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Naive Precursors of Human Regulatory T Cells Require FoxP3 for Suppression and Are Susceptible to HIV Infection

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CD4+CD25+ human regulatory T cells (T_{reg} cells), which express the transcription factor FoxP3, suppress T cell activation. In this study, we sought to define cellular and molecular mechanisms of human T_{reg} cell differentiation. A subset of human naive CD4+ T cells that are CD25+ express high levels of FoxP3. We show that upon activation through the TCR, these FoxP3-expressing naive T cells (termed T_{Nreg} cells) greatly expand in vitro. Expanded T_{Nreg} cells acquire a full T_{reg} phenotype with potent suppressive activity and display low IL-2 production upon TCR stimulation. T_{Nreg} cells in which FoxP3 expression was reduced through RNA interference lost their suppressive activity, but retained their low IL-2 secretion in response to TCR stimulation. Furthermore, in support of the notion that T_{Nreg} cells represent a separate lineage of naive cells, we found that they were more susceptible to HIV infection as compared with naive CD4+ T cells. Based on these findings, we propose that T_{Nreg} cells are precursors for human T_{reg} cells and that these cells require a high level of FoxP3 expression to maintain their suppressive function. Accordingly, modulation of T_{Nreg} cell numbers during infections such as HIV may disrupt human T_{reg} cell development, and contribute to chronic immune activation.

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response can blunt HIV-specific immune responses or reduce the effectiveness of potential vaccines (33, 35, 37). Therefore, deciphering human T<sub>reg</sub> cell biology would benefit our understanding of the perturbation of the immune system during HIV infection and potentially reveal new ways to modulate inflammation and disease.

In this study, we show that human T<sub>reg</sub> cells develop in vitro from a naive precursor subset, and that FoxP3 expression is required for maintenance of their suppressive function. In addition, our findings indicate that the precursor population of T<sub>reg</sub> cells, which we termed naive T<sub>reg</sub> cells (T<sub>reg</sub><sub>naive</sub>), are highly susceptible to HIV infection. These findings have important implications for understanding human T<sub>reg</sub> cell development and for developing therapeutic strategies to treat conditions associated with chronic immune activation such as HIV infection, or to enhance the effectiveness of vaccination.

Materials and Methods

Cell isolation and culture

The blood obtained from healthy donors for this study have been reviewed and approved by Vanderbilt University School of Medicine Institutional Review Board committees. PBMC were isolated from blood of healthy donors through Ficoll-Hypaque (Pharamcia). Resting CD4<sup>+</sup> T cells were purified using CD4<sup>+</sup> Dynabeads (Dynal Biotech) as previously described (38) and were at least 99.5% pure as determined by postpurification FACS analysis. To purify naive, memory, T<sub>reg</sub>, and T<sub>reg</sub><sub>naive</sub> subsets, purified CD4<sup>+</sup> T cells were stained with CD25 and CD45RO Abs and CD5RO CD25<sup>-</sup> (naive CD4<sup>+</sup> T cells (T<sub>reg</sub><sub>naive</sub>)), CD45RO<sup>-</sup>CD25<sup>+</sup> (memory CD4<sup>+</sup> T cells (T<sub>reg</sub>)), CD45RO<sup>-</sup>CD25<sup>high</sup> (T<sub>reg</sub><sub>naive</sub>), and CD45RO<sup>-</sup>CD25<sup>-</sup> (T<sub>reg</sub><sub>naive</sub>) subsets were sorted on a FACS Aria flow cytometer. The culture medium used in all experiments was RPMI 1640 (Invitrogen Life Technologies) and was prepared as described previously (38). All cytokines were purchased from R&D Systems. Monocyte-derived dendritic cells (DCs) were generated as previously described (38). Superantigen, staphylococcal enterotoxin B (SEB; Sigma-Aldrich) was used to stimulate resting T cells in the presence of DCs (39).

FACS analysis

T cells were stained with the relevant Ab on ice for 30 min (chomokine receptor staining performed at room temperature for 20 min to enhance staining) in PBS buffer containing 2% FCS and 0.1% sodium azide. Cells were then washed twice, fixed with 1% paraformaldehyde, and analyzed with a FACS Calibur or FACS Aria flow cytometer. Live cells were gated based on forward and side scatter properties and analysis was performed using FlowJo software (Tree Star). The following anti-human Abs were used for staining: CD4, CD45RA, CD45RO, HLA-DR, CD25, GITR, CTLA-4, CD127, CCR5, CXC4R4 (all from BD Biosciences), and CCR4 (R&D Systems). TCR V region staining performed at room temperature for 20 min to enhance receptor staining performed at room temperature for 20 min to enhance expression. The T cell proliferation and cytokine assay

To assess proliferation and quantify cell divisions, purified CD4<sup>+</sup> T cell subsets were labeled with CFSE (Molecular Probes). Purified cells were first washed and resuspended in (PBS). While vortexing the cells, CFSE was added at a final concentration of 5 μM. The mixture was vortexed for an additional 15 s and incubated at 37°C for 3 min. Labeling was quenched by addition of 50% FCS in PBS. Cells were washed once more with 50% FCS in PBS. Cells were washed twice with 1 ml of PBS buffer containing 2% FCS and 0.1% sodium azide, and washed twice with 1 ml of PBS containing 2% FCS and 0.1% sodium azide, and washed twice with 1 ml of PBS containing 2% FCS and 0.1% sodium azide and washed twice with 1 ml of PBS containing 2% FCS and 0.1% sodium azide. Cells were incubated with 10<sup>6</sup> IFU/ml). RNA was isolated from T cell subsets using an RNeasy kit (Qiagen). To synthesize cDNA, 100 ng of RNA was treated with Superscript II Reverse Transcriptase (Invitrogen GAG Technologies). real-time PCR analyses: β-actin primer mix assay ID Hs99999903_ml; FoxP3 primer mix assay ID Hs00203958_ml. Real-time PCR was performed using the ABI 7700 apparatus (Applied Biosystems). The reaction mixtures (20 ml total volume) contained 2 μl of serially diluted cDNA, 10 μl of TagMan Universal PCR Master Mix (Applied Biosystems), TaqMan Assays-on-Demand Gene Expression Primers (Applied Biosystems) were used in real-time PCR analyses: β-actin primer mix assay ID Hs99999903_ml; FoxP3 primer mix assay ID Hs00203958_ml. Real-time PCR was performed using the ABI 7700 apparatus (Applied Biosystems). The reaction mixtures (20 ml total volume) contained 2 μl of serially diluted cDNA, 10 μl of TagMan Universal PCR Master Mix (Applied Biosystems), and 1 μl of either FoxP3 or β-actin primer mix. The reactions were amplified as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 1 min and 65°C for 1 min. Expression of FoxP3 mRNA was normalized to β-actin expression in each sample.

HIV production and infections

HIV pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) enveloping cDNA, 100 ng of RNA was treated with Superscript II Reverse Transcriptase (Invitrogen GAG Technologies). To test the effectiveness of potential vaccines (33, 35, 37). Therefore, deciphering human T<sub>reg</sub> cell biology would benefit our understanding of the perturbation of the immune system during HIV infection and potentially reveal new ways to modulate inflammation and disease.

In this study, we show that human T<sub>reg</sub> cells develop in vitro from a naive precursor subset, and that FoxP3 expression is required for maintenance of their suppressive function. In addition, our findings indicate that the precursor population of T<sub>reg</sub> cells, which we termed naive T<sub>reg</sub> cells (T<sub>reg</sub><sub>naive</sub>), are highly susceptible to HIV infection. These findings have important implications for understanding human T<sub>reg</sub> cell development and for developing therapeutic strategies to treat conditions associated with chronic immune activation such as HIV infection, or to enhance the effectiveness of vaccination.

Real-time PCR

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Results

Phenotypic characterization of CD25+ human naive T cells

Recently, a subset of human naive T cells that express CD25 were shown to also express high levels of the transcription factor FoxP3 and to display some of the characteristics of Treg cells (28–30). To better establish that these FoxP3-expressing naive CD4+ T cells (termed T Nreg cells) are precursors for Treg cells, we determined their phenotype and function both ex vivo and upon expansion in vitro. We defined this T Nreg subset as CD4+ CD45RA+ CD25+, which comprises ~1–3% of adult CD4+ T cells and is enriched in neonatal cord blood compared with Treg cells (Fig. 1A).

T Nreg cells expressed high levels of FoxP3 mRNA (Fig. 1B) and protein (Fig. 1C), comparable to Treg cells. To compare the phenotype of T Nreg cells to Treg cells, purified CD4+ T cells were stained for various cell surface molecules expressed or down-regulated on Treg cells including GITR (42, 43), CTLA4 (44–46), CCR4 (47), and CCR5 (26). Similar to Treg cells, T Nreg cells expressed high levels of CTLA4 and low levels of CD127 (Fig. 1C). However, expression of other surface markers preferentially expressed on Treg cells, such as HLA-DR, GITR, CCR4, and CCR5, were much lower or negative on the T Nreg subset (Fig. 1C). The expression profile of these markers on T Nreg cells more closely resembled T N cells (Fig. 1C). The surface phenotype of T Nreg cells can therefore be classified as an intermediate between T N cells and Treg cells.

Characterization of functional and proliferative capacity of T Nreg cells

Compared with conventional T cells, Treg cells exhibit a lower proliferative capacity and produce less cytokines in response to TCR stimulation (4, 6, 48) both of which have been attributed to higher expression of FoxP3 in this subset (13, 26). Therefore, we first determined the cytokine secretion profile and proliferative ability of T Nreg cells in response to TCR signaling induced by DCs in the presence of the superantigen SEB (Fig. 2A). Similar to Treg cells, T Nreg secreted much lower levels of IL-2 compared with TN or TM cell subsets (Fig. 2A). Stimulating T Nreg cells through Abs against TCR and CD28 did not increase their IL-2 secretion (data not shown). We also determined whether T Nreg cells secreted different levels of suppressive cytokine IL-10. We found that IL-10 levels produced by T Nreg cells upon activation was not higher than other T cell subsets cells and was variable from donor to donor (data not shown).

To compare the proliferative capacity of the T Nreg cells to other T cell subsets, we labeled cells with CFSE and stimulated with DCs pulsed with SEB. Proliferation of the T cell subsets was determined 4 days postactivation through CFSE dilution. Interestingly, the T Nreg subset exhibited a proliferative response that was slightly less than that of the T N subset, but much greater than that of Treg cells (Fig. 2B).

To determine whether T Nreg cells could be expanded long-term in culture similar to T N cells, we activated these cells with DCs and SEB. The activated T cells were then cultured in IL-2-containing medium for about 2 wk and the cell numbers were assessed by counting the expanded T cells. We found that T Nreg cells had a high capacity to expand in vitro, in contrast to Treg cells (Fig. 2C), despite expression of very high levels of FoxP3 (Fig. 1, B and C). T Nreg cells proliferated and expanded comparably to T N cells, while proliferation of Treg cells was significantly less compared with T N cell proliferation (Fig. 2C).

We next evaluated the ability of T Nreg cells to suppress the activation and proliferation of CD4+ T N cells. For this experiment, highly purified T N cells were labeled with CFSE and cocultured with either unlabeled Treg, T Nreg, or control T N cells. The coculture was stimulated with allogeneic DCs and varying concentrations of SEB for 4 days, because T cells were activated with a strong TCR stimulus (SEB) overall contribution of allogeneic activation in these experiments during the course of 4 days is minimal as determined by proliferation with DCs alone (data not shown). T Nreg cells displayed a suppressive function compared with the T N subset, however, this function was not as potent as that obtained using
assessed by gating on live cells and analysis of forward scatter (cell size) the presence of SEB at a 1:1:30 ratio. Proliferation of target cells was shown in the figure. Statistical significance was determined using the Student's t test comparing TN and TNreg conditions across donors.

Supernatants were collected 18–24 h postactivation and analyzed for cytokines using the CBA assay. Results from three different donors are shown. Statistical significance was determined using the Student test comparing TN and TNreg cells (not significantly different) and TN and Treg cells across donors.

Functional analysis of T reg subsets.

FIGURE 2. Functional analysis of T reg subsets. A, Sorted T reg subsets were activated in the presence of DCs pulsed with SEB (1–10 ng/ml). Supernatants were collected 18–24 h postactivation and analyzed for cytokines using the CBA assay. Results from three different donors are shown. Statistical significance was determined using the Student t test comparing TN and TNreg conditions across donors. B, Resting, sorted T reg subsets were labeled with CFSE and activated with SEB (1–10 ng/ml) pulsed DCs. At day 4 or 5 postactivation, cells were fixed and analyzed for CFSE expression by flow cytometry. Results represent one of five donors analyzed. C, Sorted T reg subsets were activated with DCs + SEB (1–10 ng/ml) and expanded in the presence of IL-2. Cells were counted on day 12 and fold expansion was calculated for each subset. Two representative donors are shown in the figure. Statistical significance was determined using the Student t test comparing T reg and TNreg cells (not significantly different) and TN and T reg cells across donors. D, Resting naive cells were labeled with CFSE and cocultured with unlabeled T Nreg, T reg, or TN cells, and DCs in the presence of SEB at a 1:1:30 ratio. Proliferation of target cells was assessed by gating on live cells and analysis of forward scatter (cell size) and expression by flow cytometry. Results represent one of five donors analyzed.

Phenotypic characterization of expanded T Nreg cells

Because T Nreg cells can be expanded >100-fold in IL-2 after TCR stimulation (Fig. 2C), we sought to address whether their surface phenotype and suppressive capacity is altered by in vitro expansion. To ensure that T Nreg cells maintained expression of FoxP3, and to exclude the possibility that the expanded cell population was contaminated with conventional T cell subsets, we performed surface phenotype analysis in conjunction with intracellular staining of FoxP3. Almost all of the expanded T Nreg cells expressed high levels of FoxP3, whereas expanded TN cells remained low or negative for FoxP3 expression (Fig. 3A).

We next analyzed the T reg cell-associated surface markers on expanded T Nreg cultures 2 wk postactivation. Expanded T Nreg cells expressed higher levels of CD25, GITR, HLA-DR, CCR4, and CTLA-4 as compared with expanded TN cells (Fig. 3B). Similar to TN cells, T Nreg cells also lose the expression of CD45RA and express CD45RO after activation and expansion in vitro (data not shown). This differentiated cell surface phenotype of T Nreg cells is consistent with a T reg cell profile, ex vivo (Fig. 1C). In addition, expanded T Nreg cells expressed high levels of the HIV coreceptors CCR5 and CXCR4 (Fig. 3C), similar to T reg cells (26) suggesting that these cells could be potentially susceptible to HIV infection.

Functional characterization of expanded T Nreg cells

We asked whether activation and expansion of T Nreg cells resulted in full acquisition of T reg cell functions. We first determined the responsiveness of expanded T Nreg cells to TCR restimulation by measuring IL-2 secretion. Expanded T Nreg cells stimulated with DCs and SEB continued to secrete low levels of IL-2 compared with the TN subset (Fig. 4A), indicating that they maintain this T reg cell characteristic even after expansion in vitro.

We then evaluated whether expanded T Nreg cells acquired more potent suppressive function compared with freshly isolated cells. We found that expanded T Nreg cells had displayed more potent immunosuppressive activity (Fig. 4B) than freshly purified T Nreg counterparts (Fig. 2D). At suboptimal TCR stimulation (0.01–0.001 ng/ml SEB) the suppression by expanded T Nreg cells was similar (~80–90% inhibition of target cell proliferation) to T reg cells (Fig. 4B). Taken together, these findings suggest that activation and expansion of T Nreg cells induces a program leading to their differentiation into fully competent T reg cells.

TCR Vβ repertoire of T Nreg and T reg cells

Our data support the hypothesis that, upon their activation and expansion, T Nreg cells may contribute to the pool of T reg cells upon TCR Vβ repertoire of T Nreg and T reg cells

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T reg cells (Fig. 2D). Efficiency of suppression by resting T Nreg cells ranged from ~50% to ~10<sub>-</sub>1% suppression of target cells (data not shown), though T reg cells consistently suppressed 70–90% target cells (data not shown). To confirm that cell death in the T Nreg and T reg conditions was not playing a role in the lack of target cell proliferation, we assessed cell death by percent of live cells based on forward vs side scatter plots. Approximately 85–90% of cells were within the live cell gate in all conditions (data not shown). Thus, we conclude that lack of proliferation is not due to excessive cell death. Together, these results show that T Nreg cells possess an intermediate functional profile as compared with T reg cells and, importantly, that they have the capacity to expand upon Ag stimulation.
activation and expansion. As a corollary to this, we hypothesized that the TCR repertoire of T Nreg cells would be similar to that of Treg cells. To investigate the breadth of the TCR Vβ repertoire of the naive and memory T cell subsets, we stained purified CD4+ T cells with CD25 and CD45RO as subset markers, and costained with Abs directed against Vβ. 80% of the mature human TCR Vβ repertoire of T cells (49). This analysis indicated that TCR Vβ repertoire of T Nreg cells was very similar to that of other T cell subsets (Fig. 5). Furthermore, we noted that the relative expression of different Vβ families was very similar between T reg and TNreg subsets (Fig. 5). This finding supports a model whereby T Nreg differentiate into T reg cells upon activation with self or nonself Ags in vivo, acquiring a suppressive capability while maintaining the capacity to recognize similar Ags as conventional T cell subsets.

Is FoxP3 required for maintaining T Nreg cell phenotype and function?

To determine whether sustained expression of FoxP3 is required to maintain the capability of T Nreg cells to differentiate into fully suppressive Treg cells, we reduced expression of FoxP3 in these subsets by RNA interference (RNAi). Accordingly, shRNAs targeting the FoxP3 gene were stably expressed in activated T N or TNreg cells using a lentiviral delivery system in which the coexpression of GFP allows for the identification of transduced cells (50).

Using this system, we specifically and stably reduced expression of FoxP3, as shown by intracellular staining with a FoxP3 Ab (Fig. 5).
We next assessed the surface phenotype of the expanded TNreg cells in which FoxP3 expression has been reduced by RNAi. Knockdown of FoxP3 was associated with lower expression of CD25, CD62L, CTLA4, CCR4, and HLA-DR as compared with TNreg cells transduced with control shRNA vector (Fig. 6A).

Similar results were obtained by stably expressing a second distinct shRNA against FoxP3 in TNreg cells (Fig. 6A). We next evaluated the effects of FoxP3 knockdown in activated TNreg cells on their cytokine production and suppressive function. For this experiment, we first sorted the shRNA transduced expanded TNreg cells, restimulated them through the TCR, and measured secreted IL-2. TNreg cells with reduced FoxP3 were unchanged in their inability to produce IL-2 compared with control TNreg cells expressing high levels of FoxP3 (Fig. 6B). However, reduction of FoxP3 levels in TNreg cells greatly diminished their ability to suppress the proliferation of conventional TN cells (Fig. 6C). These results indicate that a high-level expression of FoxP3 is indispensable for suppressive activity of Treg cells.

Are TNreg cells susceptible to HIV infection?

TNreg cells were shown to potentially play an important role in modulating the immune response to HIV and pathogenesis associated with HIV infection (26, 33). We have shown that TNreg cells are capable of significant proliferation upon TCR stimulation and express high levels of HIV coreceptors CCR5 and CXCR4. We therefore studied the susceptibility of TNreg cells to HIV infection in vitro. T cell subsets were purified and activated with DCs and SEB and at the time of activation, these cells were also infected with either replication-defective viruses pseudotyped with VSV-G.HIV or replication-competent HIV viruses, that use CCR5 (R5.HIV) for entry, both of which also express GFP as a marker of infection (38). TNreg cells were highly infected by VSV-G.HIV and were also more susceptible to infection with R5.HIV compared with Treg cells (Fig. 7A). To determine the level of HIV replication in the TNreg cells as compared with activated Treg cells, subsets were infected with R5.HIV for 3 days and washed to remove input virus. Supernatants from infected cultures were then monitored for HIV replication by measuring the GFP expression from infected T cells and the level of HIV p24 Ag levels using an ELISA, as described (26). R5.HIV spread within TNreg cell cultures over a 2-wk period and replicated at levels comparable to memory T cells, and importantly higher than TN cells during the early stages of the infection (Fig. 7, B and C).

FIGURE 5. Vβ repertoire of TNreg subsets from peripheral blood. CD4+ T cells were stained with mAbs to CD25 and CD45RO, and gates were set for TN, TNreg, and TNreg cells. Cells were costained with the Abs against the Vβ family indicated in the figure. Percentage shown is percent of Vβ+ cells in the given population. This is a representative profile from three separate donors.

FIGURE 6. shRNA mediated knockdown of FoxP3 in TNreg cells. Sorted TN or TNreg cells were activated with DC and SEB (10 ng/ml), and concurrently infected with shRNA expressing virus at an MOI of 3–5. Cells were expanded in IL-2-containing medium for 10–14 days. A, TNreg cells were stained for FoxP3, or cell surface marker shown. Uninfected, a control non-mammalian shRNA (control sh), and two FoxP3-specific shRNAs (FoxP3 sh1 and FoxP3 sh2) transduced cells are shown. Transduced cells were identified by expression of GFP. Cells were gated on GFP-positive cells and further analyzed for the indicated surface molecule. B, TN or TNreg cells transduced with shRNA were sorted based on GFP expression, and restimulated with DC plus SEB (0.01 ng/ml), supernatants were collected 18–24 h postactivation. IL-2 production was measured using a CBA assay. C, TN or TNreg cells transduced with shRNA were sorted based on GFP expression, and a suppression assay was performed as described in Fig. 4. Target naive T cells were labeled with a red dye (SNARF). Live cells were gated and percentage of target cells having undergone at least one cell division are shown. This experiment is representative of three experiments for all panels.
Although neither resting T₇ nor T₇₆reg cells expressed detectable levels of CCR5 by Ab staining (Fig. 2C) or mRNA by quantitative real-time PCR (data not shown), we hypothesized that T₇₆reg cells more rapidly up-regulate CCR5 upon activation compared with T₇ cells. To test this hypothesis, we sorted T₇ and T₇₆reg cell populations and stained each subset for the expression of CCR5 on days 5 and 7 postactivation. We found that T₇₆reg cells contained ~8- to 10-fold higher CCR5+ T cells at these earlier time points (Fig. 7D). This result was corroborated by ~5-fold higher CCR5 mRNA expression, by real-time PCR analysis upon activation, on T₇₆reg cells compared with T₇ cells (data not shown). CCR5 expression was detectable on activated T₇ cells by surface staining 8–10 days postactivation (Fig. 3D), thus lagging several days compared with activated T₇₆reg cells.

Discussion

In this study, we demonstrate that a population of naive T cells coexpressing CD25 and FoxP3, which we called T₇₆reg cells, has the capacity to differentiate into fully functional T₇ cells in vitro. In contrast to T₇ cells, T₇₆reg cells exhibit a high proliferative capacity upon TCR stimulation, despite expressing high levels of FoxP3. In our culture system, the proliferation of T₇₆reg cells was higher compared with a prior report (28), which could be due to our activation conditions using DCs pulsed with SEB that we use to provide a more physiological cell-cell interaction. Several groups have reported expanding CD25+ T₇ cells using a multitude of stimulation conditions, including CD28-coated beads (51–53). Upon expansion, these cells retain their high expression of FoxP3. However, in these studies, the CD25+ T cells were not subdivided into naive and memory subsets. Because we have not been able to expand mature T₇ cells in vitro to any significant numbers, our present data raise the possibility that in vitro-expanded T₇ cells obtained by others were derived from the T₇₆reg cell population. This would be consistent with a recent report showing that in vitro expansion of only the CD45RA⁺ population of CD25+ T cells gives rise to a pure T₇ cell line (54).

Our results indicate that the T₇₆reg cell population does not adopt a full T₇ phenotype or exert its function until it has undergone activation and expansion and reactivation through the TCR. This observation agrees with FoxP3 overexpression studies performed by our group (26) and others (13, 55, 56) to gain insights into its possible functions in naive T cells. Mere overexpression of FoxP3 in naive CD4⁺ T cells does not render them fully suppressive and hyporesponsive until FoxP3 overexpressing cells have been re-stimulated through their TCR (26). This leads to the intriguing hypothesis that the high expression of FoxP3 is not sufficient for T₇ suppressive activity in the absence of other cell differentiation programs, some of which are possibly activated by FoxP3 after TCR stimulation.

In our experiments, we found that T₇₆reg cells were consistently less suppressive relative to T₇ cells with memory phenotype, but acquired equally potent suppressive activity after they are expanded in vitro. Prior publications (28, 29, 57), found that cells similar to T₇₆reg cells were more suppressive, quantitatively compared with our findings. It is conceivable that these quantitative differences are due to the type of stimulus used in these previous studies. Indeed, we have tried anti-CD3/28 stimulations, similar to published reports, but the suppressions from these assays were not

**FIGURE 7.** T₇₆reg cells are susceptible to HIV infection and can serve as sites of replication. Sorted T cell populations were activated using DCs and 10 ng/ml SEB, and concurrently infected with R5.HIV viruses expressing GFP at a range of 1–5 MOI. GFP expression for VSV-G.HIV or R5.HIV day 6 postinfection.

**Panel A** shows the percentage of infected cells based on GFP expression in T₇, T₇₆, and T₇₆reg samples within the same experiment. Statistical significance was determined using the Student t test comparing T₇ and T₇₆reg conditions. *p < 0.05.

**Panel B** shows the percentage of infected cells based on GFP expression in T₇, T₇₆, and T₇₆reg day 6 postinfection.

**Panel C** shows the level of HIV p24 virus production by ELISA. Data shown are infection with 1 MOI. Similar results were obtained from three different donors. Error bars represent SD of duplicate samples within the same experiment. Statistical significance was determined using the Student t test comparing T₇ and T₇₆reg conditions. *p < 0.05.

**Panel D** shows the SSC-A vs. CCR5 analysis of T₇ and T₇₆reg cells activated for 5 days under same conditions as above were stained with a CCR5 Ab. Data are represented by side scatter profile and CCR5 staining and percent positive cells are shown on top of boxes.
as consistent. This is not surprising because T<sub>reg</sub> cells require cell-to-cell contact and triggering only the TCR may have the caveat of not forming an immunological synapse with an APC and recruiting T<sub>reg</sub> cells to the site of activation. We believe our method to stimulate T<sub>reg</sub> cells and T cells through DCs and SEB is more physiological way to activate them which allows three cell (DC plus T cell plus T<sub>Nreg</sub> or T<sub>reg</sub>) clusters to form, and could be a better reflection of the T<sub>reg</sub>-mediated suppression in vivo.

In this study, we demonstrated for the first time that reduction of FoxP3 expression in human T<sub>reg</sub> cell precursors results in an altered phenotype and is required to maintain their suppressive function. Similar findings were reported for murine T<sub>reg</sub> cells, which showed that induced ablation of FoxP3 disrupted T<sub>reg</sub> suppressive functions in vivo (22). In addition, it was demonstrated that attenuated FoxP3 expression subverted T<sub>reg</sub> cell functions in vivo (23). The suppressive functions of FoxP3-attenuated mice were abrogated, but the cells retained their hypoproliferative nature (23). We show that cells with reduced expression of FoxP3 are still hyporesponsive to TCR stimulation as measured by the production of IL-2. Williams et al. (22) showed a rescue of IL-2 production upon controlled deletion of FoxP3 in vivo. In our shRNA system, FoxP3 expression in T<sub>Nreg</sub> cells is greatly reduced but not completely ablated, leaving open the possibility that residual FoxP3 levels may be sufficient to render T cells hyporesponsive to TCR signals, but insufficient to maintain their suppressive function. It is also possible that IL-2 secretion in T<sub>reg</sub> cells is independent of FoxP3 expression, but rather due to epigenetic modifications that regulate gene expression in T cells, such as in the histone tails.

The nature of the Ag recognized by T<sub>reg</sub> cells remains controversial. Studies conducted using transgenic mouse models suggest that once activated through the TCR, suppressor effector function was completely Ag independent (58, 59). These studies, however, did not exclude the possibility that T<sub>reg</sub> cells exert their suppressive function in an Ag-specific manner. We show here that both T<sub>Nreg</sub> cells and T<sub>reg</sub> cells express a full repertoire of TCR V<sub>β</sub> receptors. It was shown that Ag-mediated expansions within specific TCR V<sub>β</sub> families were associated with equal increases of the same TCR V<sub>β</sub> in the T<sub>reg</sub> cell population (60). Therefore, it is possible that T<sub>reg</sub> cells are replenished from a precursor population in the periphery. The precursor population is likely to be the T<sub>Nreg</sub> subset as suggested by our study and others (28, 29, 54, 61). Accumulating evidence in animal models indicates that T<sub>reg</sub> cells are selected by self Ag, (25, 62, 63), although it is not clear whether a naive precursor subset for T<sub>reg</sub> cells described in the present study also exists in mice. It appears that, unlike the mouse system, human FoxP3 expression can be induced in human CD4<sup>+</sup>CD25<sup>−</sup> T cells following TCR stimulation (64, 65). We propose that, at least in the human system, acquisition of suppressive function by the T<sub>Nreg</sub> subset is similar to that of the acquisition of effector functions of conventional T<sub>N</sub> cells. This suggests that during an immune response, T<sub>N</sub> cells undergo activation and differentiation to gain effector functions, and T<sub>Nreg</sub> cells undergo a parallel differentiation process leading to clonal expansion. This scenario would predict that for exogenous Ags, T<sub>reg</sub> suppression would be weaker for primary immune responses and stronger after secondary or subsequent exposures, thereby functioning to control chronic immune activation.

The role of the T<sub>reg</sub> compartment of T cells during HIV infection and pathogenesis remains a very important question and is not yet resolved. We clearly demonstrate an enhanced ability of HIV to replicate and infect T<sub>Nreg</sub> cells. Our findings also show a 10-fold higher HIV infection rate of T<sub>Nreg</sub> cells compared with T<sub>N</sub> cells with R5-tropic viruses, which use CCR5 coreceptor for entry, during the early stages of infection (Fig. 7). We found that T<sub>Nreg</sub> cells up-regulated CCR5 upon activation several days before up-regulation of CCR5 in T<sub>N</sub> cells (Fig. 7D). We believe this finding could partly explain the difference in infection in the early days after activation. This finding could be important during HIV infection, because during an immune response the preferential infection of T<sub>Nreg</sub> cells early after activation could contribute to lesser control of immune activation, and thus more cellular targets for HIV. This data also suggests that CCR5 expression is differentially regulated in T<sub>N</sub> and T<sub>Nreg</sub> cells during the early stages of T cell activation. This is also consistent with the our results that HIV spreads to a larger portion of activated T<sub>N</sub> cells after day 8 (Fig. 7B), at which time they begin to up-regulate CCR5 expression. It has been suggested that the expression of FoxP3 enhances HIV long-terminal repeat activity (21), it is therefore possible that T<sub>Nreg</sub> cells that express high levels of FoxP3, also provide a more optimal intracellular environment for HIV gene expression, similar to cells that overexpress FoxP3 during early stages of infection (21). A contradictory report demonstrated that FoxP3 represses retroviral transcription (66). Differences in these two studies may be due to the model systems to used to test HIV long-terminal repeat activity, such as transient transfection assay (66) vs a luciferase reporter HIV infection assay (21). Thus, the effect of FoxP3 on HIV infection remains to be further clarified in future studies.

The effect of T<sub>reg</sub> disruption during HIV infection has been associated with both beneficial (26, 31, 36) and detrimental outcomes (26, 32–35). It has been suggested that HIV-immune dysfunction is associated with T<sub>reg</sub> cells that may target HIV-specific effectors, leading to an inefficient immune response against HIV (32–35, 37). We and others have proposed that T<sub>reg</sub> cells may serve a protective function by limiting the overall immune activation that is a hallmark of HIV infection (26, 31, 36). In this context, the depletion of T<sub>reg</sub> cells could potentially hasten the progression to acquired immunodeficiency syndrome. At the same time, the specific depletion of T<sub>Nreg</sub> cells may prevent insufficient regeneration of the T<sub>reg</sub> cell pools during primary immune responses, and could be one of the reasons for immune hyperactivation associated with HIV infection. This is supported by data showing HIV- positive individuals with higher relative levels of FoxP3 have lower immune activation as measured by expression of HLA-DR (26, 31). We demonstrate that when compared with T<sub>N</sub> cells, T<sub>Nreg</sub> cells are highly susceptible to infection by R5.HIV during early stages post-TCR activation. This result suggests that T<sub>Nreg</sub> cells could be preferentially targeted during HIV infection. How T<sub>Nreg</sub> cells are modulated in vivo during different stages of HIV infection remains to be determined. Furthermore, it will be important to determine whether a similar subset of T<sub>Nreg</sub> cells can be identified in monkey models of SIV infection and whether this can be correlated with the presence or absence of immune activation in these models, which highly correlate with disease progression (67). Interestingly, it has been reported that administration of IL-2 to HIV-positive individuals leads to the expansion of T<sub>Nreg</sub> subset (30). Thus, enhancing this subset by IL-2 treatment could be additionally beneficial in regulating T cell homeostasis during HIV infection by dampening chronic immune activation.

In summary, our results indicate that the T<sub>Nreg</sub> cell population acquires a full T<sub>reg</sub> phenotype upon activation, and that this differentiation process is dependent upon sustained, high-level FoxP3 expression. This subset also appears to be more susceptible to HIV infection, when compared with T<sub>N</sub> cells. The T<sub>Nreg</sub> cell population could provide an exciting target cell for potential therapeutics. The ability to manipulate this population of cells could allow for the generation of homogenous, Ag-specific T<sub>reg</sub> cells that could be used for the prevention and treatment of T cell-mediated diseases,
including graft-vs-host disease or reducing chronic immune activation during HIV infection. It has been shown that the memory subset of Treg cells is highly susceptible to apoptosis and has limited replicative capacity in vivo (60, 68), suggesting that the modulation of Treg cells for therapeutic purposes may not be long-lasting. The therapeutic manipulation of Treg cells may provide a more tractable way to obtain specific and long-term protection against aberrant T cell activation, and permit effective immune modulation for vaccine responses.

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References


