Decreased T Follicular Regulatory Cell/T Follicular Helper Cell (T<sub>FH</sub>) in Simian Immunodeficiency Virus–Infected Rhesus Macaques May Contribute to Accumulation of T<sub>FH</sub> in Chronic Infection

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Decreased T Follicular Regulatory Cell/T Follicular Helper Cell (T<sub>FH</sub>) in Simian Immunodeficiency Virus–Infected Rhesus Macaques May Contribute to Accumulation of T<sub>FH</sub> in Chronic Infection

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T follicular helper cells (T<sub>FH</sub>) are critical for the development and maintenance of germinal center (GC) and humoral immune responses. During chronic HIV/SIV infection, T<sub>FH</sub> accumulate, possibly as a result of Ag persistence. The HIV/SIV-associated T<sub>FH</sub> expansion may also reflect lack of regulation by suppressive follicular regulatory CD4<sup>+</sup> T cells (T<sub>FR</sub>). T<sub>FR</sub> are natural regulatory T cells (T<sub>REG</sub>) that migrate into the follicle and, similar to T<sub>FH</sub>, upregulate CXCR5, Bcl-6, and PD1. In this study, we identified T<sub>FR</sub> as CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>CXCR5<sup>+</sup>PDI<sup>+</sup>Bcl-6<sup>+</sup> within lymph nodes of rhesus macaques (RM) and confirmed their localization within the GC by immunohistochemistry. RNA sequencing showed that T<sub>FR</sub> exhibit a distinct transcriptional profile with shared features of both T<sub>FH</sub> and T<sub>REG</sub>, including intermediate expression of FOXP3, Bcl-6, PRDM1, IL-10, and IL-21. In healthy, SIV-uninfected RM, we observed a negative correlation between frequencies of T<sub>FR</sub> and both T<sub>FH</sub> and GC B cells, as well as levels of cell-associated viral DNA. Our data suggest that T<sub>FR</sub> may contribute to the regulation and proliferation of T<sub>FH</sub> and GC B cells in vivo and that a decreased T<sub>FR</sub>/T<sub>FH</sub> ratio in chronic SIV infection may lead to unchecked expansion of both T<sub>FH</sub> and GC B cells. The Journal of Immunology, 2015, 195: 3237–3247.

Several key findings over the past few years have energized efforts toward the development of an effective HIV vaccine, including the discovery and characterization of a number of broadly neutralizing Abs (bnAbs) that develop in a subset of HIV-infected individuals. However, the mechanisms involved in shaping Ab responses to immunization with HIV Ags or natural HIV infection remain incompletely understood (1). Importantly, HIV-Env–specific bnAbs develop at relatively late stages of HIV infection and show peculiar genetic and molecular features, including a high level of divergence from germline predecessors, which indicates that they are the products of extensive somatic hypermutation within germinal centers (GCs), as well as the presence of unusually long CDR3 regions (2). Perplexingly, there appear to be no direct or predictable routes to the development of these bnAbs from the germline predecessors, and it remains unclear whether this process is driven by antigenic mutations and/or escape as opposed to specific intrinsic aspects of the B cell or Th cell response (3). A better understanding of the mechanisms responsible for the development of bnAbs is crucial to harness this type of immunity for HIV prevention and therapy in humans.

T follicular helper cells (T<sub>FH</sub>) are critical for the development and maintenance of GCs, and competition for survival signals from T<sub>FH</sub> via molecules such as CD40L and IL-21 is thought to be a key mechanism of selection of high-affinity B cells (4). The regulation of T<sub>FH</sub> frequency, and by extension the regulation of their impact on GC B cell development and function, is vital to the quality of the humoral immune response (5). Although the presence of too few T<sub>FH</sub> may lead to abortive GC formation and defective B cell responses, an overexpansion is associated with the prevalence of autoantibodies (6, 7). It is possible that an expansion of T<sub>FH</sub> also lowers the selection pressure on GC B cells and leads to the emergence of low-affinity B cells (8).

Several studies have shown that T<sub>FH</sub> accumulate during the chronic stages of HIV/SIV infection. This accumulation occurs even though these cells support high levels of viral replication and represent an important component of the persistent virus reservoir under antiretroviral therapy (9). The chronic expansion of T<sub>FH</sub> in the case of HIV/SIV infection with persistent virus replication may be...
a direct result of antigenic persistence. As expected, HIV/SIV-associated expansion of T$_{FH}$ is associated with dysregulation of B cell responses with ineffective memory cell formation and hypergammaglobulinemia (10, 11). Whether and to what extent this T$_{FH}$ expansion is also related to a deficit in the physiological regulation of specific T$_{FH}$ immune response within the lymph nodes remains unknown. However, this possibility would be consistent with the well-known observation that the chronic phase of pathogenic HIV/SIV infections is associated with a state of generalized immune activation that is resistant to the normal mechanisms of immune regulation.

Under normal circumstances, regulation of T$_{FH}$ function is mediated, at least in part, by a recently described subset of CD4$^+$ T cells termed T follicular regulatory cells (T$_{FR}$). T$_{FR}$ are thought to develop from thymic-derived regulatory T cells (T$_{REG}$) that express lineage-associated markers such as FOXP3, CD25, and low levels of CD127. These T$_{FR}$ migrate into the follicles of lymph nodes by virtue of their expression of CXCR5 (and downmodulation of CCR7) and, similarly to T$_{FH}$, express high levels of Bcl-6 and PD1 (12, 13). Notably, the role of T$_{FR}$ in the immunopathogenesis of HIV/SIV infections is currently unknown, both in terms of ability to negatively regulate HIV-specific B cell responses (including, potentially, the production of bnAbs) and to suppress local virus-induced immune activation. Indeed, none of the previous reports on T$_{FH}$ dynamics in the context of SIV or HIV infection have distinguished between cells that either do or do not coexpress T$_{REG}$-associated markers. Thus, the T$_{FR}$ subset within the broader CXCR5$^+$ Bcl-6$^+$ PD1$^+$ is not fully characterized in the setting of HIV/SIV infection.

In this study, we described and characterized phenotypically, historically, and genomically the T$_{FR}$ population that is found within the GCs of rhesus macaques (RM) and express markers associated with both T$_{FH}$ and T$_{REG}$. The hypothesis that T$_{FR}$ play a suppressive role in vivo is supported by the observation that their frequency is inversely correlated with both the levels of T$_{FH}$ and GC B cells and the percentage of proliferating CD4$^+$ T cells. In the setting of SIV infection, we found that T$_{FR}$ show a slow in vivo proliferative response after the initial infection and exhibit only a small increase in their frequency within the total CD4$^+$ T cell pool during the chronic phase. In conjunction with the large expansion of T$_{FH}$ observed post SIV infection, this phenomenon leads to a significantly decreased T$_{FR}$/T$_{FH}$ ratio in the lymph nodes of chronically SIV-infected RM. These data suggest that, during SIV infection, a lack of T$_{FR}$ expansion may allow for a progressive accumulation of T$_{FH}$ cells in the lymph nodes of chronically infected RM, thus indirectly contributing to the aberrant immune activation that characterizes this pathogenic infection.

### Materials and Methods

#### Animals

This study involved a total of 40 Indian origin female RM divided as follows: 1) 10 healthy, unvaccinated, and SIV-uninfected RM; 2) 10 healthy, SIV-immunized, but SIV-uninfected RM; 3) 11 unvaccinated SIV-infected animals; and 4) 9 vaccinated and SIV-infected RM. Animals were vaccinated with a SIVmac239 Gag-, Pol-, and Env-expressing DNA vaccine with inactivating mutations in proteases, half of which also coexpressed GM-CSF. These were followed by two boosts of a SIVmac239 Gag-, Pol-, and Env-expressing modified vaccinia Ankara (MVA) vaccine as described previously (14). All infections were a result of SIVsmE660 intravaginal challenge at 106–107 PFU per animal. Tissue collection and processing was performed as previously described (15). Tissue processing

Lymphocytes were isolated from freshly obtained lymph node and spleen by passing homogenized tissue through a 70-μm cell strainer and lysing blood cells with ACK lysis buffer. Tissue collection and processing was performed as previously described (15). Cells to be later used for sorting were cryopreserved at −80°C in PBS medium containing 10% DMSO.

#### Immunophenotyping and confocal microscopy

Immunohistochemistry and confocal microscopy

Immunohistochemistry was performed on 5-μm tissue sections mounted on glass slides, which were deparaffinized and rehydrated with double-dH$_2$O. Slides were then deparaffinized with xylene, and rehydrated with double-dH$_2$O and incubated for 10 min using Dako Protein block. Slides were then incubated with secondary Ab mixture containing donkey anti-rabbit Alexa 488 (1:500), donkey anti-mouse Alexa 594 (1:500), and donkey anti-goat Alexa 647 (1:500). Digoxigenin-dUTP (clone DG-77 (1:500)), anti-PD1 (1:500) for 1 h at room temperature. Next, slides were washed in TBS with 0.05% Tween 20. Slides were then incubated for an hour in the dark with secondary Ab mixture containing donkey anti-rabbit Alexa 488 (1:500), donkey anti-mouse Alexa 594 (1:500), and donkey anti-goat Alexa 647 (1:500). After washing in TBS with 0.05% Tween 20, Prolong Gold with DAPI was applied to all the slides. Confocal microscope images were obtained using Olympus FV10i Confocal Microscope with CellSens 1.9 Digital Imaging software.

Quantitative PCR for SIV gag DNA

Cell-associated viral DNA was measured in sorted cell populations from RM lymph nodes by RT-PCR as previously described (16–18).

RNA-Seq library preparation

Total RNA was prepared using the QIAGEN RNeasy Micro Kit. Libraries were generated using the Clontech SMARTer HV kit, barcoding, and sequencing primers were added using Nextera XT DNA kit. Libraries were validated by microelectrophoresis, quantified, pooled, and clustered on Illumina TruSeq v3 flow cell. Clustered flow cell was sequenced on an Illumina HiSeq 1000 in 100-base single-read reactions.

RNA-sequencing data analysis

RNA sequencing (RNA-Seq) data were submitted to the Gene Expression Omnibus repository at the National Center for Biotechnology Information. RNA-Seq data were aligned to a provisional assembly of Indian Macaca mulatta (MaSuRCA rhesus assembly v7.20130927) using STAR version 2.3.0e (19, 20). Transcripts were annotated using the provisional UNMC
Total RNA was prepared using the QIAGEN RNasy Micro Kit from sorted TFR, TFH, and TREG. RNA quantity was measured using Nanodrop analysis and reverse transcribed as previously described for RNA sequencing. Finally, 0.1 μl cDNA was used for real-time SYBR green PCR analysis using an ABI 7900 HT real-time PCR instrument (Applied Biosystems). Primer sequences for PCR were GAPDH: forward (Fwd) 5'-GGGACCACTCAGTTCGATAGC-3', reverse (Rev) 5'-TCTTCTGGTCCCGTATGGA-3'; IL-10: Fwd 5'-GCACCCCCCTCCGACG-3', Rev 5'-GCCCTTATCCCATTCGACG-3'; SLAMF6: Fwd 5'-TGCCGATGACGCCATGAGG-3', Rev 5'-CTCCTGGCTTCACGTTATTTCT-3'. Relative RNA transcript levels were calculated normalized to primer efficiency and housekeeping gene RNA (GAPDH).

Statistical analyses
Except for RNA-Seq data, all statistical analyses were conducted using GraphPad Prism 5.0. Comparisons of mean fluorescence intensity (MFI) between cell populations in uninfected RM were made using Wilcoxon signed rank tests (Fig. 2). Mann-Whitney U tests were used to compare frequencies of populations in uninfected, acutely infected, and chronically infected RM (Figs. 4, 5). Spearman rank correlation tests were used to analyze all correlations (Fig. 6). All p values <0.05 were defined as significant.

Results
TFR are distinct from TFH and TREG and can be found within lymph node GCs in RM
Recent studies of GC TFR have defined these cells based on their surface expression of the chemokine receptor CXCXR5 and very high levels of the co-inhibitory receptor PD1 (10). However, a fraction of these canonically defined TFR also express the lineage-specific TREG differentiation pathway to TFR. Fig. 1B and 1C also show that, as expected, non-FOXP3–expressing “true” TFH are also seen within GCs of the same animals.

TFR express markers of both TFH and TREG differentiation
We next performed a comprehensive examination of the TFR phenotype in healthy, SIV-uninfected RM. As shown in Fig. 2, our analysis of relative MFI for TFR markers confirmed that TFR express FOXP3 and CD25 at comparable levels with TREG (Fig. 2A) and both CXCXR5 and PD1 at comparable levels with TFH (Fig. 2B). We next examined in TFR the expression patterns of a series of markers (i.e., CD127, CTLA4, Bel-6, and Helios) that have been linked to either TFH or TREG phenotype and function (1, 25). CD127, the IL-7Rα chain, is expressed at low levels on TREG in humans (26–28). As expected, we found that TFR express CD127 at lower levels than the bulk of CD4+ T cells, and similar or even lower levels than those observed in TREG and TFH (Fig. 2C). CTLA4 is a key negative T cell regulator that is constitutively expressed on TREG and, upon ligation, induces downmodulation of cytokine production and inhibition of cell cycle progression (25). Consistent with previous reports in murine models (12), we observed that TFR express CTLA4 at a higher frequency and MFI than both TREG and TFH cell populations (Fig. 2D). This is consistent with a putative role of TFR as negative regulators of GC responses. Helios is a transcription factor expressed in thymus-derived natural TREG (29). As shown in Fig. 2E, TFR express Helios at levels that are even higher than those observed in TREG in terms of both frequency of positive cells and MFI, thus suggesting that TFR originate from natural TREG in RM, as well as in mice.

Transcriptome analysis of TFR reveals a distinct but overlapping transcriptional profile compared with TFH and TREG
To further define the functional features of TFR in RM, we next examined the transcriptional profiles of TFH, TFR, and TREG using RNA-Seq by Illumina technology. Splenocytes from five healthy, SIV-uninfected, and unvaccinated RM were sorted into “bulk” CD3+ CD4+ T cells, TREG, TFH, and TFR based on the following phenotypic markers: TFR (CXCXR5+PD1+CD127+), TFH (CXCXR5+PD1−CD127+/−), TREG (CXCXR5−PD1− CD127−), and TREG (CXCXR5−PD1−CD127−). As expected, non-FOXP3–expressing “true” TFH are also seen within GCs of the same animals.

TFR express markers of both TFH and TREG differentiation
The Journal of Immunology 3239

These TFR can also be readily identified within GCs of SIV-infected expression of PD1 were identified within GCs of uninfected RM. As shown in Fig. 1B, single cells with nuclear expression for FOXP3, but not PD1, are visible in the T cell zone just outside the GC (Fig. 1C). Presumably, some of these TREG migrate into the GC and upregulate TFR-like markers along their differentiation pathway to TFR. Fig. 1B and 1C also show that, as

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FIGURE 1. T_{FR} can be defined by flow cytometry and identified by confocal microscopy within the GCs of RM. (A) Representative flow-cytometry plot of lymphocytes from lymph nodes of untreated, uninfected RM showing the gating strategy used to define T_{FR}, T_{FH}, and T_{REG} populations. (B) Representative confocal microscope image showing a single T_{FR} within the lymph node of an uninfected RM. The first image shows staining for DAPI (green) and FOXP3 (red), the second image shows the same section with CD4 (green) and FOXP3 (red), the third image PD1 (blue) and FOXP3 (red), and the last image CD4 (green), PD1 (blue), and FOXP3 (red). Original magnification ×120. (C) Representative images of lymph node biopsies from SIV-uninfected and acutely infected RM showing cells stained with CD4 (green), FOXP3 (red), and PD1 (blue) within the GC regions.
in TFR than either TREG or TFH, thus suggesting that the CD25+ CXR5+PD1hi phenotype may represent a more transcriptionally active population than classical TREG or TFH. This latter set of RNA-Seq data provides strong evidence that TFR are indeed a distinct cell subset and that the somewhat hybrid transcriptional profile of TFR is not simply due to the sample being a mixture of TREG and TFH. Notably, elevated expression of IL-10 in TFR compared with TREG has been previously reported in murine studies (13).

To then compare the profile of gene expression between TFR with TREG and TFH subsets without using any a priori information, we defined TFH and TREG expression signatures by statistically contrasting RNA-Seq data from TFH and TREG with bulk CD4+ T cells. After exclusion of transcripts that had zero expression in any of the populations, a total of 88 genes made up the combined TFH and TREG signature of which 12 genes were TREG related. Many, but not all, canonical TREG and TFH genes were also identified as significantly

FIGURE 2. TFR share immunophenotypical features of both TFH and TREG populations. MFI, percent positive, representative flow-cytometry plots, and histograms (A–F) for expression of various immunophenotypical markers (i.e., FOXP3, CD25, CXCR5, PD-1, CD127, CTLA4, Bcl-6, and Helios) among TREG–non-TREG, TFR, and TFH populations from lymph node of healthy, unvaccinated, and uninfected RM. Non-TREG are defined as all CD4+CD25–FOXP3–T cells. Significance was determined by Wilcoxon signed rank tests.
upregulated compared with bulk CD4+ T cells. The lack of statistical significance for some prototypical transcripts is likely due to the presence TREG and TFH subsets within the bulk CD4+ population used as a comparator sample. Nevertheless, we found that TFR show similar levels of expression of TFH signature genes such as Bcl-6, TIGIT, CD200, LATm, and BATF (Fig. 3C). TFR cells also express mRNA for key TFH-related genes that are important for B cell help, including IL-21, SH2D1A, CD40L, and CD84. One
The dynamics of TFR in the setting of HIV or SIV infection have not been previously investigated; in fact, all published studies of TFH dynamics during HIV/SIV infection used a definition of these cells that included TFR as well. To study the kinetics of TFR, TFH, and TREG post SIV infection of RM, we measured the frequency of these cells within the lymph nodes preinfection, 2 wk postinfection, and 6 mo postinfection with SIVsmE660. The RM included in these kinetics analyses included both unvaccinated and animals that were challenged after immunization with a SIVmac239 Gag-, Pol-, and Env-expressing DNA vaccine (with or without GM-CSF) followed by two boosts of a SIV239 Gag-, Pol-, and Env-expressing MVA vaccine. As previously reported, we found a significant increase (\( p < 0.0001 \)) in frequency of TFR at 24 wk postinfection (Fig. 4A). Interestingly, the frequency of TFR measured as percent of total CD4\(^+\) T cells also showed a significant (\( p = 0.0001 \)) increase during chronic SIV infection (Fig. 4A). However, when the frequency of TFR is measured as percentage of total TFH, we found that the TFR decrease significantly at both the acute (\( p = 0.0385 \)) and the chronic (\( p = 0.0016 \)) phases of SIV infection (Fig. 4B). Accordingly, the overall ratio of TFR to TFH also decreased significantly (\( p = 0.0018 \)) at the week 24 postinfection time point as compared with baseline (Fig. 4C). The increase of both TFH and TFR as a percent of CD4\(^+\) T cells postinfection is likely the result of proliferation driven by Ag persistence and virus-mediated depletion of other CD4\(^+\) T cell subsets. However, the relative decrease in the frequency of TFR when measured as percentage of TFH suggests that the low frequency of these regulatory cells might contribute to the expansion and accumulation of TFH in chronically SIV-infected RM. Notably, we found no significant changes in TREG frequencies after SIV infection within the lymph nodes. To better define the kinetics of TFH and TFR during SIV infection, we next measured the level of cell proliferation using the well-established marker Ki67. We observed that TFH show a significant increase in proliferating cells during the acute (\( p < 0.0001 \)) and chronic (\( p = 0.0001 \)) phases of infection (Fig. 4D). TFR have a similar pattern of proliferation, with a significant increase in proliferating cells during the acute (\( p < 0.0001 \)) and chronic (\( p = 0.0376 \)) phases of infection (Fig. 4D). In contrast, the level of Ki67 expression in TREG remains relatively low throughout our analysis with a small, significant increase (\( p = 0.0141 \)) during the chronic phase of infection (Fig. 4D).

**Similar levels of SIV infection of TFR as compared with TFH and TREG despite higher CCR5 expression**

Several studies have shown that, during HIV and SIV infection, TFH are highly infected with the virus despite their relative increase within the total CD4\(^+\) T cell pool (9). Although the actual in vivo life span of TFH, either infected or uninfected, remains unknown in the setting of HIV/SIV infection, the presence of a notable fraction of these cells expressing the proliferation marker Ki67 suggests that their number could be maintained through continual replenishing from precursors located outside the GC. To measure the level of direct SIV infection of TFR, TFH, and TREG, we sorted these subpopulations from the lymph nodes of a subset of our studied animals and quantified the levels of total cell-associated SIV-DNA by RT-PCR. This analysis revealed that TFH, TFR, and TREG derived from chronically SIV-infected RM all harbor comparably high levels of cell-associated viral DNA (Fig. 5A). Interestingly, the levels of SIV infection were similarly high between TFR and TFH even though the
surface expression levels of the main SIV coreceptor CCR5 were significantly higher in TFR as compared with TFH (Fig. 5B).

**Frequency of TFR is negatively correlated with the number and proliferation of both TFH and GC B cells**

To further examine the relationship between TFR and TFH and GC B cells, we next performed a set of correlation analyses in the RM included in this study. We observed that, in healthy uninfected RM, the frequency of TFR (as fraction of the total TFH pool) is negatively correlated with the percentages of TFH (as fraction of total CD4+ T cells) and GC B cells (as fraction of total B cells) (Fig. 6A). In addition, we found that, in the same animals, the frequency of TFR (as fraction of TFH) is negatively correlated with the level of CD4+ T cell proliferation as measured by Ki67 expression (Fig. 6A).

We next performed the same correlation analyses in our cohort of SIV-infected RM. The SIV-infected RM included in these regression analyses included both unvaccinated and animals that were challenged after immunization with an SIVmac239 Gag-, Pol-, and Env-expressing DNA vaccine (with or without GM-CSF) followed by two boosts of an SIV239 Gag-, Pol-, and Env-expressing MVA vaccine. In the SIV-infected RM, similar to what was observed in uninfected animals, the frequency of TFR (as fraction of TFH) is negatively correlated with the percentages of both TFH and GC B cells (Fig. 6B). However, the negative correlation between frequency of TFR (as fraction of TFH) and the level of CD4+ T cell proliferation as measured by Ki67 expression is not seen in SIV-infected RM (Fig. 6B). The negative correlation between TFR cells (as a frequency of TFH cells) and both TFH and GC B cell frequencies is consistent with the hypothesis that TFR cells play a role in regulating TFH and GC responses under normal circumstances and in the setting of chronic SIV infection.

**Comparative analysis of the TFR transcriptome in SIV-infected and uninfected RM**

To further define the effect of SIV infection on TFR, we next compared the transcription profiles of TFR that were isolated from unvaccinated, chronically SIV-infected and uninfected RM (Fig. 7). We performed RNA-Seq analysis, and transcripts that were significantly differentially expressed in TFR sorted from SIV-infected versus uninfected RM were analyzed by Ingenuity Pathway Analysis. Unsurprisingly, a large proportion of genes induced during SIV infection in TFR (CD3G, FOS, CD4, ZAP70, PIK3CD, STAT3) were components of T cell proliferation, activation of T cell effector function, and costimulatory activation (data not shown).
The enhanced T cell activation was consistent with our observation that TFR cells express higher levels of the proliferation marker Ki67 compared with TREG (Fig. 4D). We also observed that several genes implicated in pathways regulating apoptosis or cell-cycle control were perturbed in SIV-infected RM. Of particular interest was the observation that the proapoptotic gene FASLG was 100-fold induced, whereas the antiapoptotic regulator XIAP was significantly downregulated. This latter finding was again validated by RT-PCR (Supplemental Fig. 2B). Thus, although we observed a significant increase of the proliferation marker Ki67 in TFR after SIV infection (Fig. 4D), a proapoptotic shift of gene expression may partly explain why only a modest increase in TFR frequency was observed (Fig. 4A).

TFR require IL-6 signaling and STAT3 expression for differentiation and, once mature, produce several factors that support B cell activation. Conversely, IL-2R signaling drives STAT5 to activate Blimp1/PRDM1, which ultimately blocks TFH differentiation (30). However, TFR express both Blimp/PRDM1 and Bcl-6. In this study, we find that both IL-6 and IL-2 signaling genes are enriched in TFR after SIV infection. However, several of these genes, such as MAPK1, are common to different cytokine signaling pathways, thus making it difficult to establish whether SIV infection causes a shift in the TFR/TFH differentiation pressure. Interestingly, downstream signaling for IL-10, a regulatory cytokine produced by both TFR and TREG, is also enriched in TFR postinfection. ICOS-ICOSL signaling was also enriched in TFR postinfection. These data suggest that TFR may be engaged in similar TFR-like cell-surface receptor–ligand interactions with B cells. In addition to genes that were identified with differential expression without any a priori knowledge, we also examined genes with known function in TFR and TREG. Postinfection, TFR show a significant increase in the expression of PD1, IL-6R, SLAMF6, and CD84, that is, all markers associated with TFR differentiation and function (Fig. 7B). We also found a significant decrease in STAT3 and IL-2RA in TFR after SIV infection and a nonsignificant decrease in Bcl-6 expression. Finally, as expected, we also observed several other changes in expression patterns of the TFR and TREG signature gene sets as we had previously determined (Supplemental Fig. 1). Overall, these data indicate a complex remodeling of gene expression in TFR post SIV infection of RM.

**Discussion**

**T**FH are critical to the development of the humoral response to infections, and their role in the setting of HIV and SIV infection (and vaccination) is the subject of intense investigation. However, some aspects of the complex TFR response to HIV/SIV infection remain poorly understood, including: 1) their role in promoting the development of broadly neutralizing HIV/SIV-specific Abs, and 2) their role in the immunopathogenesis of the infection. In particular, the mechanisms by which TFR accumulate during the chronic stage of infection despite high levels of direct virus infection are unclear. Importantly, a series of recent studies have shown that TFR include a subset of cells that are derived from thymic TREG precursors, express the classical TREG markers (i.e., FOXP3 and CD25, as well as low levels of CD127), and acquire TFR markers (i.e., PD1, CXCR5, and Bcl-6) while migrating into the GC of lymph nodes, where they are thought to act as regulators of the host humoral immune response. To the best of our knowledge, this study, together with the independently generated set of data that are included in the accompanying article by the group of Franchini and Vaccari (31), represents the first description of the main features of TFR in a nonhuman primate species. In this work, we also investigated the dynamics of this cell subset during SIV infection of RM.

The main findings of this study are the following: 1) TFR show distinct yet overlapping phenotype as compared with TREG and TFR based on a combination of flow cytometric, histological, and transcriptional analyses; 2) in healthy, SIV-uninfected RM, the frequencies of TFR are negatively correlated with the levels of both TFR and GC B cells; 3) post SIV infection, the TFR/TFH ratio is reduced; and 4) TFR sorted from SIV-infected RM harbor comparable levels of cell-associated viral DNA as compared with TFR and TREG. Collectively, these data indicate that although TFR closely resemble TFR in several aspects, they are also clearly distinguishable from...
DECREASED TFR/TFH RATIO IN SIV-INFECTED RHESUS MACAQUES

Although CD4+ T cells are the main target for HIV and SIV infection, substantial differences exist between various CD4+ T cell subsets in terms of their relative levels of direct virus infection in vivo (34–36). In this study, we tested the possibility that the decrease in the TFR/TFH ratio observed during SIV infection of RM was associated with higher level of virus infection in TFR as compared with TFH. However, our comparative analysis of the cell-associated viral burdens in sorted TFR, TFH, and TREG revealed similar levels of SIV-DNA in the three CD4+ T cell subsets, even though TFR exhibited higher levels of the SIV co-receptor CCR5 as compared with the other two subsets.

In summary, to our knowledge, the presented data provide the first comprehensive description of TFR in healthy, uninfected RM, as well as the first examination of the kinetics of these cells in the setting of pathogenic SIV infection. These results support the hypothesis that these cells play an important immune-regulatory role in vivo, and that a relative decline of the TFR/TFH ratio may be involved in establishing a state of chronic immune activation in the B cell areas of lymph nodes during pathogenic HIV and SIV infection.

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Disclosures
The authors have no financial conflicts of interest.

References


Corrections


The second author’s name was incorrect in the published article. The correct name is Perla Mariana Del Rio Estrada.

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