Decreased T Follicular Regulatory Cell/T Follicular Helper Cell (T_{F H}) in Simian Immunodeficiency Virus–Infected Rhesus Macaques May Contribute to Accumulation of T_{F H} in Chronic Infection

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

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Abbreviations used in this article: bnAb, broadly neutralizing Ab; GC, germinal center; MFI, mean fluorescence intensity; MVA, modified vaccinia Ankara; RM, rhesus macaque; RNA-Seq, RNA sequencing; TFH, T follicular helper cell; TFR, T follicular regulatory cell; TREG, regulatory T cell.

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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 (TFH) are critical for the development and maintenance of GCs, and competition for survival signals from TFH 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 TFH 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 TFH 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 TFH 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 TFH 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 persisting virus reservoir under antiretroviral therapy (9). The chronic expansion of TFH in the case of HIV/SIV infection with persistent viral 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 defect 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 SIVmac660 intraregional challenge at 2.06 × 10^5 50% tissue culture infective dose grown in RM PBMCs. Lymph node biopsies were collected for measurement of a number of immunological parameters at day −35 preinfection and days 14 and 168 postinfection (i.e., acute and chronic phase, respectively). Spleen and lymph node biopsies were collected at necropsies performed at 6 mo postinfection. All animals were housed at Yerkes National Primate Center at Emory University and were cared for in accordance with National Institutes of Health guidelines and following protocols approved by the Institutional Animal Care and Use Committee.

Tissue processing

Lymphocytes were isolated from freshly obtained lymph node and spleens 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 media containing 10% DMSO.

Immunophenotyping and flow cytometry

Multicolor flow-cytometric analysis was performed on mononuclear cells isolated from blood and lymph nodes according to standard procedures using mAbs directed against RM markers and human markers that also cross-react with the same markers in RM. For optimal staining of intracellular markers, permeabilization of cells using the eBioscience Foxp3-permeabilization buffer was performed as recommended by the manufacturers. Predetermined optimal concentrations of the following Abs and reagents were used: CD3–Alexa 700 (clone SP54-2), CD4-allophycocyanin-Cy7 (clone OKT-4), Bcl-6–PE–Texas Red (clone K112-91), Ki67–FITC (clone B56), CCR5–PE (clone 3A9), and CTLA4–BV421 (clone BN3) from BD; CXCR5–PerCP eFlour710 (clone MUSBEE), PD1–PeCy7 (clone J105), and CD127–PeCy5 (clone eBioRDR5) from eBioscience; and CD20–BV650 or PE–CF594 (clone 2H7), CD25–BV711 (clone BC96), Helios–FITC (clone 22F6), and FOXP3–allophycocyanin (clone 15D0) from BioLegend; and Live/Dead Fixable Aqua from Invitrogen. Flow-cytometric data were acquired using LSRII flow cytometer using BD’s FACS Diva software. Acquired data were analyzed using FlowJo version 9.3.2 following the gating strategy described in Fig. 1. Further analyses were performed using PRISM (GraphPad) and Excel (Microsoft Office 2011) software.

Cell sorting

Cryopreserved cells were thawed in a 37°C water bath and rested overnight for 8–10 h and then stained for sorting. Splenocytes from five SIV-uninfected and unvaccinated RM, as well as five unvaccinated SIV-infected animals were used for sorting of T~FR~, T~FH~, and T~REG~. Cells populations were sorted using FACS Aria II flow cytometer. Cells were first gated based on light scatter followed by positive gating on cells negative for Live/Dead Fixable Aqua and positive for CD3 and CD4. After collecting bulk CD4^+ cells the following three populations were collected: T~FR~ (CXCR5^+PD1^+CD127^−CD25^−), T~FH~ (CXCR5^+PD1^+CD127^+CD25^−), and T~REG~ (CXCR5^+PD1^+CD127^+CD25^+).

Immunohistochemistry and confocal microscopy

Immunohistochemistry was performed on 5-μm tissue sections mounted on glass slides, which were deparaffinized and rehydrated with double-dH2O. Ag retrieval was performed in 1× Dako Target Retrieval Solution (pH 6.0) in a pressure cooker heating slides to 122°C for 30×. Slides were then rinsed in ddH2O and incubated for 10 min using Dako Protein block. Slides were then incubated with rabbit anti-CD4 (1:200), mouse anti-FOXP3 (1:100), and goat anti-PDI (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 microscopy images were obtained using Olympus FV100 ConfoScan 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 HiSeqation 1000 in 100-base single-read reactions.

RNA-seq 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 v.7_20130927) using STAR version 2.3.0e (19, 20). Transcripts were annotated using the provisional UNMC
RT-PCR validation of RNA-sequencing data

Total RNA was prepared using the QIAGEN RNasey Micro Kit from sorted T_{FR}, T_{FH}, and T_{REG}; 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 7900HT real-time PCR instrument (Applied Biosystems). Primer sequences for PCR were GAPDH: forward (Fwd) 5’-GCACACACTGCTTGA-3’; reverse (Rev) 5’-TCCTTGGCCACTGGTGTTC-3’; IL-2RA: Fwd 5’-GGCTTCATTTTCCCACGGT-3’; Rev 5’-CTC TGC AGTATC CAG TTG AGA AG-3’; IL-6R: Fwd 5’-TTGCGACCGACGTGTTC-3’; Rev 5’-GCAGCTGGCGGAC-3’; SLAMF6: Fwd 5’-TGG AAC ATC TCT GTC TTG CAT CAT AG-3’; Rev 5’-GTG TAG TAG AGT CCA GCA CTT G-3’; IL-10: Fwd 5’-AGACCTCTCCGCTTGAGG-3’; Rev 5’-TCCACGCCTTGCTTTG-3’; IL-21: Fwd 5’-TGTAAGTGACTTGGACCCTGAAA-3’; Rev 5’-AAACAGGAAATAGCTGACCACTCA-3’.

Relative 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

T_{FR} are distinct from T_{FH} and T_{REG} and can be found within lymph node GCs in RM

Recent studies of GC T_{FH} have defined these cells based on their surface expression of the chemokine receptor CXCR5 and very high levels of the co-inhibitory receptor PD1 (10). However, a fraction of these canonically defined T_{FH} also express the lineage-specific T_{REG} marker FOXP3 and have been therefore defined as T_{FR} as proposed previously (22–24). In this article, we identified CD4^+ T_{FR} by flow cytometry by their coexpression of CXCR5, PD1, FOXP3, and CD25 within lymph nodes of uninfected RM. The gating strategy used to define T_{FR}, T_{FH}, and T_{REG} throughout this study is shown in Fig. 1A. Notably, the gating strategy for T_{REG} includes both CXCR5^+ and CXCR5^− cells. To confirm the presence of T_{FR} within GCs, we conducted an immunohistochemistry analysis. As shown in Fig. 1B, single cells with nuclear expression for FOXP3 and surface expression of PD1 were identified within GCs of uninfected RM.

The expected, non-FOXP3–expressing “true” T_{FH} are also seen within GCs of the same animals.

T_{FR} express markers of both T_{FH} and T_{REG} differentiation

We next performed a comprehensive examination of the T_{FR} phenotype in healthy, SIV-uninfected RM. As shown in Fig. 2, our analysis of relative MFIs for T_{FR} markers confirmed that T_{FR} express FOXP3 and CD25 at comparable levels with T_{REG} (Fig. 2A) and both CXCR5 and PD1 at comparable levels with T_{FH} (Fig. 2B). We next examined in T_{FR} the expression patterns of a series of markers (i.e., CD127, CTLA4, Bel-6, and Helios) that have been linked to either T_{FH} or T_{REG} phenotype and function (1, 25). CD127, the IL-7Rα chain, is expressed at low levels on T_{REG} in humans (26–28). As expected, we found that T_{FR} express CD127 at lower levels than the bulk of CD4^+ T cells, and similar or even lower levels than those observed in T_{REG} and T_{FH} (Fig. 2C). CTLA4 is a key negative T cell regulator that is constitutively expressed on T_{REG} 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 T_{FR} express CTLA4 at a higher frequency and MFI than both T_{REG} and T_{FH} cell populations (Fig. 2D). This is consistent with a putative role of T_{FR} as negative regulators of GC responses. Helios is a transcription factor expressed in thymus-derived natural T_{REG} (29). As shown in Fig. 2F, T_{FR} express Helios at levels that are even higher than those observed in T_{REG} in terms of both frequency of positive cells and MFI, thus suggesting that T_{FR} originate from natural T_{REG} in RM, as well as in mice.

Transcriptome analysis of T_{FR} reveals a distinct but overlapping transcriptional profile compared with T_{FH} and T_{REG}

To further define the functional features of T_{FR} in RM, we next examined the transcriptional profiles of T_{FH}, T_{FR}, and T_{REG} using RNA-Seq by Illumina technology. Splenocytes from five healthy, SIV-uninfected, and unvaccinated RM were sorted into “bulk” CD3^+ T_{FR}, T_{FH}, and T_{REG} based on the following phenotypic markers: T_{FR} (CXCR5^+PD1^hiCD127^hiCD25^hi), T_{FH} (CXCR5^+PD1^-CD127^-CD25^-), and T_{REG} (CXCR5^-PD1^-CD127^-CD25^-). As expected, non-FOXP3–expressing “true” T_{FH} are also seen within GCs of the same animals.

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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 $\times120$. (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 T_{FR} than either T_{REG} or T_{FH}, thus suggesting that the CD25^{+} CXR5^{+}PD1^{hi} phenotype may represent a more transcriptionally active population than classical T_{REG} or T_{FH}. This latter set of RNA-Seq data provides strong evidence that T_{FR} are indeed a distinct cell subset and that the somewhat hybrid transcriptional profile of T_{FR} is not simply due to the sample being a mixture of T_{REG} and T_{FH}. Notably, elevated expression of IL-10 in T_{FR} compared with T_{REG} has been previously reported in murine studies (13).

To then compare the profile of gene expression between T_{FR} with T_{REG} and T_{FH} subsets without using any a priori information, we defined T_{FH} and T_{REG} expression signatures by statistically contrasting RNA-Seq data from T_{FH} and T_{REG} 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 T_{FH} and T_{REG} signature of which 12 genes were T_{REG} related. Many, but not all, canonical T_{REG} and T_{FH} genes were also identified as significantly

**FIGURE 2.** T_{FR} share immunophenotypical features of both T_{FH} and T_{REG} 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 T_{REG}, non-T_{REG}, T_{FR}, and T_{FH} populations from lymph node of healthy, unvaccinated, and uninfected RM. Non-T_{REG} 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 TFR signature genes such as Bcl-6, TIGIT, CD200, LATm, and BATF (Fig. 3C). TFR cells also express mRNA for key TFR-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 \( p = 0.0141 \) and the chronic \( p = 0.0001 \) stages 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 induced, whereas the antiapoptotic regulator XIAP was 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 TFR 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**

TFH 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 TFR and TREG 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 TFH 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

**FIGURE 7.** RNA expression within TFR cells in uninfected and infected RM. (A) Enrichment of gene pathways in TFR derived from the lymph nodes of unvaccinated, SIV-infected RM as compared with TFR from uninfected animals as determined by Ingenuity Pathway Analysis. (B) Log 2 fold change of expression of TFRJFH and TREG-related gene transcripts in TFR sorted from unvaccinated SIV-infected RM and uninfected RM. Significantly upregulated genes are in red, and significantly downregulated genes are in blue.
this cell subset in terms of both their immunophenotypic and transcriptional profile. It is therefore important that, in future studies of TFR, a distinction be made between TFR and true "non-TFR TFH" to fully take into account the complexity of the different CD4^+ T cell subsets that are present in the GC.

The observation that TFR express proteins typically expressed by TFH, such as CD40L, as well as proteins typically expressed by TREG, like IL-10 and CTLA4, is consistent with studies in mice showing that TFR are thymic-derived TREG that migrate into the follicle and, in a manner similar to TFH, upregulate CXCR5, Bcl-6, and PD1 in a B cell-dependent manner (24). The production of IL-21 by TFR is an intriguing new finding and suggests that TFR play a more complex role in RMs than has been described in mice. In addition, principal component analysis suggests that the transcriptional profile of TFR tends to be more similar to TFH than TREG. Further studies are required to fully investigate the functional role played by TFR in RMs and, specifically, in the context of SIV infection. The finding that >90% of TFR express Helios as measured by flow cytometry is also consistent with the natural TREG origin of these cells. Importantly, these immunophenotypic and RNA-Seq data were complemented by histological analyses showing that TFR are found within GCs of both uninfected and infected RM. Although CD4^+FOXP3^+ TREG were found in abundance outside the GC, CD4^+PD1^+ TFH and CD4^+PD1^+FOXP3^+ TFR were both only seen within the GC. The hypothesis that TFR play an immune-regulatory role in vivo is supported by the observation that their frequency is inversely correlated with the levels of both TFH and GC B cells. These data are consistent with mouse studies indicating that: 1) TFR suppress TFH in vitro and prevent the outgrowth of non--Ag-specific B cells (13); and 2) TFR may inhibit Ab production without an effect on T cell activation, thus indicating an ability to directly regulate B cells (32).

In the setting of in vivo SIV infection, we found that TFR exhibited only a small increase in their frequency within the total CD4^+ T cell pool. In conjunction with the large expansion of TFH that is consistently observed post SIV infection, the minor expansion of TFR leads to a significantly decreased TFR/TFH ratio in the lymph nodes of chronically SIV-infected RM. We confirmed this trend in the ratio of TFR/TFH cells after SIV infection by quantifying the number of TFH and TFR cells by immunohistochemistry (Supplemental Table I). Although the present set of results does not allow us to determine whether and to what extent the kinetics of TFR and TFH during SIV infection are mechanistically linked, it is conceivable that the limited TFR expansion facilitates progressive accumulation of TFH in chronically SIV-infected RM, thus indirectly contributing to the aberrant immune activation that characterizes this pathogenic infection. In contrast, it is also possible that the strong proliferation of TFH and associated increase in PD1 expression post SIV infection hampers the development or differentiation of TFR as suggested previously (32).

Comparison of the transcriptional profiles of TFR cells before and post SIV infection showed a significant upregulation of transcripts typically expressed by activated TREG including FOXP3, FABP5, USP2, and USP13 (data not shown) (33), thus suggesting that TFR may be involved in the generalized immune activation associated with pathogenic SIV infection. Interestingly, we also observed a downregulation of several TFH and TREG signature genes as established by our own algorithm. This somewhat unexpected observation indicates that SIV infection has a complex effect on the in vivo phenotype and function of TFR. A better understanding of the overall contribution of TFR to the immunopathogenesis of AIDS, in terms of causing either the virus-associated B cell dysfunction or the changes in the lymph node architecture and function, will require further in vitro and in vivo investigation of the suppressive effect by these TFR on the function of either TFH or GC B cells.

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 coreceptor CCR5 as compared with the other two subsets.

In summary, to our knowledge, the present 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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