Generation of Potent and Stable Human CD4+ T Regulatory Cells by Activation-independent Expression of FOXP3

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Therapies based on enhancing the numbers and/or function of T regulatory cells (Tregs) represent one of the most promising approaches to restoring tolerance in many immune-mediated diseases. Several groups have investigated whether human Tregs suitable for cellular therapy can be obtained by *in vitro* expansion, *in vitro* conversion of conventional T cells into Tregs, or gene transfer of the FOXP3 transcription factor. To date, however, none of these approaches has resulted in a homogeneous and stable population of cells that is as potently suppressive as *ex vivo* Tregs. We developed a lentivirus-based strategy to ectopically express high levels of FOXP3 that do not fluctuate with the state of T-cell activation. This method consistently results in the development of suppressive cells that are as potent as Tregs and can be propagated as a homogeneous population. Moreover, using this system, both naïve and memory CD4+ T cells can be efficiently converted into Tregs. To date, this is the most efficient and reliable protocol for generating large numbers of suppressive CD4+ Tregs, which can be used for further biological study and developed for antigen-specific cellular therapy applications.

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INTRODUCTION

$CD4^+CD25^+$ T regulatory cells (Tregs) have a central role in establishing and maintaining immune tolerance. Because defects in these cells are known to underlie autoimmunity, allergy, and graft rejection,[1–3] it is thought that enhancing the number and/or function of Tregs might be an ideal route to the therapeutic restoration of tolerance to defined antigens *in vivo*.[4,5] The development of methods that rely on the use of Tregs as cellular therapy has been limited, at the technical level, by difficulties in obtaining large numbers of homogeneous populations *in vitro* and, at the theoretical level, by an incomplete understanding of the development and functioning of these cells.

Several strategies have been explored for obtaining human Tregs suitable for cell therapy. Besides our group, others too have shown that polyclonal or alloantigen-specific Tregs sorted on the basis of high CD25 expression can be expanded *in vitro* in the presence of exogenous interleukin-2 (IL-2) and strong T cell receptor (TCR)–mediated activation.[6–9] Although these methods result in a population of cells that are potently suppressive in the short term, non-suppressive T effector (Teff) cells outgrow suppressive cells in the long term.[10,11] Several groups of researchers have also attempted to improve sorting strategies on the basis of differential expression of other markers such as CD127, CD45RA, and HLA-DR.[10–13] Although these methods may result in more homogeneous populations, they require large numbers of cells for initial sorting and significantly reduce the number of Tregs that can be isolated, requiring subsequent expansion by several-thousand fold in order to generate numbers sufficient for therapy.

Another way to generate large numbers of regulatory cells involves conversion of conventional Teff cells into "inducible" Tregs. In humans, TCR-mediated stimulation *in vitro* can lead to the development of a subpopulation of suppressive inducible Tregs,[14] a process that is greatly enhanced by the addition of exogenous tumor growth factor-β (TGF-β)[15–17] or rapamycin.[18] Although inducible Tregs resemble *ex vivo* Tregs phenotypically and functionally, the stability of their suppressive capacity is not well-established, and the possibility that they may re-convert to Teff cells remains. Methods based on the over-expression of the FOXP3 transcription factor have also been developed. In mice, the expression of FoxP3 is necessary and sufficient for the development of Tregs, and retrovirus-mediated overexpression of FoxP3 generates regulatory cells that ameliorate autoimmune disease.[19] In human cells, however, the expression patterns and function of FOXP3 are more complex,[19] and retrovirus-mediated overexpression of FOXP3 does not consistently result in the generation of potent suppressive T cells *in vitro*.[20–22]

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Given these limitations, we sought to understand why retroviral overexpression of FOXP3 in human T cells does not lead to robust development of Tregs, and attempted to develop a more efficient protocol for generating a homogeneous population of Tregs *in vitro*. Here we report that lentivirus-mediated expression of FOXP3 under the control of the human elongation factor (EF)1α promoter can efficiently convert Teff cells to functional Tregs. A comparison with previously described retrovirus-based expression systems revealed that Treg conversion requires that expression levels of FOXP3 do not fluctuate with the state of T-cell activation. Furthermore, using this method, both naïve and memory CD4$^+$ T cells acquire suppressor activity when transducted with FOXP3. These findings establish, for the first time, a straightforward and reliable method for generating large numbers of suppressive CD4$^+$ Treg cells.

RESULTS

Retroviral LTR–driven, but not EF1α-driven, FOXP3 expression fluctuates depending on T-cell activation

We previously demonstrated that, although expression of FOXP3 under the control of the moloney murine leukemia retroviral long terminal repeat (LTR) caused cell-autonomous blocking of proliferation and cytokine production, it did not consistently result in the differentiation of potent suppressive T cells.[20] We hypothesized that this could be because the LTR-driven expression of FOXP3 is influenced by T-cell activation,[23–26] and may not mimic the pattern of endogenous FOXP3 expression in human Tregs. Moreover, recent data demonstrate that, in humans, FOXP3 can also be expressed transiently in non-suppressive Teff cells.[27–31] This suggests that the conditions in which FOXP3 expression would bring about full Treg conversion might be more stringent in humans than in mice. In order to test this possibility, we developed a new bi-directional lentiviral vector[32] in which expression of FOXP3 was placed under the control of the EF1α promoter, and ΔNGFR was included as a cell-surface marker for tracking and sorting of transduced cells (**Figure 1a**). ΔNGFR was chosen as a marker gene because it is known to be safe and non-immunogenic in humans.[33] The pCCL.FP3 vector drives high, co-ordinate FOXP3 and ΔNGFR expression in stably transduced primary human CD4$^+$ T cells (**Supplementary Figure S1a**).

In order to compare our previous retroviral FOXP3 vector (pLX.FP3) with the new lentiviral FOXP3 vector (pCCL.FP3), we examined the kinetics of FOXP3 expression in Jurkat cells, which do not express endogenous FOXP3. Jurkat cells were transduced with the empty pLX control retroviral vector (data not shown), pLX.FP3, the empty pCCL control lentiviral vector, or pCCL.FP3, and purified on the basis of ΔNGFR expression after 5 days. The intensity of FOXP3 expression was determined by fluorescence-activated cell sorting analysis (**Figure 1b**). The resultant data demonstrated that the pCCL.FP3 lentiviral vector drove higher levels of FOXP3 protein than the pLX.FP3 retroviral vector did. The monoclonal antibody used for detecting FOXP3 resulted in some background staining of control pCCL-transduced Jurkat cells, despite the fact that these cells do not express FOXP3 messenger RNA (mRNA) (**Figure 1c**).

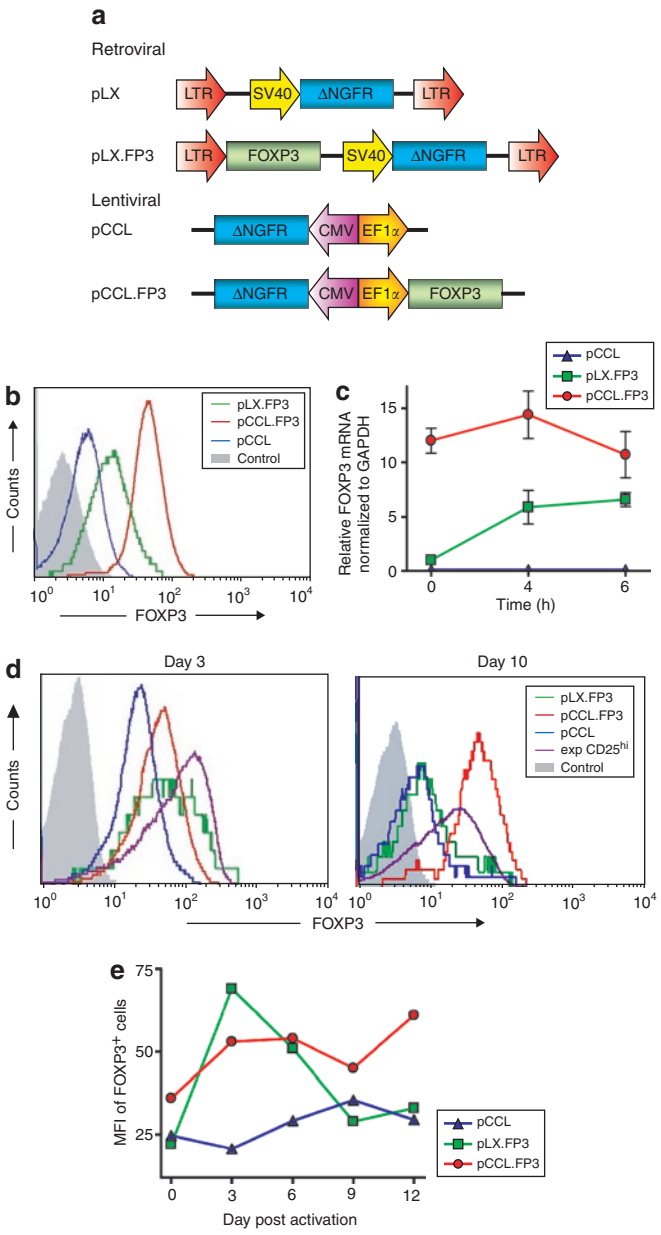

Figure 1 Comparison of retrovirus- and lentivirus-based vectors for expression of FOXP3 in human T cells. (**a**) Illustration of control or FOXP3-expressing retro- and lentiviral vector constructs. (**b**) Fluorescence-activated cell sorting analysis of FOXP3 expression (with clone 236A/E7) in Jurkat cells that expressed ΔNGFR after transduction with retro- or lentiviruses. (**c**) Transduced Jurkat cells were stimulated with phorbol 12-myristate 13-acetate (1 ng/ml) and Ca^{2+} ionophore (50 ng/ml) for the indicated times, and the amounts of FOXP3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) were determined by quantitative polymerase chain reaction. Normalized levels of FOXP3 mRNA are plotted relative to pLX.FP3-transduced Jurkat cells at time point = 0. The data represent the average of three independent determinations, and error bars indicate the SD. (**d**) CD4$^+$CD25$^-$ T cells were transduced with pLX.FP3, pCCL.FP3, or pCCL viruses and purified for ΔNGFR expression. The expression of FOXP3 (with clone PCH101) was determined 3 and 10 days after re-stimulation. CD4$^+$CD25^{hi} T-regulatory cells were cultured and analyzed in parallel. (**e**) The intensity of FOXP3 expression in retro- versus lentivirally transduced CD4$^+$CD25$^-$ T cells during a 12-day expansion period is plotted as the MFI of FOXP3$^+$ cells at the indicated time points. **d** and **e** are representative of data from four different donors. Control indicates unstained or fluorescence-minus-one-stained cells.

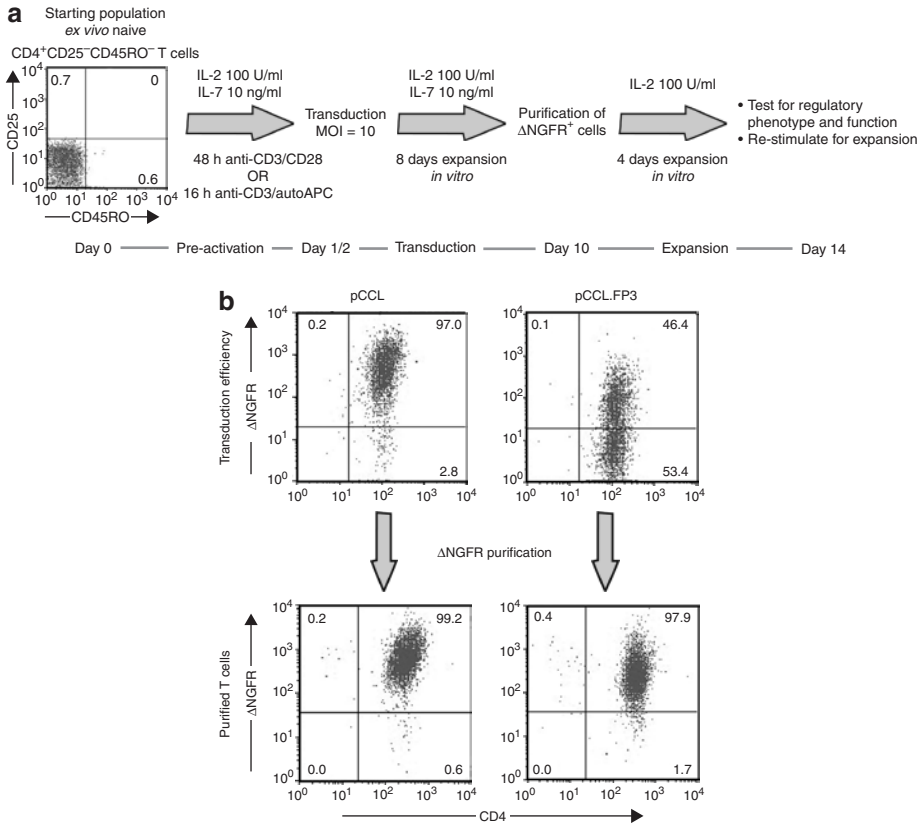

Figure 2 Optimized method for lentiviral transduction of human CD4$^+$CD25$^-$CD45RO$^-$ T cells. (**a**) Schematic diagram of transduction protocol: T cells were pre-activated with either anti-CD3/28 (for 48 hours) or anti-CD3/APCs (for 16 hours) in the presence rhIL-2 and rhIL-7, and transduced with concentrated lentiviral supernatants. ΔNGFR$^+$ cells were purified 8 days later and assayed for phenotype and function in the resting phase (approximately day 14), and re-stimulated for expansion. (**b**) Transduction efficiencies of pCCL and pCCL.FP3 vectors assessed 6 days following infection of CD4$^+$CD25$^-$CD45RO$^-$T cells pre-activated with anti-CD3/APCs, and ΔNGFR expression following purification. Representative data from a minimum of 4 different donors. IL, interleukin; MOI, multiplicity of infection.

In order to assess whether vector-driven gene expression was influenced by the state of T-cell activation, control and FOXP3-transduced Jurkat cells were stimulated with phorbol 12-myristate 13-acetate and Ca^{2+} ionophore, and the levels of FOXP3 mRNA were determined by quantitative polymerase chain reaction. In contrast to lentiviral EF1α-driven FOXP3 expression, retroviral-LTR-driven expression was influenced by the activation state of the cells. After 4 hours, stimulated pLX.FP3-transduced cells upregulated FOXP3 mRNA by an average of 6.4 ± 2.0-fold, in contrast to only 1.5 ± 0.4-fold in p.CCL.FP3-transduced cells (P = 0.007, n = 4). The difference between the retroviral and lentiviral vector expressions is probably due to the fact that the promoter activity of retroviral LTRs is significantly increased by T-cell activation.[24,26] Control-transduced Jurkat cells were consistently negative for FOXP3 mRNA (**Figure 1c**).

We found a similar difference in expression kinetics between the two vector systems in human CD4$^+$ T cells. At the peak of activation, pLX.FP3- and pCCL.FP3-transduced T cells expressed equal levels of FOXP3 protein, which were comparable to those in CD4$^+$CD25^{hi} Tregs. At this time point, control pCCL-transduced T cells were also FOXP3$^+$, on account of activation-induced expression of this protein.[31] As T-cell activation waned, however, LTR-driven FOXP3 expression declined, whereas EF1α-driven expression remained consistent (**Figure 1d** and **e**). In order to assess the performance of the lentiviral vector independent of endogenous activation-induced FOXP3, we transduced T cells with a lentivirus that encoded hemagglutinin-tagged FOXP3 (**Supplementary Figure S1b**), and confirmed that high expression of ectopic FOXP3 does not depend on the endogenous protein.

Optimized transduction and culture conditions for expression of FOXP3 in CD4$^+$CD25$^-$CD45RO$^-$ T cells

Viral supernatants from control pCCL and pCCL.FP3 vectors were used for transducing CD4$^+$CD45RO$^-$CD25$^-$ T cells, pre-activated with either anti-CD3/28 monoclonal antibodies for 48 hours (n = 5 donors) or with autologous irradiated antigen-presenting cells (APCs) and soluble anti-CD3 for 16 hours (n = 4 donors) (**Figure 2a**). Optimal transduction efficiency was achieved when pre-activated with anti-CD3 monoclonal antibodies and APCs: 76–97% (mean 86 ± 12%, n = 4) of cells were transduced with the control pCCL virus and 28–71% (mean 47 ± 18%, n = 4) of cells with the pCCL.FP3 virus (**Figure 2b**). As in other systems,[20,32] the additional sequence (the FOXP3 complementary DNA) in the pCCL.FP3 vector resulted in a lower transduction efficiency because of less efficient packaging of the larger viral genome.

Eight days after transduction, ΔNGFR$^+$ T cells were purified using anti-ΔNGFR magnetic beads, and expanded.[9] The yield of ΔNGFR$^+$ cells following magnetic sorting ranged from 20 to 44%

(mean 29.5 ± 9.5%), and the resulting cells were always >95% pure. It has been reported that transduction of naïve T cells is optimal following exposure to IL-7,[34] and accordingly we found that addition of IL-2 and IL-7 during T-cell pre-activation led to a greater recovery of transduced cells, as a result of increased cell viability, proliferation, and more distinct populations of ΔNGFR$^+$ T cells (data not shown). Although the addition of IL-7 improved the overall transduction efficiency and cell growth, it was detrimental to the preservation of the Treg phenotype in the long term. Culture of *ex vivo* CD4$^+$CD25^{hi} Tregs in IL-7 completely abrogated their phenotype and functioning after 4 weeks (**Supplementary Figure S2**). This possibly reflects the preferential outgrowth of non-suppressive CD127$^+$ Teff cells in the presence of IL-7.[11,12] Therefore, after purification of ΔNGFR$^+$ cells, the transduced cells were expanded in the presence of IL-2 alone so as to minimize the potential for outgrowth of ΔNGFR$^-$ cells in pCCL.FP3-transduced cultures.

EF1α-driven expression of FOXP3 results in upregulation of Treg markers and anergy

Previous studies have reported variable changes in expression of Treg-associated molecules such as CD25, GITR and CTLA4 after FOXP3 transduction.[20,22,35] pCCL.FP3-transduced T cells expressed significantly higher amounts of FOXP3, CD25, CTLA4, and GITR, and lower amounts of CD127 than the control cells did (**Figure 3a**). With the exception of CTLA-4, expression levels of these molecules did not significantly differ between pCCL.FP3-transduced cells and CD4$^+$CD25^{hi} Tregs expanded in parallel (**Supplementary Figure S3**). Although CTLA-4 expression was consistently higher in pCCL.FP3-transduced cells than in control cells (1.8 ± 0.5-fold higher mean fluorescence intensity, $p = 0.0005$, $n = 16$), it was never expressed as highly as in expanded CD4$^+$CD25^{hi} Tregs (5.6 ± 2.4-fold higher mean fluorescence intensity as compared to controls). In addition to altering the cell-surface phenotype of CD4$^+$ T cells, lentivirus-mediated transfer of FOXP3 induced a hyporesponsive phenotype. Upon stimulation with immobilized anti-CD3 (**Figure 3b**), pCCL.FP3-transduced T cells proliferated significantly less than control T cells did [7.6 ± 3.6-fold-less ($P = 0.0001$, $n = 8$) at 1 μg/ml anti-CD3]. Hyporesponsiveness was reversed with addition of exogenous IL-2 (**Figure 3c**), and at no point did the FOXP3-transduced cells become independent of growth factor.

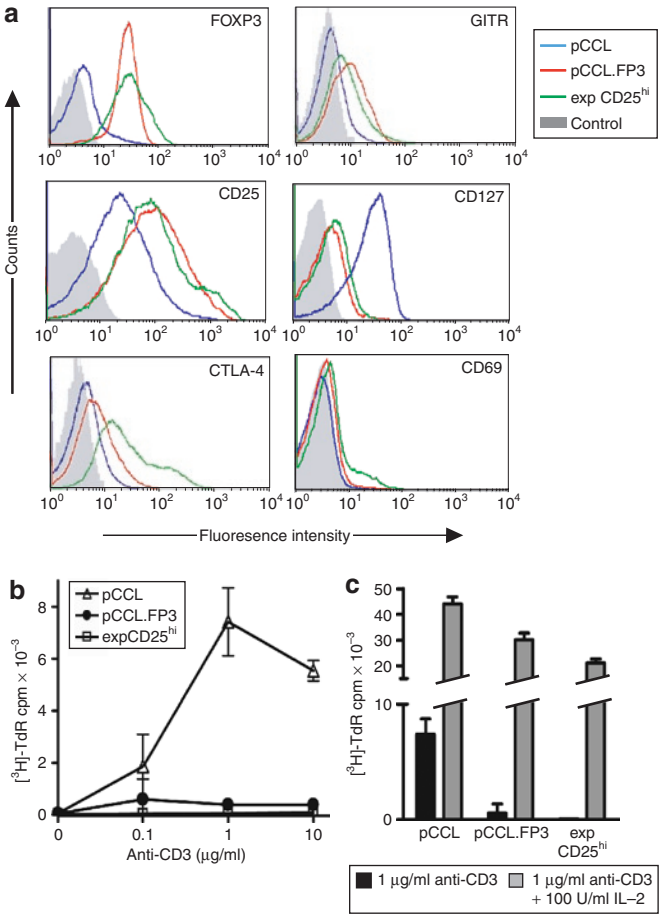

Figure 3 Phenotype and proliferative potential of transduced T cells. (**a**) Expression of the indicated markers was determined by fluorescence-activated cell sorting analysis of control and FOXP3-expressing T cells in the resting phase. Donor-matched CD4$^+$CD25^{hi} T-regulatory cells were expanded (exp CD25^{hi}) in parallel. The data are representative of a minimum of nine independent experiments with cells derived from at least six different donors. FOXP3 staining was performed with clone 236A/E7. Control indicates unstained or fluorescence-minus-one-stained cells. (**b**) Transduced T cells were tested for their ability to proliferate in response to increasing amounts of immobilized anti-CD3. (**c**) The capacity of exogenous interleukin-2 (IL-2) to induce proliferation of transduced T cells was assessed in the presence of immobilized anti-CD3 (1 μg/ml). Error bars indicate SD of values from triplicate wells. **b** and **c** are representative data from a minimum of six independent experiments performed with transduced T cells derived from at least four donors.

EF1α-driven expression of FOXP3 suppresses cytokine production in CD4$^+$ T cells

In accordance with our previous data,[20] over-expression of FOXP3 resulted in significantly reduced production of IL-2, interferon-γ (IFN-γ), and IL-4 in culture supernatants after TCR-mediated stimulation (**Figure 4a**). IL-10 production by the pCCL.FP3-transduced cells was reduced, although this difference did not reach statistical significance. Expression of TGF-β was equivalent in control and pCCL.FP3-transduced cells. Results for IL-2 and IFN-γ were confirmed by intracellular staining, after re-stimulation with either anti-CD3/28-coated beads or phorbol 12-myristate 13-acetate and Ca^{2+} ionophore (**Figure 4b**). The percentage of cytokine-producing cells was significantly lower among pCCL.FP3-transduced T cells than among control-transduced T cells, irrespective of the activation condition (**Figure 4c**). phorbol 12-myristate 13-acetate and Ca^{2+} ionophore stimulation has previously been shown to reverse the energy and suppressive function of CD4$^+$CD25$^+$ Tregs,[36] and in our study this stimulus was sufficient to overcome a blocking of IL-2 production. Interestingly, in comparison with the expanded CD4$^+$CD25^{hi} Tregs tested in parallel, the pCCL.FP3-transduced cells consistently showed a more profound suppression of cytokine production, thereby demonstrating superior preservation of a Treg phenotype after *in vitro* expansion. As further evidence that EF1α-driven lentiviral FOXP3 expression enforces a more consistent Treg phenotype than LTR-driven retroviral FOXP3 does, we compared the proliferative capacity and cytokine production of pCCL.FP3- and pLX.FP3-transduced cells in parallel. As predicted,

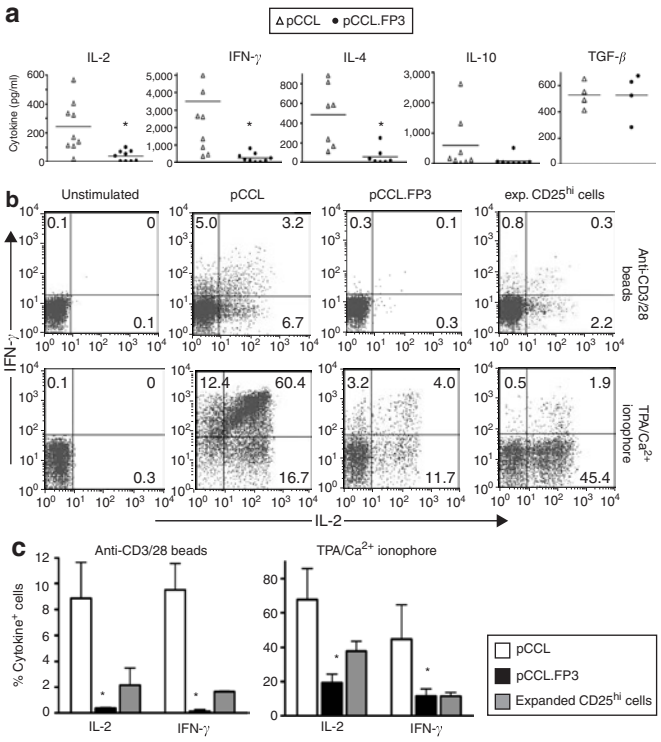

Figure 4 Cytokine production by FOXP3-transduced T cells. (**a**) Cytokine production of transduced T cells was determined by enzyme-linked immunosorbent assay or cytometric bead array 24 hours (IL-2), 48 hours (IFN-γ, IL-4, and IL-10), or 72 hours (TGF-β) after re-stimulating resting T cells with anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml). Each point represents a single determination; asterisk indicates $P \leq 0.02$. (**b**) Cytokine production by control cells or by FOXP3-transduced cells was determined by intracellular staining after 6 hours of stimulation with anti-CD3/28 coated beads or 4 hours TPA and Ca^{2+} ionophore. Data in **a** and **b** represent a minimum of four experiments with transduced T cells derived from at least three different donors, with results of intracellular cytokine analysis ± SD averaged in **c**. Asterisk indicates $P \leq 0.04$ for comparison of pCCL- and pCCL.FP3-transduced cells. IFN, interferon; IL, interleukin; TGF, tumor growth factor.

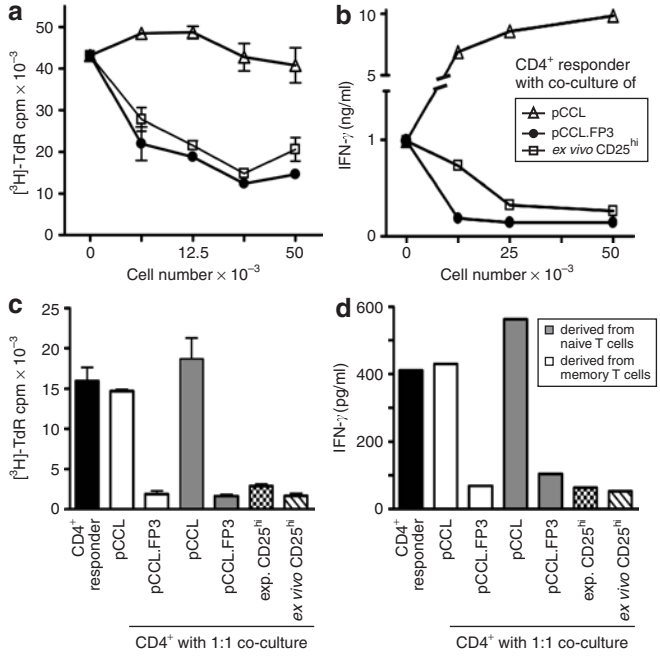

Figure 5 Suppressive capacity of transduced T cells. (**a**) $CD4^+$ responder T cells were stimulated with soluble anti-CD3 (1 μg/ml) in the presence of antigen-presenting cells (APCs), with or without the indicated numbers of transduced T cells. (**b**) After 72 hours, the culture supernatants from suppression assays were collected and analyzed by the cytometric bead assay for interferon-γ (IFN-γ) concentrations. (**c**) The regulatory capacity of transduced cells derived from either naïve (white bars) or memory (gray bars) T cells was determined as in **c** by co-culture of a 1:1 ratio of transduced T cells to $CD4^+$ responder cells. (**d**) After 72 hours, the culture supernatants from suppression assays were collected and analyzed by the cytometric bead array for IFN-γ concentrations. **a** and **b** are representative of a minimum of eight experiments with transduced T cells derived from at least six donors, and **c** and **d** a minimum of three experiments with transduced T cells from three different donors. Error bars indicate SD between a set of three wells.

transduction with pCCL.FP3 led to a more profound suppression of proliferation and cytokine production in $CD4^+$ T cells than with the pLX.FP3 vector (**Supplementary Figure S4**).

EF1α-driven FOXP3 expression confers potent suppressor function upon memory $CD4^+$ T cells

pCCL.FP3-transduced T cells consistently and potently suppressed the proliferation (**Figure 5a**) and cytokine production (**Figure 5b**) of $CD4^+$ responder T cells. Their suppressive capacity was comparable to that of *ex vivo* $CD4^+CD25^{hi}$ Tregs. At a 1:1 ratio, pCCL.FP3-transduced T cells suppressed proliferation by an average of 75 ± 8% ($n = 12$), while for *ex vivo* $CD4^+CD25^{hi}$ Tregs the extent of suppression was 78 ± 11% (P = NS, $n = 12$). Similarly, production of IFN-γ by $CD4^+$ responder T cells was inhibited by an average of 81 ± 15% by pCCL.FP3-transduced T cells, and by 83 ± 12% (P = NS, $n = 8$) by *ex vivo* $CD4^+CD25^{hi}$ Tregs. If the pCCL.FP3-transduced cells remained >90% $\Delta NGFR^+$, their capacity to suppress did not decrease over time in culture (tested up to 8 weeks, data not shown). Control-transduced cells did not suppress proliferation or cytokine production by responder $CD4^+$ T cells.

It has been found that ectopic expression of FOXP3 is not capable of converting human memory T cells into Tregs.[35] In view of the fact that our method was more efficient than the one described for inducing Tregs from naïve cells, we investigated whether EF1α-driven FOXP3 expression would also convert memory Teff cells into Tregs. $CD4^+CD25^-$ T cells were expanded in culture for 2 weeks prior to transduction. We were able to establish that our method for ectopic expression of FOXP3 was sufficient to induce a suppressor phenotype in $CD4^+CD45RO^+$ T cells (**Figure 5c** and **d**); however, their suppressive capacity was more variable than when naïve T cells were transduced. Depending on the donor, memory T cells transduced with pCCL.FP3 suppressed proliferation by 32–90% (mean value = 61.6%, $n = 5$) and IFN-γ by 52–75% (mean value = 65%, $n = 3$). The reduced and more variable suppressive capacity of pCCL.FP3-transduced memory cells could not be linked to lower FOXP3 expression; when compared to naïve cells transduced with FOXP3 in parallel, the difference in FOXP3 mean fluorescence intensity expression between the two populations was not significant ($P = 0.09$, $n = 4$). These data indicate that memory T cells are biologically capable of acquiring suppressor function, provided they continuously express high levels of FOXP3.

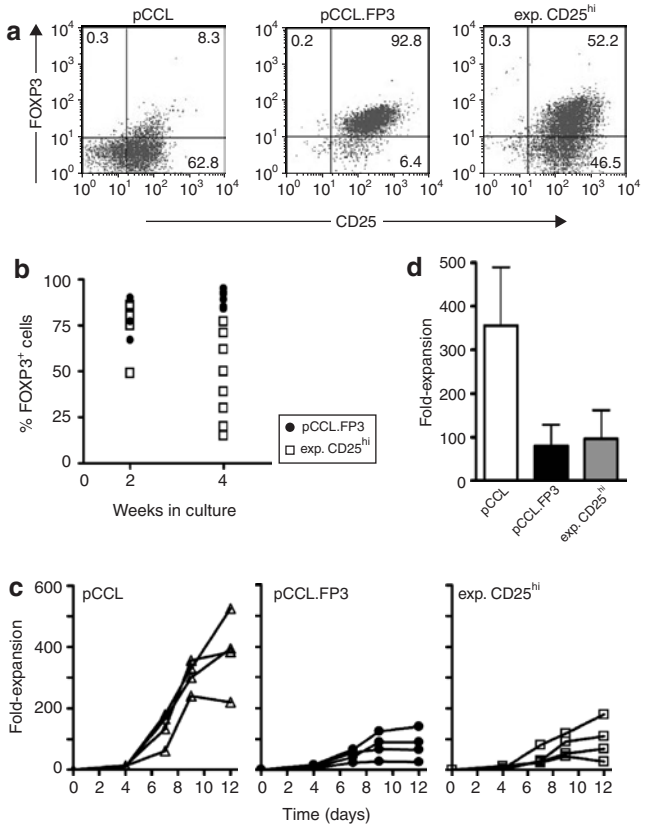

Figure 6 Comparison of *in vitro* expansion of FOXP3-transduced and CD4$^+$CD25^{hi} T cells. (**a**) Control or pCCL.FP3-transduced cells were expanded in parallel with CD4$^+$CD25^{hi} T cells for 4 weeks, and expressions of CD25 and FOXP3 were determined in the resting phase. (**b**) The percentage of FOXP3$^+$ cells in pCCL.FP3-transduced T cells and in donor-matched CD4$^+$CD25^{hi} T regulatory cells (Tregs) expanded in parallel was compared. Each symbol represents an independent T cell line. (**c**) The multiples of expansion of transduced T cells and fluorescence-activated cell sorting–sorted CD4$^+$CD25^{hi} T cells were determined over a 12-day culture period. Each line represents T cells derived from a different donor, with average results ± SD shown in **d**.

EF1α-driven FOXP3 expression induces a stable population of suppressive T cells

A major limitation of protocols involving *in vitro* expansion of CD4$^+$CD25^{hi} Tregs for the purpose of cellular therapy is that isolation on the basis of CD25 expression does not result in a homogeneous population of FOXP3$^+$ cells[11] and that, depending on cell numbers, extensive expansion *in vitro* may be necessary (**Figure 6a**). While pCCL.FP3-transduced T cells maintained a high percentage of FOXP3 expressors over several weeks, the expression of FOXP3 in *ex vivo* Tregs, sorted on the basis of high CD25 expression, declined with each round of expansion (**Figure 6b**). The FOXP3-transduced T cells could be efficiently expanded *in vitro* in the presence of exogenous IL-2. After 12 days, the average multiple of expansion of purified FOXP3-expressing cells was 80 ± 48, which was less than that of pCCL-transduced cells (356 ± 133), but comparable to that of CD4$^+$CD25^{hi} Tregs (96 ± 65) (**Figure 6c** and **d**).[6,7,9] The number of Tregs generated in each experiment varied, depending on the donor and pCCL.FP3 transduction efficiency. In an average experiment, if 0.5×10^6 T cells were transduced, following purification and one round of expansion (*i.e.*, 3 weeks of *in vitro* culture), ~1.5×10^7 Tregs were generated.

Expression of FOXP3 does not affect TCR diversity

We also investigated whether the transduction process and/or expression of FOXP3 affects TCR diversity. Accordingly, the diversity of the TCR repertoire was analyzed *ex vivo* and in expanded control and pCCL.FP3-transduced T cells derived from two donors (#207 and #266). Specific subsets of the TCR β chain were amplified, including the CDR3 clonotypic determinant, and multiple (n = 27–34) independently derived clones were sequenced[37,38] (**Tables S1** and **S2**). As expected, *ex vivo* CD4$^+$ T cells were polyclonal, with 28 unique clonotypes observed in 29 TRBV7 sequences, and 31 unique clonotypes (100%) in TRBV30. In contrast, after 4 weeks of culture, both control cells and pCCL.FP3-transduced T cells from donor #207 showed loss of polyclonality in TRBV7 and TRBV30. Results from donor #266 were similar, with the exception that there was more pronounced oligoclonality, probably because the transduced cells were analyzed after 8 weeks of culture (**Supplementary Table S2**). These data indicate that while there is a loss of polyclonality with time in culture, there is no apparent difference between control cells and pCCL-FP3-transduced cells. This loss of TCR diversity is more likely to be related to the process of *in vitro* expansion rather than to transduction, as previously reported with retroviral gene transfer.[39]

DISCUSSION

We describe here the first robust and reliable method for the *in vitro* generation of potently suppressive human CD4$^+$ T cells using lentivirus-mediated gene transfer of FOXP3. In comparison to other methods involving LTR-driven expression of FOXP3, or expansion of *ex vivo* Tregs on the basis of cell-surface marker expression, this method generates a more homogeneous population of cells with stable suppressive capacity equal to that of CD4$^+$CD25^{hi} Tregs. The phenotype of FOXP3-transduced cells also mimics Tregs with respect to high expression of several Treg-associated molecules, repression of CD127,[11] lack of cytokine production, and hypo-responsiveness to TCR stimulation. Analysis of surface marker expression in FOXP3-transduced cells confirms previous findings that CD127 is a target of FOXP3 repressor activity, but suggests that factors in addition to FOXP3 are required for producing the high CTLA-4 expression found in naturally occurring Tregs. This method allows functional Tregs to be generated from memory T cells, and does not rely on the preservation of Treg-associated cell-surface markers that are conventionally used for sorting populations of resting or *ex vivo* T cells. A key advantage of our protocol over expansion of CD4$^+$CD25^{hi} Tregs is the ability to re-sort populations of pCCL.FP3-transduced T cells on the basis of surface ΔNGFR expression [which is transcriptionally coupled to that of FOXP3 (ref. 32)] so as to maintain a pure population of suppressive FOXP3^{hi} cells. The numbers of Tregs that are required for successful therapeutic applications is currently unknown. Because an unlimited number of CD4$^+$CD25$^-$ T cells can be transduced, and because, once purified, they can be expanded by an average of 80-fold in <2 weeks, this protocol should be useful for generating large

numbers of Tregs that preserve a diverse TCR repertoire. Studies will need to be carried out to determine the capacity of the pCCL.FP3-transduced cells to survive and expand *in vivo*.

The fact that IL-10 and TGF-β are not produced at high levels by FOXP3-transduced cells indicates that expression of this transcription factor alone is not sufficient to induce their expression, and that high expression of these cytokines is not required for the *in vitro* suppressive function of these Tregs. Moreover, the addition of TGF-β-blocking antibodies to *in vitro* suppression assays does not abrogate the inhibitory function of pCCL.FP3-transduced cells (data not shown), and this result is similar to the behavior of *ex vivo* human Tregs.[9,40] These findings strengthen our conclusion that FOXP3 transduction recapitulates a Treg phenotype and not a Tr1-like phenotype. It is possible, however, that *in vivo* FOXP3$^+$ Tregs may mediate cytokine-dependent suppressive activity.

LTR-driven retroviral expression of Foxp3 in mouse CD4$^+$ T cells efficiently drives the development of Tregs.[19] As we have demonstrated here, potently suppressive human Tregs can be consistently induced by gene transfer only when FOXP3 is under the control of an activation-independent promoter. Although retroviral LTR-driven FOXP3 expression was actually higher than that driven by EF1α at the peak of TCR-mediated activation, it varied with activation state and was less potent than EF1α-driven expression in inducing suppressive cells. This would suggest that stable expression of FOXP3 throughout the cycles of activation, expansion, and resting is necessary to efficiently convert human T cells to suppressor cells. Because the function of FOXP3 is influenced by its ability to physically interact with nuclear factor of activated T cell,[41] the balance of these factors during the initial stages of T cell activation may influence the molecular activities of the protein, and determine whether functional suppression is the biological outcome of its expression.

Importantly, our findings indicate that populations of CD4$^+$CD25RO$^+$ T cells can be transduced with pCCL.FP3 and switched into functional suppressor cells. Thus memory T cells in the periphery may be biologically capable of acquiring regulatory function, provided high, constitutive FOXP3 expression is induced. On the basis of this evidence, it is possible to conclude that antigen-specific suppressor cells can be generated by *ex vivo* transduction of Teff cells specific for a defined antigen. In this manner, small numbers of antigen-specific cells originating from a patient could be propagated *in vitro* so as to achieve therapeutically useful populations of antigen-specific FOXP3^{hi} Tregs. Importantly, lentiviral vectors such as pCCL.FP3, which contain self-inactivating LTRs, pose significantly lower risks of genotoxicity than conventional expression vectors, in which transgene expression is driven by a retroviral LTR.[42] Our strategy would therefore alleviate concerns relating to insertional mutagenesis and cell transformation, and ensure that engineered Tregs can be used safely for *in vivo* transplantation. Furthermore, ΔNGFR could be replaced with a gene encoding a selective marker, such that pCCL.FP3-transduced cells could be manipulated *in vivo*. More *in vivo* experiments will need to be conducted in order to determine whether Tregs generated by pCCL.FP3 transduction have the *in vivo* suppressive efficacy required for successful cellular therapy applications.

MATERIALS AND METHODS

Construction and production of retroviral and lentiviral vectors. The moloney murine leukemia virus–based retroviral vectors encoding FOXP3 and ΔNGFR have been previously described.[20] Third-generation lentiviral vectors were constructed from a previously characterized vector, MA1, in which two transgenes are efficiently transcribed from a common bi-directional origin: ΔNGFR from the minimal cytomegalovirus promoter, and a second transgene from the human phosphoglycerate kinase promoter.[32] In order to construct pCCL.FP3, hFOXP3 was cloned into MA1as the second transgene, and the phosphoglycerate kinase promoter was excised and replaced with the human EF1α promoter. The control pCCL vector carries the mCMV-ΔNGFR cassette and the EF1α promoter without a second transgene. Lentiviral vectors were produced by transient 4-plasmid overnight transfection of HEK 293T cells.[32] The titers of concentrated virus were determined by limiting dilution on 3T3 or 293T cells, and titers of ~2×10^9/ml for retroviral and ~2×10^8/ml for lentiviral supernatants were routinely obtained.

Cell purification and expansion of T cell lines. Following approval by the University of British Columbia Clinical Research Ethics Board, peripheral blood was obtained from healthy volunteers after obtaining their written informed consent. Peripheral blood mononuclear cells were isolated from buffy coats by Ficoll separation, and CD4$^+$ T cells were purified by negative selection (Stemcell Technologies, Vancouver, Canada). CD4$^+$CD25^{hi} Tregs were purified by positive selection over two columns (Miltenyi Biotec, Auburn, CA) so as to ensure 85–90% purity for *ex vivo* suppression experiments, and by fluorescence-activated cell sorting for ≥99% purity for *in vitro* expansion. Fluorescence-activated cell sorting-sorted CD4$^+$CD25^{hi} Tregs (which remained >60% FOXP3$^+$) were expanded and tested in parallel. In order to obtain pure (≥99%) CD4$^+$CD25$^-$ T cells, the CD25$^-$ fraction was either passed over an LD depletion column (Miltenyi Biotec, Auburn, CA) or fluorescence-activated cell sorting sorted. For isolation of CD4$^+$CD25$^-$CD45RO$^-$ T cells, CD4$^+$CD25$^-$ T cells were incubated with CD45RO beads (Miltenyi Biotec, Auburn, CA) and passed over an LD depletion column to achieve purities ≥95%. APCs were purified from peripheral blood mononuclear cells by depletion of CD3 cells (Stemcell Technologies, Vancouver, Canada). For expansion of T cell lines, a minimum of 200,000 T cells were re-stimulated every ~14 days with a feeder mixture consisting of 1×10^6 allogeneic peripheral blood mononuclear cells/ml, 1×10^5 Epstein-Barr virus–transformed JY cells/ml, 1 μg/ml phytohemagluttinin, and 100 U/ml rhIL-2 (Chiron, Ville Saint-Laurent, Canada). In some experiments, 10 ng/ml rhIL-7 (BD Biosciences, Mississauga, Canada) was added.

Retroviral and lentiviral transduction of primary and Jurkat T cells. Prior to transduction, CD4$^+$CD25$^-$CD45RO$^-$ T cells were activated in complete medium (X-VIVO 15; Cambrex Corp., Walkersville, Canada) supplemented with 5% pooled human serum (Cambrex Corp., Walkersville, Canada), and penicillin/streptomycin (Invitrogen Corp., Burlington, Canada), containing rhIL-2 and rhIL-7 (10 ng/ml; BD Biosciences, Mississauga, Canada). Pre-activation consisted of either 48 hours of exposure to immobilized anti-CD3 (1 μg/ml OKT3; Orthoclone; Ortho Biotech Products, Bridgewater, NJ) and soluble anti-CD28 (1 μg/ml; BD Biosciences, Mississauga, Canada) or, for optimal transduction efficiency, 16 hours of exposure to soluble anti-CD3 monoclonal antibodies (1 μg/ml OKT3) and autologous-irradiated APCs, at a 1:5 ratio of T cells to APCs. For retroviral transduction, two successive rounds of infection were performed on retronectin-coated plates using a multiplicity of infection of 20 virus particles per cell and 8 μg/ml polybrene (Sigma-Aldrich, Oakville, Canada). For lentiviral transduction, T cells were infected once with lentiviral supernatant (multiplicity of infection = 10). For lentiviral transduction of CD4$^+$CD45RO$^+$ memory T cells, cells were first expanded *in vitro* with a standard feeder cell mixture for 14 days (see above). T cells were then re-stimulated for 16 hours with the standard feeder cell mixture

prior to a single round of infection with lentiviral supernatant (multiplicity of infection = 10). ΔNGFR$^+$-transduced T cells were purified 8 days after transduction using ΔNGFR-select beads (Miltenyi Biotec, Auburn, CA), and expanded in IL-2-containing medium. Prior to testing in functional and phenotypic assays, which was typically performed following two rounds of expansion (*i.e.,* after 4 weeks in culture), T cells in the resting phase (12–14 days after activation) were washed and rested in IL-2 free medium overnight. Untransduced cells, which were tested in parallel, were equivalent to control-transduced cells in all aspects of phenotype and function (data not shown).

Proliferation and suppression assays. In order to test proliferative capacity, 50,000 T cells/well were cultured in the presence of immobilized anti-CD3 (1 μg/ml OKT3) in the presence or absence of IL-2. Proliferation was assessed 72 hours after the incorporation of [^{3}H]thymidine (1 μCi/well, Amersham Biosciences, Oakville, Canada), which was added for the final 16 hours of culture. In order to test for suppressive capacity, *ex vivo* CD4$^+$CD25$^-$ T cells were stimulated at 50,000 cells/well in the presence of anti-CD3 (1 μg/ml OKT3) and 50,000 irradiated APCs. Transduced T cells or CD4$^+$CD25$^+$ T cells were added in numbers indicated in the figures, and suppression was assessed by measuring the amount of [^{3}H]thymidine incorporation in the final 16 hours of a 96 hour culture period, and/or by determination of IFN-γ concentration in culture supernatants after 72 hours using a Th1/Th2 cytometric bead array (BD Biosciences, Mississauga, Canada).

Flow cytometric analysis. Staining for cell-surface markers ΔNGFR (American Type Culture Collection), CD69, CD25, CD127 (all from BD Biosciences, Mississauga, Canada), and GITR (R&D Systems, Minneapolis, MN) was carried out prior to intracellular staining for CTLA-4 (BD Biosciences, Mississauga, Canada) or FOXP3 (clone PCH101 or 236A/E7), (eBiosciences, San Diego, CA), in accordance with the manufacturer's instructions. For analysis of intracellular cytokine staining, activated cells were fixed in 2% formaldehyde, permeabilized in 0.5% saponin, and stained with IL-2 and IFN-γ antibodies (BD Biosciences, Mississauga, Canada). Samples were acquired on a BD FACSCanto and analyzed with FCS Express Pro Software Version 3 (De Novo Software, Thornhill, Canada).

Quantitative polymerase chain reaction. For quantitative reverse transcriptase-polymerase chain reaction, amounts of FOXP3 mRNA and glyceraldehyde 3-phosphate dehydrogenase were determined as previously described.[43] All samples were run in triplicate, and the relative expression of FOXP3 was determined by normalizing to glyceraldehyde 3-phosphate dehydrogenase so as to calculate the multiple of change in value.

Determination of cytokine concentration. In order to quantitate amounts of IL-2, IFN-γ, IL-4, and IL-10, capture enzyme-linked immunosorbent assays or Th1/Th2 cytometric bead arrays (both from BD Biosciences, Mississauga, Canada) were performed on supernatants after activation with immobilized anti-CD3 (10 μg/ml) and soluble anti-CD28 (1 μg/ml), for 24 hours (for IL-2) or 48 hours (for IFN-γ, IL-4, and IL-10). Enzyme-linked immunosorbent assays for detection of TGF-β1 (R&D Systems, Minneapolis, MN) were used in analyzing supernatants after 72 hours of activation with immobilized anti-CD3 and soluble anti-CD28. For analysis of intracellular cytokine production, T cells were activated with 10 ng/ml PMA and 500 ng/ml Ca^{2+} ionophore (both from Sigma-Aldrich, Oakville, Canada) for 4 hours, or a 1:1 ratio of anti-CD3/CD28-coated beads (Invitrogen, Burlington, Canada) for 6 hours, and brefeldin A (10 μg/ml; Sigma-Aldrich, Oakville, Canada) was added half-way through activation.

TCR β-chain cloning and diversity analysis. Total RNA was extracted from a minimum of 2×10^6 *ex vivo* CD4$^+$ T cells or transduced T cells using Trizol, and complementary DNA was synthesized using oligo$(dT)_{12-18}$ primers and reverse transcriptase (all from Invitrogen, Burlington, Canada). TRBV7-3 and TRBV30 TCR β-chains were amplified, cloned, and sequenced, as described.[38] Identification of TRBV and TRBJ segments and determination of CDR3 length and amino-acid sequences were performed using the IMGT/V-QUEST webtool (http://imgt.cines.fr/IMGT_vquest/)[44] and were verified manually. CDR3 length was computed according to reference 45. Nomenclature of TRBV and TRBJ segments is according to reference 46.

Statistics. All analyses for statistically significant differences were performed with one-tailed paired Student's *t*-test. *P* values of less than 0.05 were considered significant.

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SUPPLEMENTARY MATERIAL

Figure S1. Co-ordinate expression of FOXP3 and ΔNGFR by the pCCL. FP3 lentivirus vector.

Figure S2. Phenotype and function of Tregs expanded for 4 weeks with or without IL-7.

Figure S3. Expression of Treg-associated markers in transduced T cells and expanded CD4$^+$CD25^{hi} T cells.

Figure S4. Phenotype of T cells transduced with FOXP3-expressing retroviral or lentiviral vectors.

Table S1. Diversity analysis of the TCR repertoire in ex vivo and transduced CD4$^+$ T cells from donor 207.

Table S2. Diversity analysis of the TCR repertoire in ex vivo and transduced CD4$^+$ T cells from donor 266.

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