We did not find a correlation between the level of SIV Env Abs and the level of autoreactive anti-dsDNA Abs (r^2 = +0.3493, r = +0.5910, p = 0.2167) or anti-phospholipid Abs (r^2 = +0.1151, r = +0.3393, p = 0.5106).

To investigate the relationship between viral loads and the levels of autoreactive Abs, T_{FH} cells, T_{FR} cells, T_{REGs}, and GC B cells in peripheral blood, we performed a series of correlation analyses in chronically infected macaques and did not find any significant correlations (Supplemental Fig. 4).

Discussion

GCs of peripheral lymphoid tissues are a central hub of humoral immune responses. Within GCs, T_{FH} cells and T_{FR} cells regulate the survival and maturation of non-self-antigen–specific B cells while eliminating autoreactive B cells (13–16). GCs of LTs are also a major interaction site of the host immune system and HIV where T_{FH} cells are productively infected (41, 42), follicular dendritic cells are deposited with abundant virions (43, 44), and B cells are dysfunctional (2, 3). A high prevalence of autoreactive Abs has been observed in individuals with chronic HIV infection (4–7) and in SIV-infected RMs (39); however, it remains largely unknown what role T_{FH} and T_{FR} cells play in the generation of autoreactive Abs in individuals who are chronically infected with HIV. In this study, we addressed this question using a well-established RM SIV model of human HIV infection.

Our study provides direct evidence that the ratio of T_{FH}/T_{FR} cells in LTs correlates with autoreactive Abs levels in naive RMs and in RMs in the acute or chronic stage of SIV infection. We measured plasma IgG autoreactive Abs (anti-dsDNA and anti-phospholipids), because it has been reported that there is an elevation in autoreactive Abs to dsDNA and phospholipids in macaques chronically infected with SIV (39). We found that the concentration of autoreactive Abs was significantly elevated in RMs chronically infected with SIV compared with SIV-naive or acutely infected RMs. The majority of chronically infected macaques had anti-dsDNA (83.3%) and anti-phospholipid (66.7%) Abs. We then determined the frequency of T_{FH} cells, T_{FR} cells, and GC B cells in LTs from uninfected RMs and in RMs in the acute and chronic stages of SIV infection. We found that there was an altered T_{FH}/T_{FR} cell ratio in LTs of chronically infected RMs, which is consistent with a previous study (35); however, that study did not investigate the relationship between the T_{FH}/T_{FR} cell ratio and autoreactive Ab production. In this study, we found that the frequency of GC B cells did not correlate with anti-phospholipid autoreactive Abs, and it correlated only weakly with anti-dsDNA autoreactive Abs, indicating that the production of autoreactive Abs is not a direct consequence of GC B cell expansion. The frequency of T_{FH} cells alone did not correlate with anti-phospholipid autoreactive Abs, and it correlated only weakly with anti-dsDNA autoreactive Abs, whereas the frequency of T_{FR} cells alone did not correlate with either autoreactive Ab tested; however, the ratio of T_{FH}/T_{FR} cells correlated

strongly with anti-dsDNA and anti-phospholipid Abs. To our knowledge, our study is the first to demonstrate the potential role of the T_{FH}/T_{FR} cell ratio in regulating autoreactive Ab production in chronic SIV infection, which may be mediated through reduced peripheral tolerance for autoreactive B cells. T_{FR} cells express CXCR5, PD-1, and ICOS, which are also expressed on T_{FH} cells; however, T_{FR} cells also express CD25, Foxp3, and Helios, which are characteristic of suppressive T_{REGs} (25, 26). T_{FR} cells have been shown to cooperate with T_{FH} cells in regulating humoral immunity in GCs to fight against pathogens (14, 25–28) while also preventing the development of autoimmunity (29–31, 45). Therefore, an adequate ratio of T_{FH}/T_{FR} cells is important to maintain homeostasis of the humoral immune response.

Of note, HIV bNAbs are usually generated in chronically infected individuals and are often autoreactive to self-antigens (8–12). This production of autoreactive HIV bNAbs temporally coincides with an altered T_{FH}/T_{FR} cell ratio during chronic infection. The strong correlation that we observed between the emergence of autoreactive Abs and an increased ratio of T_{FH}/T_{FR} cells provides clues for future studies testing the possible enhanced induction of protective Abs by modulating the ratio of T_{FH}/T_{FR} cells during HIV-1 vaccination. We would like to point out that our T_{FH} cell frequency in acute SIV infection compared with no infection differs from previous studies (32, 46), which may be due to the fact that the frequency was calculated based on different denominators and different dpi. There are several differences between the study by Petrovas et al. (32) and our study: their T_{FH} cell frequency was calculated based on central memory $CD4^+$ T cells as the denominator, whereas our T_{FH} cell ($CD4^+$ $CXCR5^+$ $PD\text{-}1^{hi}$ $CD25^-$ $FOXP3^-$) frequency was calculated based on total lymphocytes and $CD4^+$ T cells; their acute cases were pooled samples from 3, 7, 10, 14, and 21 dpi, whereas we used 28 dpi; and our mean plasma viral load is 3.9×10^6 copies per milliliter, whereas they did not provide plasma viral load data. There are also differences between the study by Hong et al. (46) and our study: their T_{FH} cell ($CD4^+$ $CXCR5^+$ $PD\text{-}1^+$) frequency was calculated based on $CD4^+$ $CXCR5^+$ cells as the denominator, and their acute stage was 14 dpi, whereas we used 28 dpi.

Although this study revealed a direct association between autoreactive Abs and an altered T_{FH}/T_{FR} cell ratio in LTs of chronic SIV infection, our sample size was relatively limited, and the association is not direct causal evidence. Therefore, future studies are needed to elucidate the molecular mechanisms relating to an altered T_{FH}/T_{FR} cell ratio and its impact on autoreactive B cells.

In conclusion, we report that an altered T_{FH}/T_{FR} cell ratio in LTs correlates strongly with autoantibody levels in chronic SIV infection, and this altered T_{FH}/T_{FR} cell ratio could be one important mechanism leading to increased autoantibody production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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W.F., A.J.D., and Q.L. designed the experiments; W.F., A.J.D., and Y.W. performed experiments and analyzed data; W.F. and A.J.D. wrote the manuscript; and Q.L. revised the manuscript. All authors commented on and approved the manuscript.

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Abbreviations used in this article

bNAb	broadly neutralizing Ab
dpi	d postinfection
GC	germinal center
LT	lymphatic tissue
RM	rhesus macaque
T_{FH}	T follicular helper
T_{FR}	T follicular regulatory
T_{REG}	regulatory T cell

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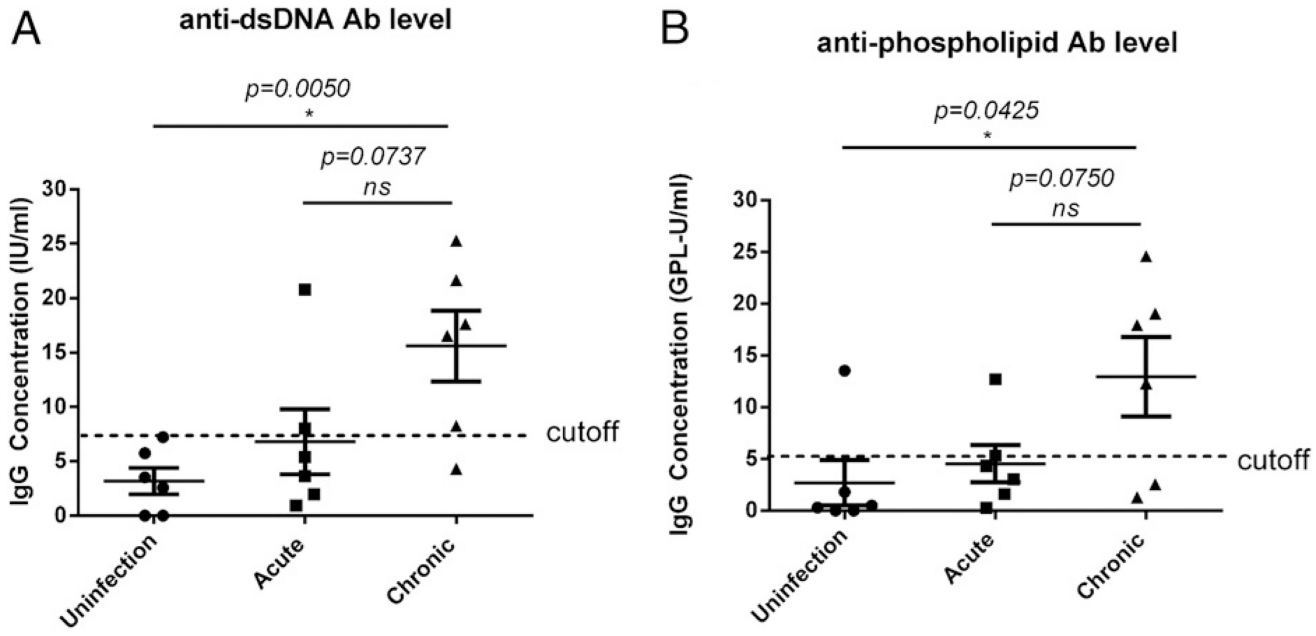


FIGURE 1.

Increased autoreactive Abs during chronic SIV infection. Anti-dsDNA (**A**) and anti-phospholipid (**B**) autoreactive Abs increased in chronic SIV infection (180 dpi) compared with no infection (0 dpi). Cutoff value for anti-dsDNA Ab is 7.46 IU/ml, and the cutoff value for anti-phospholipid Ab is 5.04 GPL-U/ml. Cutoff values were determined based on recommendations from the manufacturer or 2.5 times the OD_{450} value of the negative control. $*p < 0.05$ indicates statistically significant differences between the compared groups, Mann–Whitney nonparametric *t* test. ns, not significant.

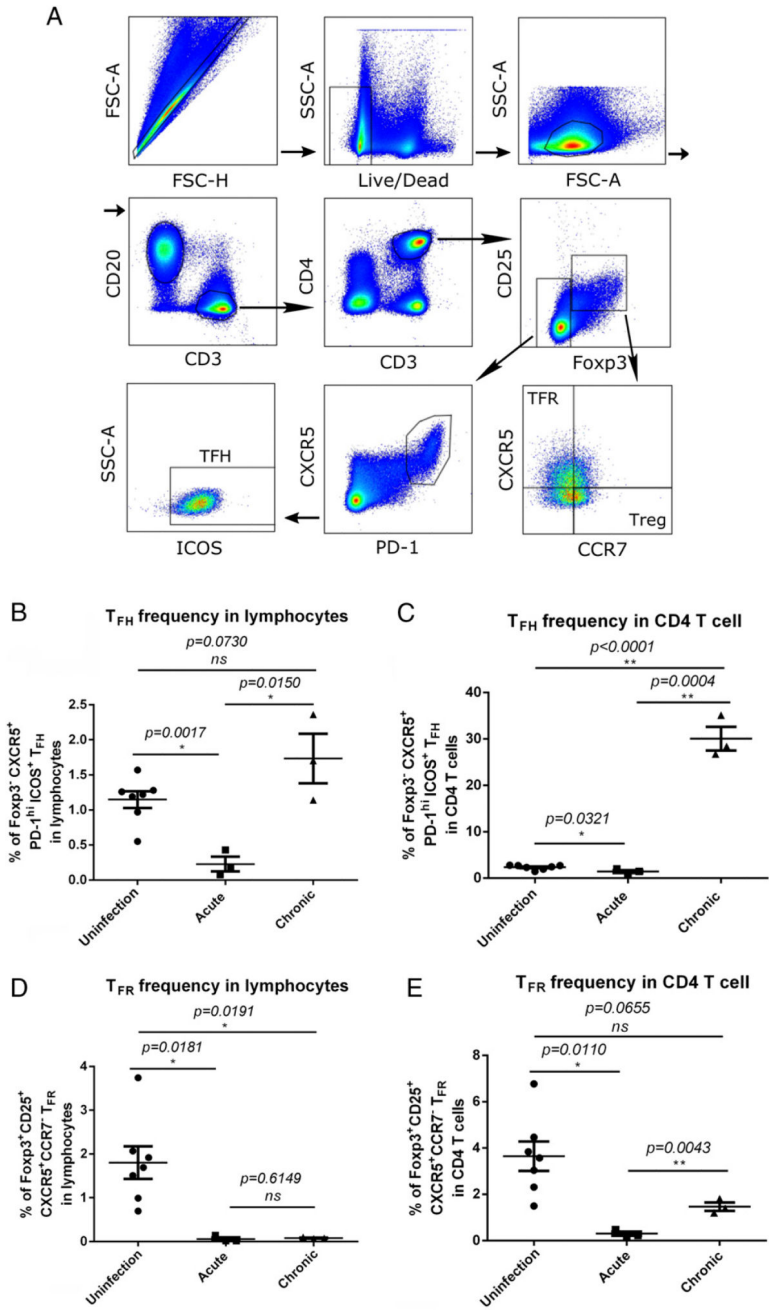


FIGURE 2.

Frequency of T_{FH} and T_{FR} cells in peripheral lymph node tissues following SIV infection. (**A**) Representative image of flow cytometry gating strategy for T_{FH} and T_{FR} cells. Frequency of T_{FH} ($Foxp3^{-}$ $PD^{-}1^{hi}$ $CXCR5^{+}$ $ICOS^{+}$) cells in total lymphocytes (**B**) and in CD4 T cells (**C**) following SIV infection. Frequency of T_{FR} ($Foxp3^{+}$ $CD25^{+}$ $PD\text{-}1^{hi}$ $CXCR5^{+}$ $CCR7^{-}$) cells in total lymphocytes (**D**) and in CD4 T cells (**E**) following SIV infection. Data are mean ± SEM. *$p < 0.05$, **$p < 0.001$, Mann–Whitney nonparametric *t* test. ns, not significant.

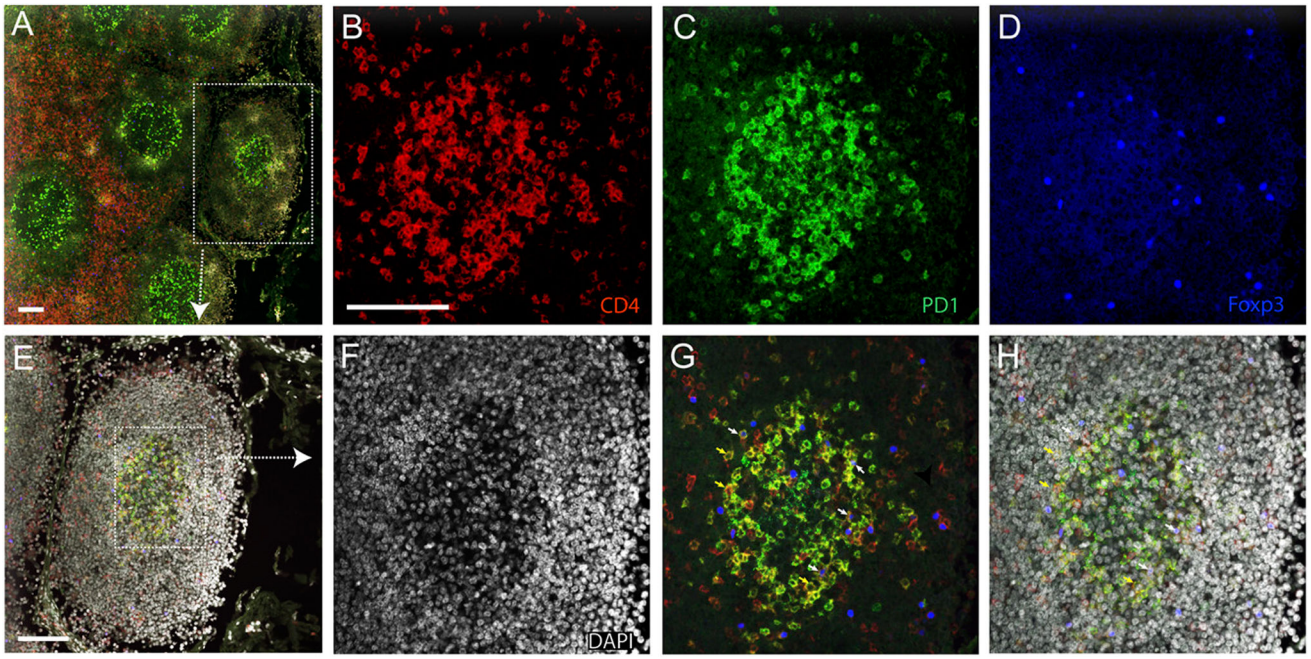


FIGURE 3.

Confocal photomicrographs of T_{FH} and T_{FR} cell localization in lymph node tissues. (**A**) B cell and T cell zones in LTs of an SIV-naive macaque are characterized by the distinct density of CD4 T cells (red). The box highlights a B cell follicle. Immunofluorescence staining of CD4 [(**B**), red], PD1 [(**C**), green], FoxP3 [(**D**), blue], and nuclei [(**F**), DAPI] in the GC shown in the box in (**E**). (**G** and **H**) Merged images; white and yellow arrows indicate representative T_{FH} and T_{FR} cells, respectively. Scale bars, 100 μm.

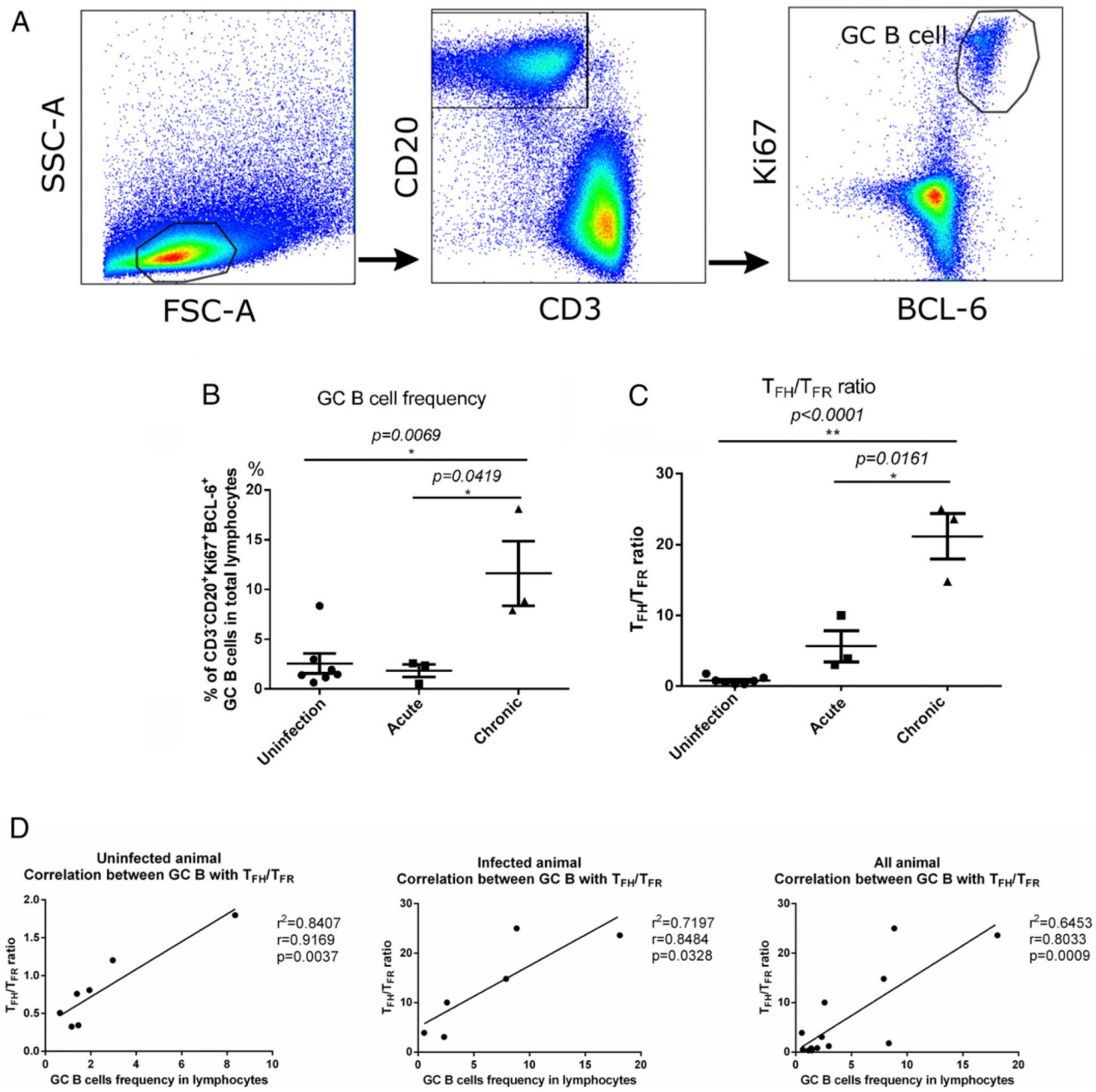


FIGURE 4.

Frequency of GC B cells following SIV infection. (**A**) Representative image of flow cytometry gating strategy for GC B ($CD3^-$ $CD20^+$ $Bcl\text{-}6^+$ $Ki67^+$) cells. (**B**) Frequency of GC B cells. (**C**) T_{FH}/T_{FR} cell ratio. (**D**) Correlation between the frequency of GC B cells in LTs and the T_{FH}/T_{FR} cell ratio. Data are mean ± SEM. Pearson *r* correlation was performed for correlation between the frequency of GC B cells and the T_{FH}/T_{FR} cell ratio. $*p < 0.05$, $**p < 0.001$, Mann–Whitney nonparametric *t* test.

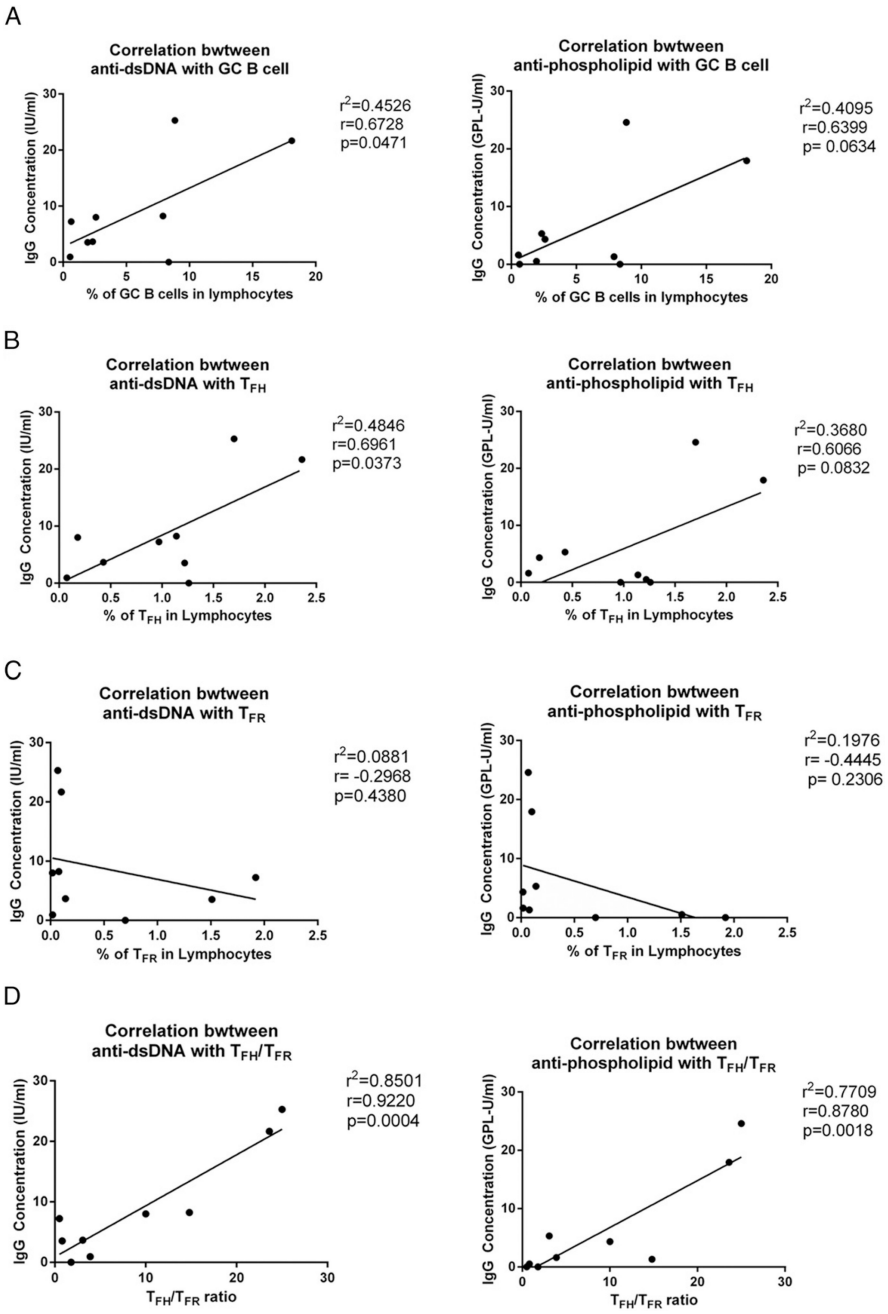
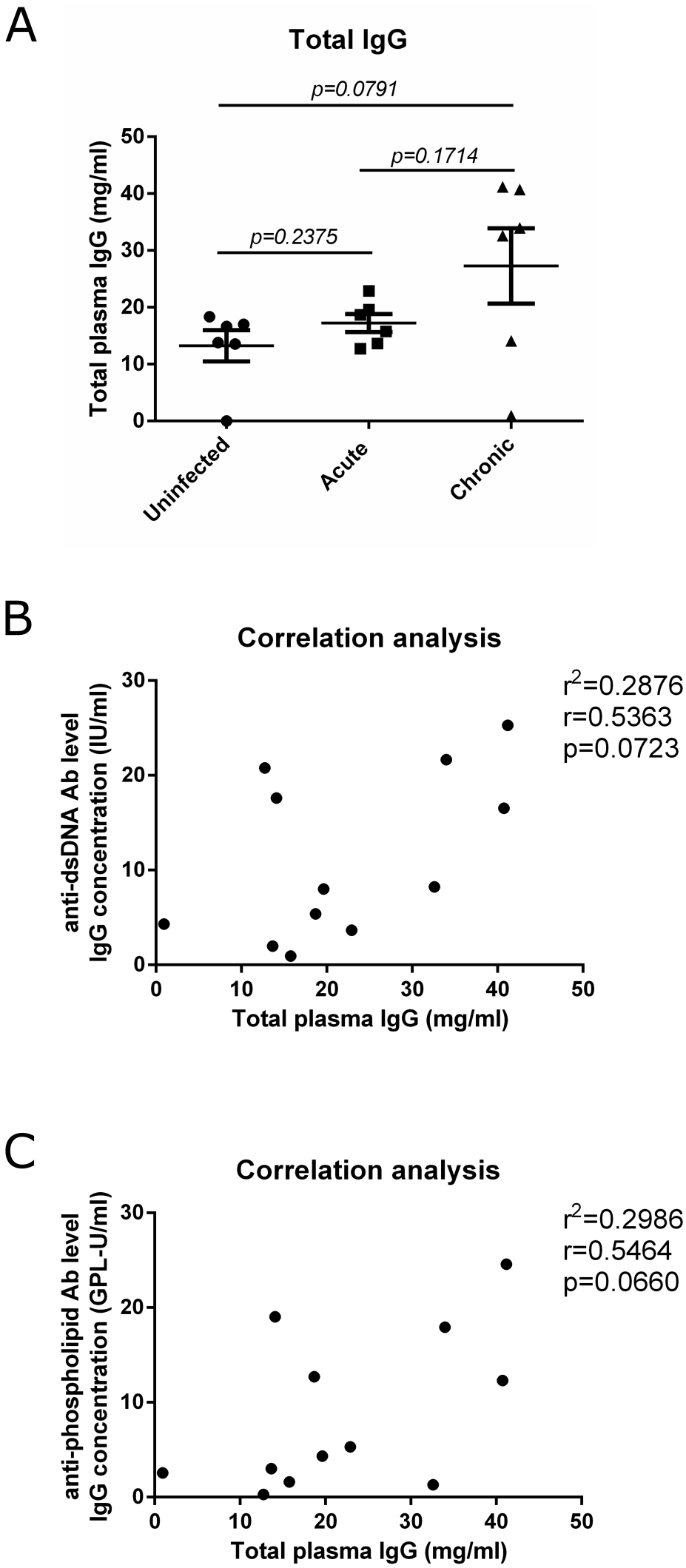
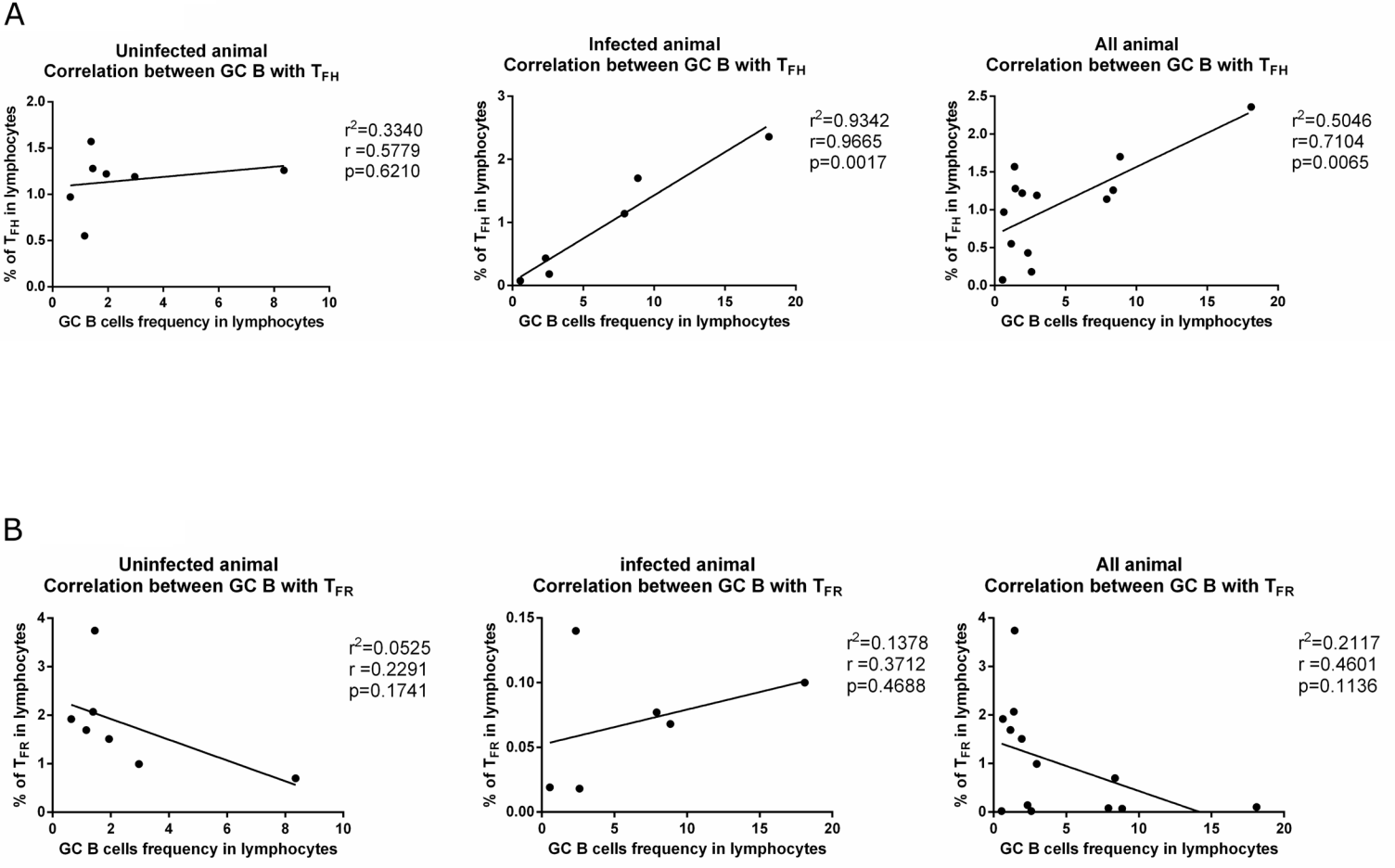


FIGURE 5.

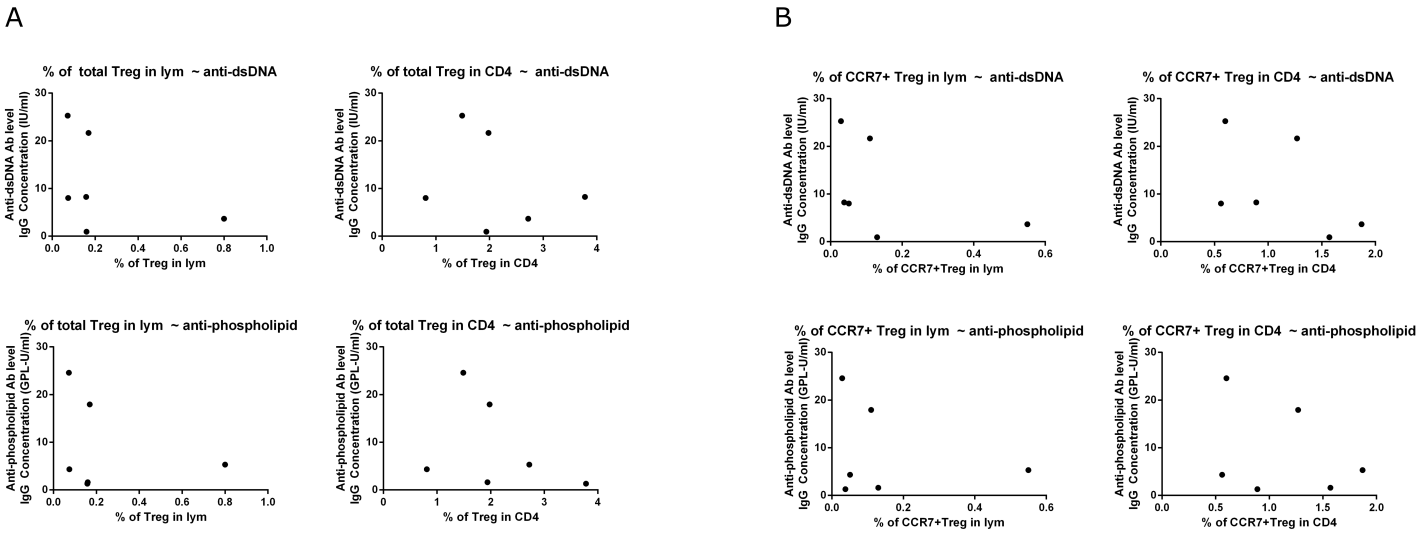
Correlation analyses between autoreactive Abs and the frequency of T_{FH} cells or T_{FR} cells or the T_{FH}/T_{FR} cell ratio. Correlation between the levels of anti-dsDNA and anti-phospholipid autoreactive Abs and the frequency of GC B cells (**A**), the frequency of T_{FH} cells (**B**), the frequency of T_{FR} cells (**C**), and the T_{FH}/T_{FR} cell ratio (**D**), using Pearson *r* correlation.



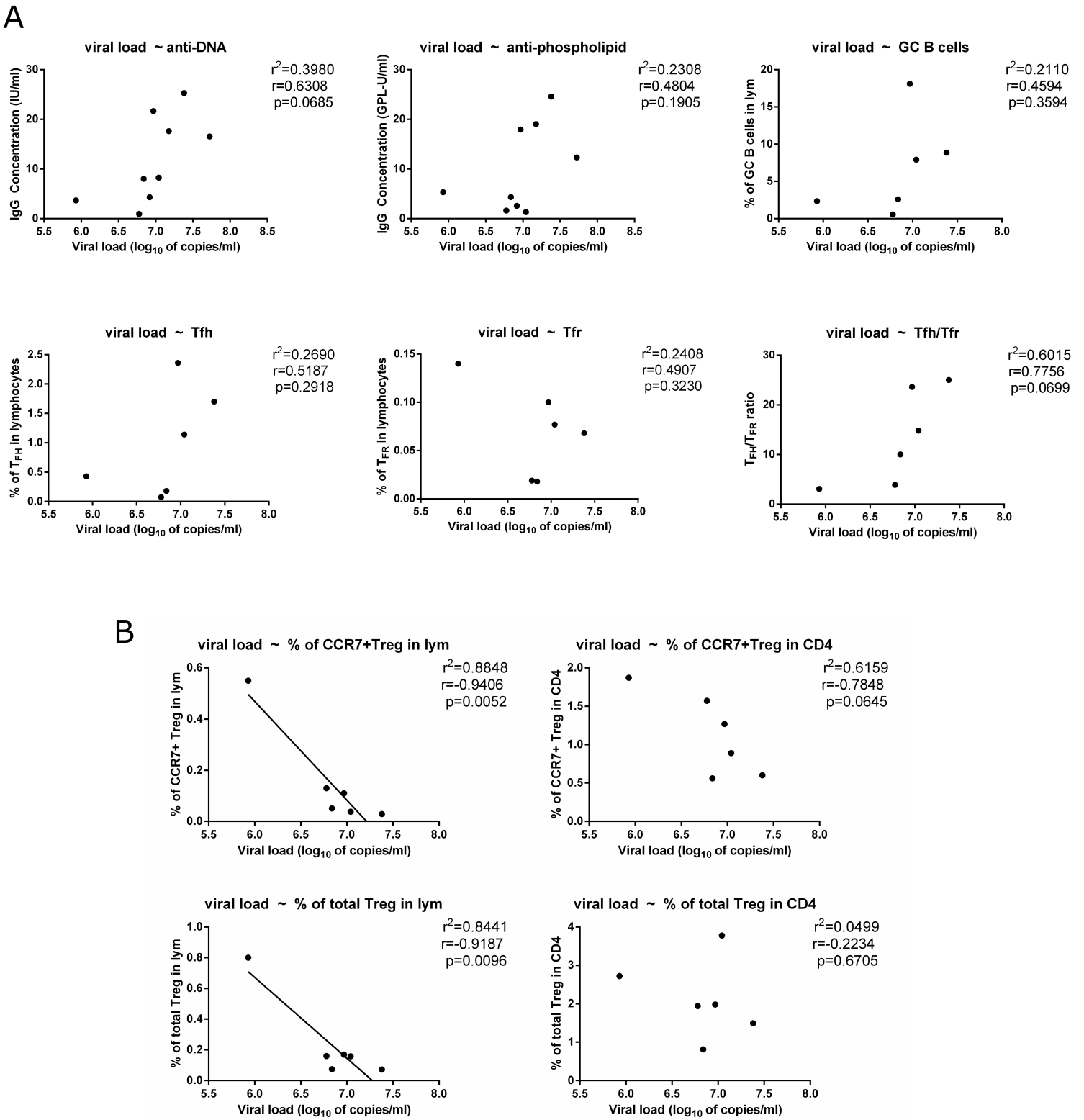
Supplemental Figure 1. Total IgG in plasma and its correlation with autoreactive antibodies. (A) Total IgG measurement. (B) Correlation analysis with anti-dsDNA autoreactive antibody. (C) Correlation analyses with anti-phospholipid autoreactive antibody.



Supplemental Figure 2. Correlation analyses of TFH and TFR cells with GC B cells. (A). TFH cells correlate with GC B cells in SIV infected RMs, but not uninfected rhesus macaques. (B) TFR cells correlate with GC B cells neither in SIV infected nor uninfected rhesus macaques.



Supplemental Figure 3. Correlation analyses of auto-reactive antibodies with Treg cells. (A) No correlation with Treg cells (CD4+Foxp3+CD25+CXCR5-). (B) No correlation with CCR7+ Treg cells (CD4+Foxp3+ CD25+CXCR5-CCR7+).



Supplemental Figure 4. Correlation analyses of plasma viral load with autoreactive antibodies, TFH, TFR, TFH/TFR ratio, TREGS and GC B cells in peripheral blood. (A) No correlation with autoreactive antibodies, TFH, TFR, TFH/TFR ratio and GC B cells. (B) No correlation with Treg cells (CD4+Foxp3+CD25+CXCR5-) and CCR7+ Treg cells (CD4+ Foxp3+CD25+CXCR5-CCR7+).