Harnessing immune regulation for treatment of human diseases : CD4+CD25+ regulatory T cells & antibody glycosylation

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CHAPTER 4

De novo generation and enhanced suppression of human $CD4^+CD25^+$ regulatory T cells by retinoic acid

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Abstract

Therapies based on $CD4^+CD25^+FOXP3^+$ T regulatory (Treg) cells offer promise for the restoration of tolerance in many immune-mediated disorders. However, it has been proven difficult to obtain large numbers of Treg cells with potent and stable suppressive ability from adult human peripheral blood due to the lack of specific markers for Treg isolation/characterization, compromised function of isolated $CD4^+CD25^{++}$ T cell populations, and the difficulty to convert conventional T cells, for example by TGF-β, into Treg cells in a consistent manner. Here we show that: 1) in the presence of TGF-β, all-*trans* retinoic acid (ATRA) efficiently converts adult human peripheral blood naive $CD4^+$ T cells into $FOXP3^+$ Treg cells with stable and potent suppressive ability; 2) memory $CD4^+$ T cells are resistant to FOXP3 induction and, moreover, inhibit Treg-conversion of naive T cells that can be partially reversed by anti-IL-4; and 3) treatment of isolated $CD4^+CD25^{++}$ T cells with ATRA/TGF-β enhances their suppressive potential during expansion. Our results indicate that ATRA/TGF-β can be employed to generate highly suppressive $CD4^+FOXP3^+$ Treg cells from adult human peripheral blood and are relevant for the development for Treg-based therapies.

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Introduction

$CD4^+CD25^+$ Treg cells play a critical role in establishing and maintaining self tolerance by damping the immune responses of many types of immune cells. Therefore, enhancing the number and/or function of Treg cells represents a potential treatment for patients with autoimmune disorders or that undergo transplant rejections. Indeed, adoptive transfer of *in vitro* expanded $CD4^+CD25^+$ Treg cells has been successfully used in several animal models of graft rejection, inflammation, and autoimmunity (1-5). However, Treg-based immunotherapy has been hampered in the clinic because of the difficulties in obtaining large numbers of homogenous Treg cell populations *in vitro* that display stable and potent suppressive function (6-8).

So far, several approaches have been explored to obtain Treg cells suitable for the clinical settings. As in mice, Treg cells, isolated on the basis of high CD25 expression can be expanded efficiently *in vitro* by strong TCR-mediated stimulation in the presence of high amounts of exogenous IL-2 (9, 10). However, because of the lack of specific markers to identify and isolate human Treg cells (11, 12), the contaminating effector cells in the starting population can out-compete the "genuine" Treg cells during expansion. Hereby the suppressive capacity of the expanded T cell population is decreased (6-8). Although the combination of CD25 with other markers, such as CD127, CD45RA, or membrane-bound TNFα (6, 7, 13–15), can lead to a more homogenous T cell population with regulatory ability, the lower number of cells obtained will also lead to a longer expansion period, during which the function of "genuine" Treg cells could wane (16). Moreover, purified $CD4^+CD25^{++}FOXP3^+$ T cells may also differentiate into IL-17-producing cells during *in vitro* culture as reported recently (17). Furthermore, the compromised suppressor function of $FOXP3^+$ Treg cells reported in several autoimmune situations could additionally hamper the ability to obtain sufficient numbers of Treg cells with powerful suppressive function from these patients (18-21).

De novo generation of Treg cells from conventional effector cells represents a promising strategy to obtain sufficient Treg cells suitable for immunotherapy. Because FOXP3 is necessary for the suppressive functions, several groups attempted to generate Treg cells by forced overexpression of FOXP3 in $CD4^+CD25^-$ T cells (8, 22, 23). In this way, homogenous FOXP3-expressing T cell population can be obtained *in vitro* and targeted specifically *in vivo* by introducing "suicide" markers/genes. Nonetheless, conflicting data on the suppressor capacity of these $FOXP3^+$ T cells have been reported, which might be attributed to the stability and expression levels of FOXP3 (8, 22, 23). In addition, the clinical use of these cells will be compromised by the regulatory constrains associated with gene transfer. Therefore, a method to generate Treg cells without gene transfer would greatly increase their clinical possibilities.

Recently, the methods to reprogram human $CD4^+CD25^-$ T cells to Treg cells *in vitro* have been investigated intensively. It has been reported that *in vitro* TCR-mediated stimulation of $CD4^+CD25^-$ T cells can result in the development of suppressive $CD4^+FOXP3^+$ Treg cells in the absence or presence of exogenous TGF-β (24–27). However, several reports indicate that even in the presence of exogenous TGF-β, TCR-stimulation of human $CD4^+CD25^-$ T cells does not consistently generate Treg cells despite high FOXP3 expression within these cells (28-30). Moreover, the stability of the suppressive capacity of these *in vitro* generated Treg cells has not been fully established.

All-*trans* retinoic acid (ATRA), the metabolite of vitamin A, has been recently shown to be capable of inducing murine $CD4^+Foxp3^+$ Treg cells from conventional $Foxp3^-$ effector cells, either directly by enhancing TGF-β-driven Smad3 signaling in naive cells and/or indirectly by relieving the inhibition from murine memory effector cells (31-39).

Such approaches show great promise as these T cells have been shown effective to combat several immune-mediated disorders in mice (31-33). In humans, it has been shown that ATRA can induce $FOXP3^+$ cells with suppressive function from $CD4^+CD25^-$ T cells isolated from cord blood (40). However, the effect of ATRA on adult peripheral blood T cells, i.e., its effect on the induction of $CD4^+FOXP3^+$ Treg cells from naive/memory effector cells or on the suppressive function of isolated $CD4^+CD25^{++}$ Treg cells, has not been investigated.

In this study, we show that ATRA, together with TGF-β, consistently induces *de novo* generation of $CD4^+FOXP3^+$ Treg cells with potent and stable suppressive ability from $CD4^+CD25^-CD45RA^+$ naive T cells in adult human peripheral blood. In contrast, $CD4^+CD25^-CD45RA^-$ memory T cells are not only resistant to FOXP3 induction but also actively inhibit FOXP3 expression in cocultured naive T cells. Furthermore, addition of ATRA and/or TGF-β during the expansion of purified peripheral blood $CD4^+CD25^{++}$ Treg cells significantly enhances their suppressive capacity. These data indicate that ATRA can be used to generate highly suppressive $CD4^+CD25^+FOXP3^+$ Treg cells suitable for Treg-based therapy because it not only induces the *de novo* generation of $CD4^+FOXP3^+$ Treg cells but also enhances the suppressive function of isolated $CD4^+CD25^{++}$ Treg cells during expansion.

Materials and Methods

Culture medium. Human T cells were cultured in complete medium consisting of IMDM (Cambrex) supplemented with 10% heat-inactivated fetal calf serum (FCS, BD biosciences), 100 U/ml penicillin, 100 μg/ml streptomycin (Cambrex) and 2 mM GlutaMAX (Invitrogen).

Antibodies. The following antibodies were used: pacific blue-conjugated anti-CD4 (RPA-T4), phycoerythrin (PE)-conjugated anti-CD25 (M-A251), anti-CD127 (hIL-7R-M21), fluorescein isothiocyanate (FITC)-conjugated anti-CD45RA (L48), AlexaFluor 647 (Alexa 647)-conjugated anti-CCR7 (3D12), allophycocyanin (APC)-conjugated anti-CD62L (Dreg 56) (all from BD Biosciences), and anti-$CD25^{Alexa\ 700}$ (BC96; Biolegend). Anti-$CTLA4^{PE}$ (eBio20A), anti-$FOXP3^{PE/APC}$ (236A/E7), anti-$FOXP3^{FITC/Alexa\ 700}$ (PCH101), and neutralization Abs to human IL-4 (MP4-25D2), IL-6 (MQ2-13A5) and IFN-γ (NIB42) were bought from eBiosciences. Adalimumab and Tocilizumab were used to block TNF-α and IL-6R, respectively.

Cell isolation. Human peripheral blood was obtained from healthy blood bank donors after informed consent in accordance with procedures approved by the local human ethics committee. PBMC were prepared by centrifugation over Ficoll-Hypaque gradients and $CD4^+$ T cells were purified by positive selection with the Dynabeads FlowComp Human CD4 T cell isolation kit (Invitrogen). $CD4^+$ T cells were then labeled with anti-$CD4^{APC}$ or anti-$CD4^{Pacific\ Blue}$, anti-$CD25^{PE}$ and anti-$CD45RA^{FITC}$ for 30 minutes at 4 °C. Cells were washed, and $CD25^-CD45RA^+$ naive, $CD25^-CD45RA^-$ memory effector cells, and $CD25^{++}$ (top 5%) Treg cells were sorted *via* a FACS Aria cell sorter (Becton Dickinson, California) with greater than 95% purity. The analysis and sort gates were restricted to the small lymphocyte gate as determined by their characteristic forward and side scatter properties. CD4-depleted PBMC irradiated with 4000 rads were used as feeders in the proliferation/suppression assay.

Activation of human T cells in vitro. Purified $CD4^+CD25^-CD45RA^+$ naive, $CD4^+CD25^-CD45RA^-$ memory effector cells and $CD4^+CD25^{++}$ T cells were activated with 5 μg/ml plate-bound anti-CD3 (OKT-3; BD Biosciences), 1 μg/ml soluble anti-CD28 (CLB-CD28/1; Sanquin), and 50 or 300 (for $CD4^+CD25^{++}$) U/ml IL-2 for 5 days as described previously (28). ATRA (Sigma) and/or recombinant human TGF-β (PeproTech Inc) were also included in some wells at the concentration of 10 nM and 5 ng/ml, respectively. ATRA was first dissolved in DMSO at 10 mM and further diluted in complete medium. After extensive washing, activated T cells were rested in complete medium supplemented with 100 U/ml IL-2 with or without 10 nM ATRA before further functional tests.

Flow cytometric analysis. Single-cell suspensions were prepared and surface molecules were stained for 30 minutes at 4 °C with optimal dilutions of each antibody. After fixation and permeabilization, cells were incubated with anti-FOXP3 or anti-CTLA4 antibodies using the eBioscience intracellular staining kit. Expression of cell surface and intracellular markers were assessed by using FACSCalibur or LSRII. Data were analyzed by CellQuest Pro for FACSCalibur or FACSDIVA for LSRII, respectively (BD Biosciences). Activation of human $CD4^+CD25^-$ T cells in the presence of TGF-β leads to the appearance of a distinct population (M2 in supplementary Figure 1) of $FOXP3^{high}$-expressing cells as well as cells displaying lower levels of FOXP3 (M1 in supplementary Figure 1). Although identical results were obtained with other anti-FOXP3 Abs (data not shown) (28, 41, 42), only $FOXP3^{high}$ cells were designated as $FOXP3^+$ cells in this study as it has been debated whether these $FOXP3^{low}$ cells are genuine $FOXP3^+$ cells (30, 41, 42).

Proliferation and suppression of T cells. Cells were plated at 2×10^4 cells/well in 96-well plates in the presence of 1 μg/ml phytohemagglutinin (PHA) and 5×10^4 cells/well feeders. Cells were pulsed with ^{3}H-thymidine (0.5 μCi/well) on day 4 and proliferation was assessed 18 hours later using a liquid scintillation counter. To test for suppressive capacity, $CD4^+$ responder T cells were stimulated as described above. Differently stimulated $CD4^+CD25^-CD45RA^+$, $CD4^+CD25^-CD45RA^-$, or $CD4^+CD25^{++}$ T cells were added, and suppression was assessed by determining ^{3}H-thymidine incorporation.

Statistical analysis. The Mann-Whitney paired test was used to compare the difference among different groups by using GraphPad Prism 5.00 software (GraphPad, San Diego, CA), and values at $P < 0.05$ were considered significant.

Results

No effect of ATRA on the proliferation/survival of $CD4^+$ T cells

Recently, several reports have shown that ATRA can enhance the TGF-β-induced differentiation of murine T cells into $CD4^+FOXP3^+$ Treg cells (31-39). Moreover, it was demonstrated that ATRA alone could induce the *de novo* generation of $FOXP3^+$ Treg cells from $CD4^+CD25^-$ T cells purified from human cord blood (40). However, it is not known whether adult peripheral blood T cells can also be converted by ATRA into $FOXP3^+$ Treg cells. Before addressing this issue, we first wished to determine potential adverse effects of ATRA by examining its effect on the proliferation and viability of $CD4^+$ T cells. To this end, $CD4^+$ T cells were purified from human peripheral blood and subsequently activated with plate-bound anti-CD3, soluble anti-CD28 in the absence or presence of TGF-β, and different concentrations of ATRA. ATRA had no effect on the proliferation of cells as measured by ^{3}H-incorporation and CFSE dilution in the absence or presence of exogenous

TGF-β (supplementary Figure 2A & B). Furthermore, ATRA did not affect the viability of cells as measured by trypan blue exclusion (data not shown) and propidium iodide (PI) staining (supplementary Figure 2B).

Inconsistent induction of Treg cells from $CD4^+CD25^-$ effector T cells in adult human peripheral blood by TGF-β and ATRA

To investigate whether ATRA can induce the *de novo* generation of Treg cells from effector cells in human adult peripheral blood, FACS-purified $CD4^+CD25^-$ T cells (> 98% $FOXP3^-$ cells) were activated in the absence/presence of TGF-β, ATRA, or both for 5 days. After extensive washing, cells were rested for differentiation in IL-2 with or without ATRA for an additional 4-6 days. Next, their proliferative and suppressive capacity was analyzed. Although the addition of ATRA during the first 5 days' stimulation did not affect cell proliferation (supplementary Figure 2 and data not shown), when restimulated, cells cultured with TGF-β, ATRA, or both displayed a significantly decreased proliferative potential as compared to cells activated with neither of them in all tested individuals (Figure 1A). Furthermore, cells preconditioned with both ATRA and TGF-β significantly inhibited the proliferation of autologous responder T cells (Figure 1B, *upper panel*) in one donor. Taken together, these data indicate that in the presence of exogenous TGF-β, ATRA can convert $CD4^+CD25^-$ T cells obtained from adult peripheral blood into Treg cells. However, data obtained from 3 other donors were different as no suppression was observed in any of the conditioned cells (Figure 1B, *lower panel*). Therefore, ATRA does not consistently induce *de novo* generation of Treg cells from adult peripheral blood $CD4^+CD25^-$ T cells, even in the presence of exogenous TGF-β.

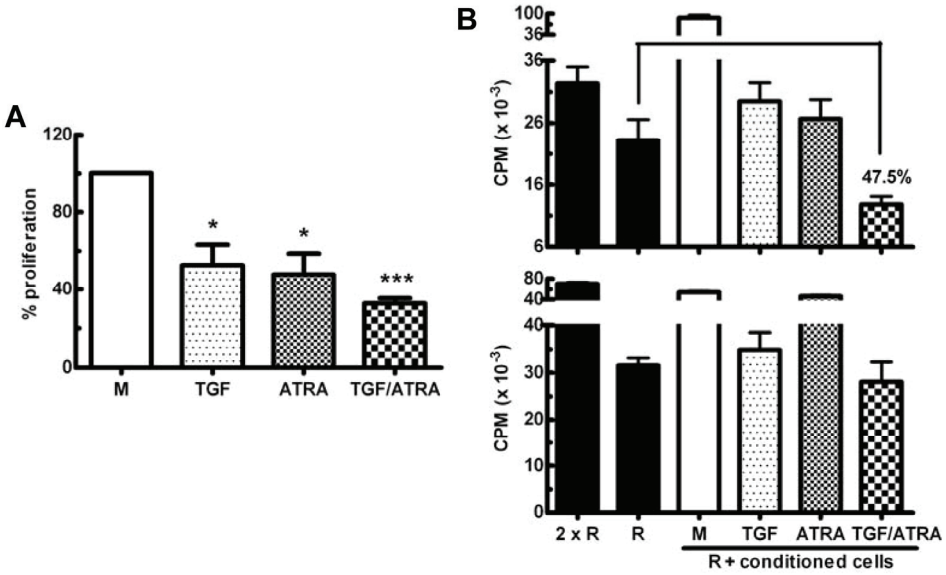


Figure 1. Inconsistent induction of Treg cells from $CD4^+CD25^-$ T cells in adult human peripheral blood by ATRA. $CD4^+CD25^-$ T cells were purified from adult peripheral blood by FACS-sorting and activated with plate-bound anti-CD3, soluble anti-CD28 and IL-2, in the absence (M) or presence of TGF-β (TGF), ATRA (ATRA) or both (TGF/ATRA) for 5 days. These cells were subsequently rested in IL-2 for additional 4-6 days. **A**, proliferative response of the conditioned cells relative to cells cultured in medium only was determined by ^{3}H-thymidine uptake after stimulation with PHA and feeders. Combined data from four different donors with means ± SEM are shown. **B**, suppressive capacities of the conditioned cells were examined by co-culture with autologous $CD4^+$ responder T cells (R) at a ratio of 1:1. Two times responders (2 x R) was included as a negative control. Suppression mediated by TGF/ATRA-conditioned cells was observed in one donor (*upper panel*), but not in 3 other individuals (*lower panel*). Results were expressed as means ± SEM of triplicate cultures and are representative of 3 experiments (*lower panel* in B). *, $P < 0.05$; ***, $P < 0.001$.

Differential effect of TGF-β and ATRA on naive vs. memory effector T cells in adult human peripheral blood

Given the recent data in mice showing that memory cells dampen the induction of Treg cells from naive cells (38), the inconsistent induction of Treg cells by ATRA and TGF-β prompted us to investigate whether ATRA has differential effects on naive *vs.* memory $CD4^+CD25^-$ T cells. Therefore, naive and memory $CD4^+CD25^-FOXP3^-$ T cells were purified on the basis of surface CD45RA expression as depicted in Figure 2A. These cells were subsequently stimulated in the absence or presence of TGF-β, ATRA, or both. We observed that memory $CD4^+CD25^-CD45RA^-$ T cells are refractory to TGF-β-induced FOXP3 expression, even in the presence of ATRA (Figure 2B, *top panel*). In contrast, although ATRA alone only slightly increases activation-induced FOXP3 expression, together with TGF-β it induces the highest FOXP3 expression in activated naive T cells. This is reflected both in the percentage of $FOXP3^+$ cells as well as in the expression levels per cell as measured by mean fluorescence intensity (MFI) of $FOXP3^+$ cells (Figure 2B, *middle panel*).

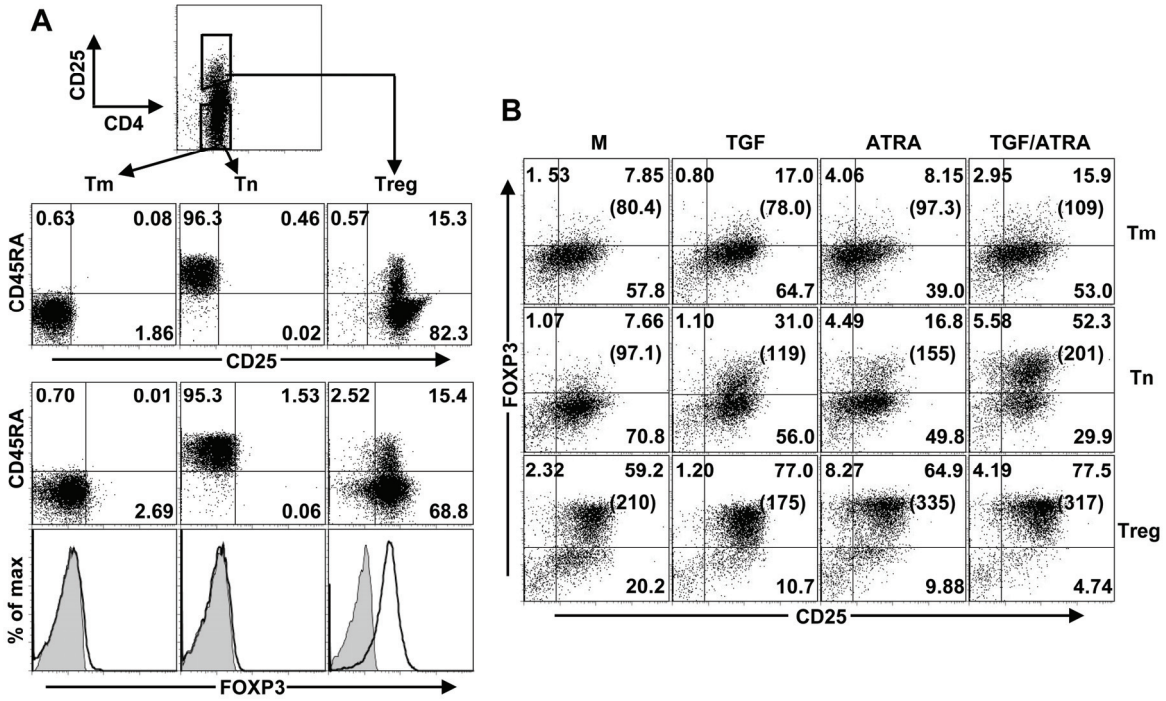


Figure 2. Expression of FOXP3 in FACS-sorted subsets of human $CD4^+$ T cells. A, $CD4^+$ T cells were purified from adult peripheral blood and subsequently stained with anti-$CD4^{Pacific\ Blue}$, anti-$CD25^{PE}$ and anti-$CD45RA^{FITC}$ for 30 min at 4˚C. $CD4^+CD25^-CD45RA^-$ memory effector T cells (Tm) and $CD4^+CD25^-CD45RA^+$ naive effector T cells (Tn) were sorted. $CD4^+CD25^{++}$ Treg cells (top 5%) were sorted on the basis of high CD25 and lower CD4 expression (top box in the *upper panel*). CD25 or FOXP3 *vs.* CD45RA expression on the post-sorted cells is shown in the *middle two panels*. The overlays of FOXP3 (black line) *vs.* corresponding isotype (filled gray) stainings in post-sorted cells are shown in the *bottom panel*. Numbers indicate the percentage of positive cells in each quadrant. **B**, FACS-sorted Tm, Tn or Treg were activated with plate-bound anti-CD3, soluble anti-CD28, and IL-2, in the absence (M) or presence of TGF-β (TGF), ATRA (ATRA), or both (TGF/ATRA) for 5 days. After resting in IL-2 for an additional 4-6 days, cells were collected and processed for analysis of FOXP3/CD25 expression. Numbers in corners indicate the percentage of positive cells in each quadrant, and numbers in brackets represent the MFI of $FOXP3^+$ cells in the upper two quadrants. Results are representative of 6 different donors.

Similar to unfractioned $CD4^+CD25^-$ cells (Figure 1A), both naive and memory $CD4^+CD25^-$ cells cultured with TGF-β and/or ATRA display reduced proliferative potential upon restimulation *in vitro* (data not shown). However, no suppression was

observed by the "conditioned" memory $CD4^+CD25^-CD45RA^-$ T cell population (Figure 3A & B), whereas TGF-β/ATRA-conditioned naive $CD4^+CD25^-CD45RA^+$ peripheral blood T cells displayed a significant suppression in all 6 donors tested (Figure 4). The average percent inhibition of these conditioned naive cells on the proliferation of autologous responder T cells was 55.6% (55.6 ± 9.5%, n=6) at a ratio of 1:1 (Figure 4C & D). Furthermore, addition of ATRA and/or TGF-β did not reduce the yield of cultured $CD4^+CD25^-CD45RA^+$ naive cells after activation and expansion in the presence of exogenous IL-2 (cells cultured in medium, TGF-β, ATRA, and TGF-β/ATRA expanded on average 38.0 ± 7.78, 40.7 ± 5.98, 39.6 ± 5.81, and 41.7 ± 5.46 times, respectively; n = 6). Together, these data indicate that naive $CD4^+CD25^-CD45RA^+$ peripheral blood T cells can be converted into Treg cells efficiently and consistently in the presence of both ATRA and TGF-β, whereas memory $CD4^+CD25^-CD45RA^-$ T cells are resistant to such conversion under these conditions (Figure 2-4).

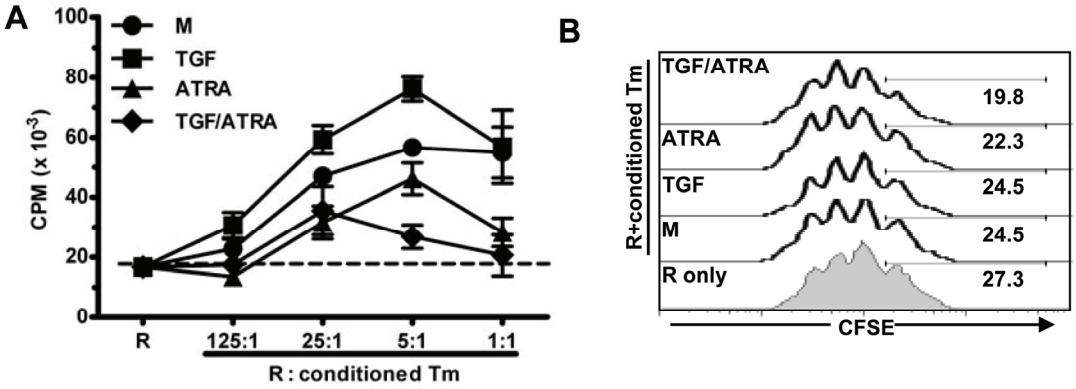


Figure 3. Memory effector T cells can not be converted into suppressors. FACS-purified $CD4^+CD25^-CD45RA^-$ memory effector cells (Tm) were activated with plate-bound anti-CD3, soluble anti-CD28, and IL-2, in the absence (M) or presence of TGF-β (TGF), ATRA (ATRA), or both (TGF/ATRA) for 5 days. These cells were subsequently rested in IL-2 for an additional 4-6 days. To analyze their suppressive abilities, autologous $CD4^+$ responder T cells (R) were activated with PHA and feeders in the absence or presence of conditioned Tm at a ratio of 125:1, 25:1, 5:1, or 1:1 (responders to conditioned Tm). **A,** proliferation after 5 days was determined by ^{3}H-thymidine incorporation. Results are expressed as means ± SEM of triplicate cultures and represent 6 different individuals. The dotted line indicates the CPM value of responder T cells only. **B,** in some parallel experiments, proliferation of CFSE-labeled autologous $CD4^+$ responder T cells (R), cultured alone or in the presence of conditioned Tm at a ratio of 1:1, was analyzed by flow cytometry after 96 h. Numbers indicate the percentage of undivided cells. No inhibition on the division of CFSE-labeled responder cells was observed.

Memory cells inhibit ATRA/TGF-β induced FOXP3 expression in naive cells

The different susceptibility of $CD4^+CD25^-$ T cell populations from different donors to suppression induction (Figure 1) prompted us to investigate whether the presence of memory cells could influence the Treg conversion of naive cells. As adult human peripheral blood $CD4^+CD25^-$ T cells comprise 20-80% $CD45RA^+$ naive T cells, depending on the age and immune status of the individuals, naive $CD4^+CD25^-CD45RA^+$ cells were isolated, labeled with CFSE, and then mixed with memory $CD4^+CD25^-CD45RA^-$ cells at different ratios (1:0, 4:1, 1:1, 1:4, and 0:1). Cells were subsequently stimulated with plate-bound anti-CD3, soluble anti-CD28, and IL-2 in the absence or presence of TGF-β and ATRA. As shown in Figure 5, addition of memory cells dose-dependently inhibited the FOXP3 expression in naive cells, both in the percentage and absolute numbers of FOXP3-expressing cells (Figure 5A-C). It is unlikely that the reduced levels of FOXP3 expression can be attributed to less cell activation / proliferation because 1) there is no significant difference in the CFSE profile of naive cells among different cultures (supplementary

Figure 3A); and 2) the expression of FOXP3 in undivided naive cells is already inhibited by cocultured memory cells (gate M0 in supplementary Figure 3B).

To gain some mechanistic insights underlying this phenomenon, we added neutralizing antibodies to cytokines that have been reported to dampen FOXP3 expression in mice (19, 38, 43-45). Although addition of antibodies, either separately or all together, to cultured naive cells alone does not further enhance TGF-β/ATRA-induced FOXP3 expression, neutralization of IL-4 in cocultures does reverse, albeit partially, the inhibitory effect of memory cells on TGF-β/ATRA-induced FOXP3 in naive cells (Figure 5D). Likewise, higher percentage of FOXP3 induced by anti-IL-4 is associated with more FOXP3-expressing cells as it does not alter the CFSE profile of naive cells in cocultures (data not shown). No effect was observed when TNF-α, IFN-γ, or IL-6 was blocked in cocultures, neither can they augment the effect of anti-IL-4 when used together (Figure 5D). As the blocking abilities of these antibodies were confirmed in other bioassays (data not shown), our results indicate that TNF-α, IFN-γ, and IL-6 are not involved in the regulation of FOXP3 expression in these co-cultures.

In summary, these data indicate that although naive $CD4^+CD25^-CD45RA^+$ T cells in adult peripheral blood can be switched to become Treg cells by ATRA and TGF-β (Figure 4), the presence of "conversion-resistant" memory $CD4^+CD25^-CD45RA^-$ cells within the $CD4^+CD25^-$ cell population not only dilutes the percentage of the induced Treg cells but also actively inhibits the conversion of naive T cells (Figure 3 & 5A-C). This inhibition can be partially reversed by neutralization of IL-4 (Figure 5D).

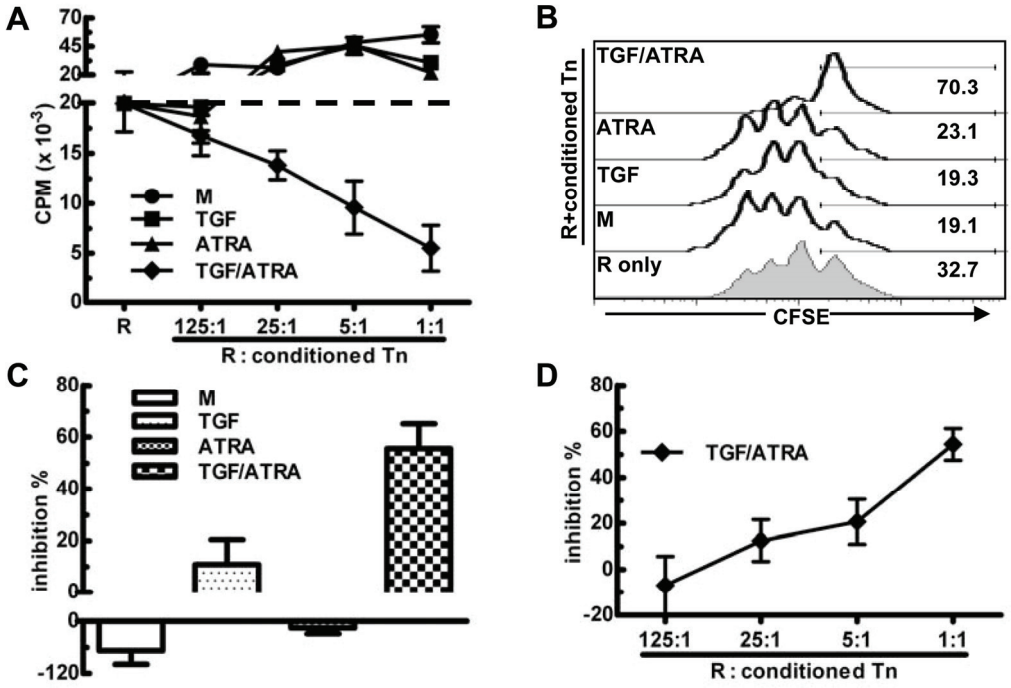


Figure 4. Consistent induction of Treg cells from naive effector T cells by ATRA and TGF-β. $CD4^+CD25^-CD45RA^+$ naive effector cells (Tn) were purified from adult peripheral blood by FACS-sorting as depicted in Figure 2A and cultured as described in the legend to Figure 3 before analyses of their suppressive function. **A,** only cells cultured with both TGF-β and ATRA (TGF/ATRA) dose-dependently inhibited the proliferation of autologous $CD4^+$ responder T cells (R). Results are expressed as means ± SEM of triplicate cultures and represent 6 different individuals. The dotted line indicates the CPM value of responder T cells only. **B**, CFSE profiles of cultured responders on day 4, alone or in the presence of conditioned Tn at a ratio of 1:1. **C & D,** percent inhibition on the proliferation of autologous responder T cells by conditioned Tn at a ratio of 1:1 (**C**) or indicated ratios (**D**). Results were expressed as means ± SEM of 6 different individuals.

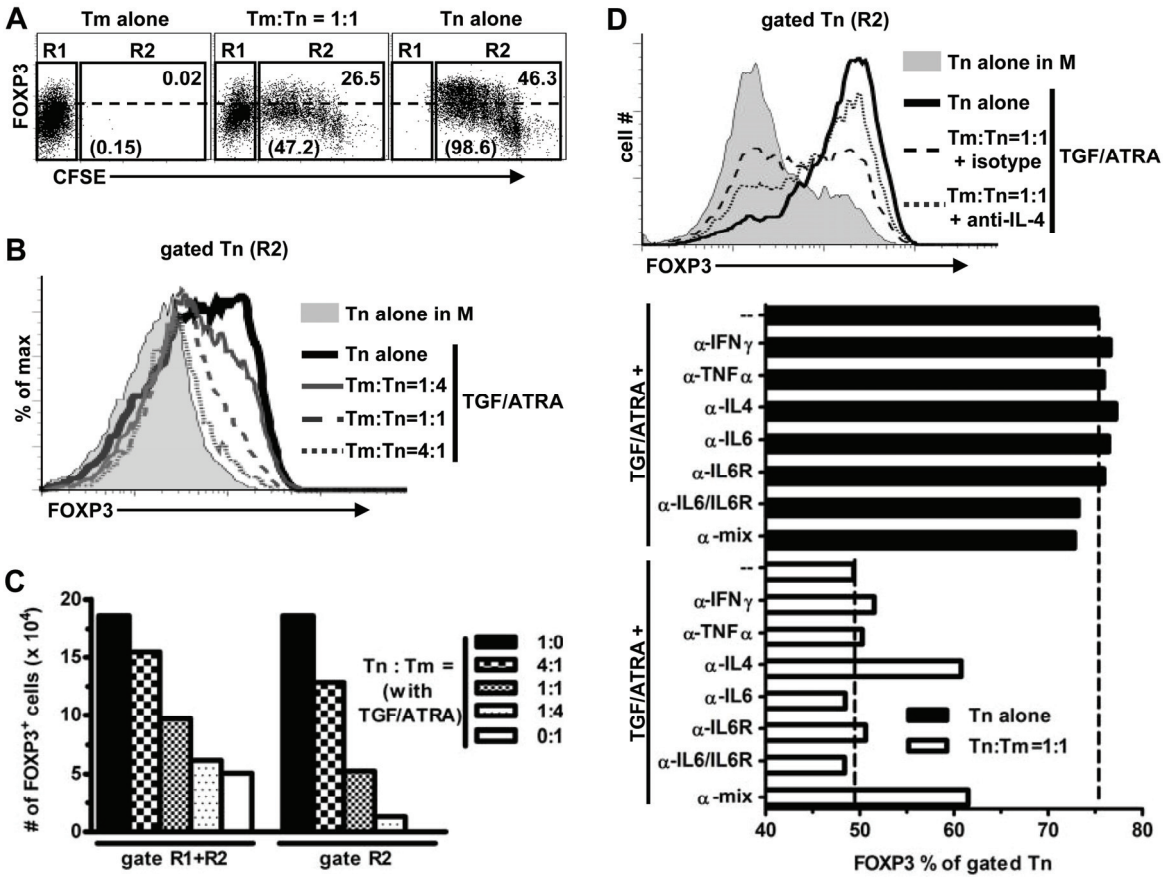


Figure 5. Memory cells inhibit ATRA/TGF-β induced FOXP3 expression in naive cells. $CD4^+CD25^-CD45RA^-$ memory (Tm) and $CD4^+CD25^-CD45RA^+$ naive effector T cells (Tn) were purified from adult peripheral blood by FACS-sorting. Tn were labeled with CFSE, mixed with Tm at different ratios (total 1 x 10^5 cells/condition), and then stimulated with plate-bound anti-CD3, soluble anti-CD28, and IL-2 in the absence (M, filled gray) or presence (TGF/ATRA, open lines) of exogenous TGF-β and ATRA. After 5 days, FOXP3 expression in activated Tm and Tn was analyzed by gating CFSE negative (R1) and positive (R2) cells, respectively. **A**, representative dot plots show CFSE *vs*. FOXP3 staining in Tm alone (*left*), Tm : Tn = 1:1 (*middle*) and Tn alone (*right*). Numbers in the upper right corner indicate the percentage of $FOXP3^+$ cells within gate R2, while numbers in brackets represent the percent of Tn (gate R2). **B**, representative histograms show FOXP3 expression in gated Tn (gate R2) in different cultures. No significant differences were observed in gated Tm cells (gate R1) among different cultures (data not shown). **C,** absolute numbers of $FOXP3^+$ cells in total (gate R1+R2) or derived from Tn (gate R2) in different conditions. **D,** representative histogram (*upper panel*) or bar graph (*lower panel*) shows the expression of FOXP3 in gated Tn cells cultured alone or together with Tm at the ratio of 1 : 1 in the absence or presence of 20 μg/ml of neutralizing Abs. No effect was observed with corresponding isotype Abs (data not shown). "Anti-mix" means the mixture of anti-IFN-γ, TNF-α, IL-4, IL-6, and IL-6R (20 μg/ml of each). Similar results were obtained in three (**A-C**) or two (**D**) different experiments with different donors.

Phenotype and stability of ATRA/TGF-β induced Treg cells

As previously reported (40), ATRA reduces the surface expression of lymphoid-homing receptors CD62L, and to a lesser extent, CCR7, while TGF-β enhances their expressions on cultured T cells (data not shown). Although cells cultured with both ATRA and TGF-β express the highest levels of FOXP3 (Figure 2B), expression of FOXP3 *per se* does not necessarily associate with suppression in human activated cells (11, 12, 26, 28-30). Given that expression of CTLA-4 has been recently reported to correlate with the acquisition of suppressive function in human activated $CD4^+CD25^-$ T cells (41), we compared the expression of CTLA-4 in our cultured cells with or without suppressive function. Naive cells cultured with TGF-β express higher levels of CTLA-4 than those cultured in medium

or ATRA only; however, no significant difference was observed between cells cultured with TGF-β only or with both TGF-β and ATRA (Figure 6A, *upper panel*), albeit that only the latter cells bear suppressive function (Figure 4).

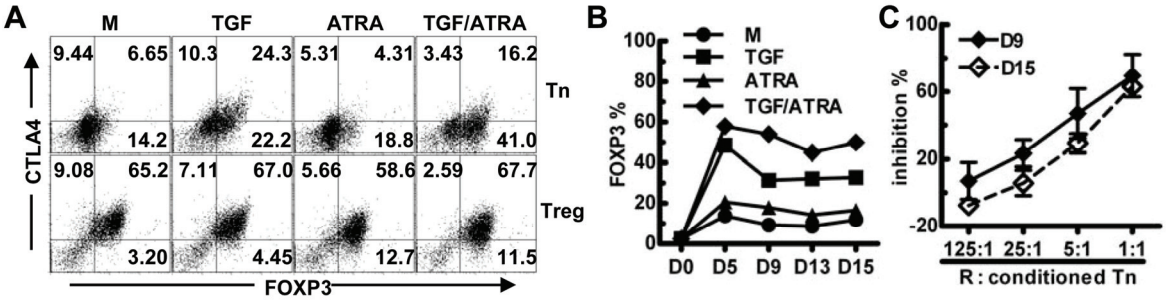


Figure 6. Phenotype and stability of ATRA/TGF-β induced Treg cells. FACS-sorted $CD4^+CD25^-CD45RA^+$ naive (Tn) or $CD4^+CD25^{++}$ regulatory (Treg) cells were stimulated with plate-bound anti-CD3, soluble anti-CD28, and IL-2 in the absence (M) or presence of TGF-β (TGF), ATRA (ATRA), or both (TGF/ATRA) for 5 days, after which cells were rested in medium supplemented with only IL-2. **A**, cells were collected and processed for analysis of CTLA4 (intracellular) *vs.* FOXP3 expression on day 9. Numbers indicate the percentage of positive cells in each quadrant. **B**, line graph shows percent of $FOXP3^+$ cells at indicated days. **C**, graph shows the percent inhibition, expressed as mean ± SEM of triplicate cultures, of TGF/ATRA conditioned Tn cultured for 9 (filled diamond) or 15 (open diamond) days on the proliferation of autologous $CD4^+$ T cells. No suppression was observed by cells conditioned with M, TGF, or ATRA on day 9 and 15 (data not shown). Results are representative of 3 experiments with different individuals.

As a stable phenotype is important for the application of cells in the clinic, we next wished to know whether Treg cells propagated by TGF-β/ATRA from naive T cells bear stable suppressive ability. To this end, we rested the cells for an additional week after the initial ~10 days' culture as described in the legends to Figure 4. Subsequently, FOXP3 expression and suppressive ability were reanalyzed. Our results show that addition of TGF-β/ATRA during the activation of naive cells leads to highest levels of FOXP3 expression, both in the percentage (Figure 6B) and the absolute numbers (supplementary Figure 4A). More importantly, these TGF-β/ATRA conditioned naive cells on day 15 inhibit the proliferation of autologous responder T cells to a comparable extent as those on day 9 (Figure 6C). No inhibition was detected on cells pre-activated with TGF-β or ATRA only (data not shown).

Together, these data indicate that Treg cells, propagated by ATRA and TGF-β from naive $CD4^+CD25^-$ T cells in adult peripheral blood, bear stable FOXP3 expression and suppressive function, at least within the 2-week *in vitro* culture period (Figure 6B & C).

ATRA and/or TGF-β enhance the suppression of cultured $CD4^+CD25^{++}$ T cells

Besides the *de novo* generation of Treg cells from $CD4^+CD25^-$ effector cells *in vitro*, *ex vivo* expansion of $CD4^+CD25^{++}$ Treg cells isolated from peripheral blood, represents another important approach to obtain sufficient numbers of Treg cells suitable for Treg-based immunotherapy. Therefore, we wished to know whether addition of ATRA during culture could enhance the function of expanded $CD4^+CD25^{++}$ T cells. FACS-sorted $CD4^+CD25^{++}$ Treg cells (*right panel* in Figure 2A) were activated in the absence or presence of TGF-β and/or ATRA, after which their FOXP3 expression and suppressive ability were tested. Addition of ATRA and/or TGF-β increased the expression of FOXP3 (*lower panel* in Figure 2B), and more importantly, significantly enhanced the suppressive capacity of cultured $CD4^+CD25^{++}$ Treg cells on day 9 (Figure 7A & B). In keeping with the results obtained from naive cells, this enhanced suppressive capacity was not

associated with elevated CTLA-4 expression (Figure 6A, *lower panel*), and moreover, ATRA alone or together with TGF-β did not reduce the expansion of cultured $CD4^+CD25^{++}$ cells (the fold expansions of cells cultured in medium, TGF-β, ATRA, and TGF-β/ATRA on day 9 are 19.8 ± 5.39, 22.4 ± 5.80, 27.1 ± 6.15, and 29.8 ± 7.91, respectively; n = 8).

Given that isolated Treg cell populations may lose FOXP3 expression and suppressive function during the *in vitro* culture (6, 7, 16), we next investigated whether ATRA could preserve the suppressive ability of *in vitro* expanded $CD4^+CD25^{++}$ T cells. As shown in Figure 7C & D, $CD4^+CD25^{++}$ Treg cell populations, pre-activated with anti-CD3 and anti-CD28 without TGF-β or ATRA, gradually lost FOXP3 expression and suppressive capacity, whereas cells stimulated in the presence of TGF-β and ATRA maintained high levels of FOXP3. The latter was associated with more $FOXP3^+$ cells (supplementary Figure 4B), and, more importantly, stronger suppressive capacity, at least within the 2-week *in vitro* culture analyzed (Figure 7C & D).

On the basis of these data, we conclude that in the presence of TGF-β, ATRA not only consistently induces Treg cells from naive $CD4^+CD25^-CD45RA^+$ peripheral blood T cells with potent and stable suppressive function (Figure 4 & 6) but also enhances/preserves FOXP3 expression and suppressive function of isolated $CD4^+CD25^{++}$ Treg cells during the 2-week *in vitro* culture (Figure 7).

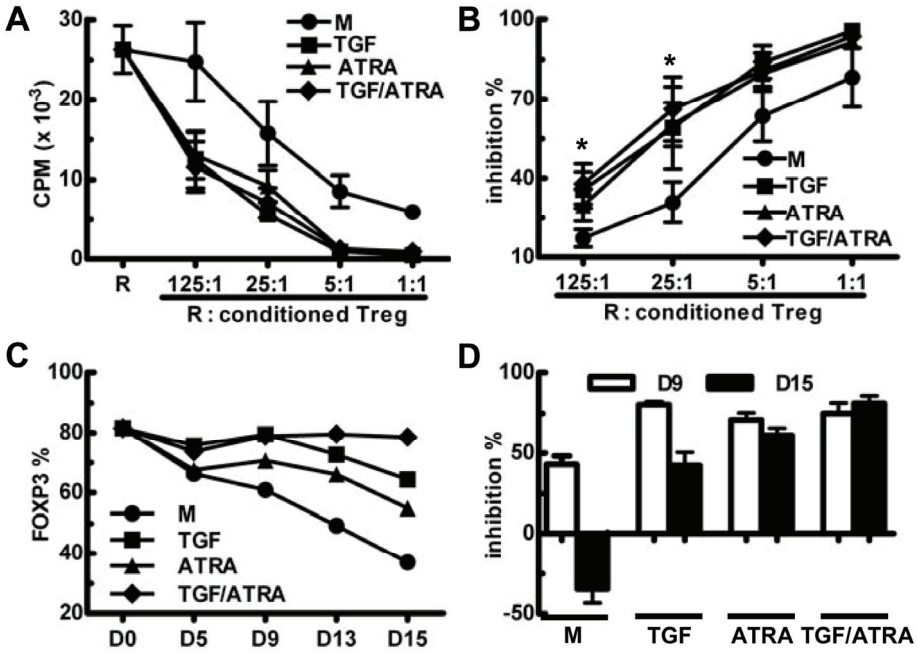


Figure 7. Enhanced suppressive capacity of $CD4^+CD25^{++}$ Treg cells cultured with ATRA and/or TGF-β. $CD4^+CD25^{++}$ Treg cells were purified from adult peripheral blood by FACS-sorting as depicted in Figure 2A. The cells were subsequently activated with plate-bound anti-CD3, soluble anti-CD28 and IL-2, in the absence (M) or presence of TGF-β (TGF), ATRA (ATRA), or both (TGF/ATRA) for 5 days. After resting in IL-2 for additional 4-10 days, cells were collected and processed for the analysis of FOXP3 expression (**C**) or suppressive ability (**A**, **B**, **& D**). **A**, cells cultured with TGF-β and/or ATRA possess increased capacity to inhibit the proliferation of autologous $CD4^+$ responder T cells (R) on day 9. **B**, graph shows the average percent inhibition of differently cultured $CD4^+CD25^{++}$ Treg cells on day 9. Results were expressed as means ± SEM of 8 different individuals. *, significant difference ($P < 0.05$) between cells cultured with TGF, ATRA, and TGF/ATRA *vs.* M. **C**, graph shows percent of $FOXP3^+$ cells at indicated days. **D**, bar graph shows the percent inhibition of conditioned $CD4^+CD25^{++}$ Treg cells cultured for 9 (open bar) and 15 (black bar) days on the proliferation of autologous $CD4^+$ responder T cells at a ratio of 25:1 (responder cells to conditioned Treg). Results are expressed as means ± SEM of triplicate cultures and represent 3 experiments with different donors.

Discussion

Immunoregulatory $CD4^+CD25^+$ Treg cells are of great interest for immunotherapy to prevent transplant rejection and on patients with immune-mediated diseases. Adoptive transfer of $CD4^+CD25^+$ Treg cells has been successfully used in several animal models (1-5). In humans, TGF-β-induced conversion of conventional T cells and the expansion of purified $CD4^+CD25^{++}$ T cells represent two important strategies to generate $FOXP3^+$ T cells suitable for immunotherapy in the clinic (9, 10, 27). However, contradicting data on the suppressive ability of these cells have been reported (6, 7, 30). In this study, we investigated the role of ATRA, a natural metabolite of vitamin A, on the *de novo* generation of Treg cells as well as on the function of *in vitro* expanded $CD4^+CD25^{++}$ T cells in adult peripheral blood. Our results show that in the presence of exogenous TGF-β, ATRA efficiently and consistently induces Treg cells with potent and stable suppressive abilities from naive peripheral blood $CD4^+CD25^-CD45RA^+$ cells. We also show that memory $CD4^+CD25^-CD45RA^-$ cells are not only resistant to FOXP3 induction but also inhibit Treg conversion of naive cells induced by TGF-β and ATRA. Moreover, addition of TGF-β and ATRA during the expansion of isolated $CD4^+CD25^{++}$ T cells not only preserves FOXP3 expression but, more importantly, also enhances their suppressive capacity. Taken together, these data indicate that ATRA is a useful supplement in the generation of large numbers of Treg cells in human settings.

Recently, it was demonstrated that ATRA on itself can induce $FOXP3^+$ cells with regulatory function from human cord blood $CD4^+CD25^-$ cells (40). In contrast, we did not observe suppressive function of ATRA-cultured adult peripheral blood $CD4^+CD25^-$ cells, either unfractionated or separated into naive $CD4^+CD25^-CD45RA^+$ and memory $CD4^+CD25^-CD45RA^-$ T cell fractions (Figure 1, 3 & 4). Moreover, contrary to the report showing no direct effect of ATRA on murine naive T cells (38), we found that human naive cells, only after activation in the presence of both TGF-β and ATRA, dose-dependently inhibited the proliferation of autologous responder T cells (Figure 4). These data are relevant not only because they show that peripheral blood cells from adults can be converted into Treg cells, but also because they suggest that both TGF-β and ATRA are needed to achieve this in a consistent fashion across donors, presumably due to the enhanced Smad3 signaling (37, 39).

In line with the previous reports describing that memory cells are resistant to TGF-β-induced FOXP3 expression (30), we now show that memory T cells do not acquire regulatory function even in the presence of both TGF-β and ATRA (Figure 2B & 3). Moreover, when mixed with naive cells, they dose-dependently inhibited TGF-β and ATRA-induced FOXP3 expression in cocultured naive cells, which can be partially reversed by blocking the action of IL-4 (Figure 5). Therefore, our data indicate that the presence of large numbers of memory cells within the total $CD4^+CD25^-$ T cell population not only dilutes the percentage of converted $FOXP3^+$ cells with regulatory function from naive cells, but also actively inhibits such conversion in naive cells. This could possibly explain the inconsistent results across donors (Figure 1). It would be interesting to study whether the number of memory T cells in peripheral blood, and/or their potential to produce IL-4, could serve as a marker to predict the ability of TGF-β/ATRA to convert total $CD4^+CD25^-$ T cells into Treg cells.

The reason why memory T cells can not be converted by TGF-β and ATRA into Treg cells is currently unknown. Given the fact that Th1/2 lineage transcription factors T-bet/GATA-3 antagonize Foxp3 induction in mice (43), it is conceivable that the precommitted Th1/2 cells present in the isolated memory cell population are resistant to Treg-conversion. Moreover, it has been shown recently in mice that memory cells can

inhibit the conversion of naive cells into Treg cells by producing proinflammatory cytokines, such as IFN-γ and IL-4 (38). In this study, we show that in humans, blocking IL-4, but not IFN-γ/TNF-α/IL-6, partially reverses the inhibitory effect of memory cells on FOXP3 expression in cocultured naive cells (Figure 5). Given that almost all IL-4-producing cells bear a memory phenotype and exogenous IL-4 strongly decreases TGF-β/ATRA-induced FOXP3 expression in human naive cells (data not shown), our data indicate that the inhibitory effect of memory cells is mediated, at least partially, *via* IL-4 (Figure 5D). Indeed, IL-4 has been described to reduce FOXP3 expression through Stat6 activation (43, 45).

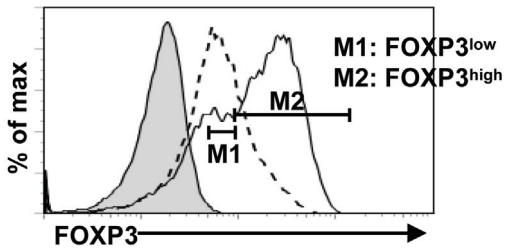
Although expression of FOXP3 itself does not necessarily associate with Treg function in humans (11, 12), it has been reported that ectopic expression of stable and high levels of FOXP3 confers a regulatory phenotype to human $CD4^+CD25^-$ cells, indicating that the levels of FOXP3 determine whether cells acquire Treg function (8, 46). This is in line with the suggestion that the balance between the NFAT-binding partners, FOXP3 and AP-1, determines the function of cells (47). As AP-1 is upregulated during cell activation and promotes the expression of activation-associated genes by cooperation with NFAT, it is conceivable that higher levels of FOXP3 are needed to convert effector cells into Treg cells. This could also contribute to the difficulty to convert memory cells into Treg cells. Our data reveal that ATRA alone only slightly increases activation-induced FOXP3 expression, but it significantly enhances TGF-β-induced FOXP3 expression in human naive peripheral blood $CD4^+CD25^-CD45RA^+$ T cells (Figure 2B). Although this combined effect is still not sufficient to confer memory cells a regulatory phenotype (Figure 2B & 3), it is clearly sufficient to convert naive cells into regulatory cells (Figure 2B & 4).

Besides the *de novo* induction of Treg cells from conventional effector cells, *in vitro* expansion of isolated $CD4^+CD25^{++}$ T cells represents another promising approach to obtain large numbers of Treg cells. However, as a result of the presence of contaminated effector cells, and/or the loss of function of "genuine" Treg cells during expansion, it is difficult to obtain, in a reproducible manner, a population with potent and stable suppressive capacity (6, 7, 16). Moreover, since the Treg function in patients with autoimmunity may already be compromised (18-21), an efficient approach to preserve/enhance the suppressive capacity during the expansion is highly desired. Our data indicate that the addition of TGF-β and ATRA during stimulation prevents the decline of FOXP3 levels without impeding cell expansions (Figure 7C and supplementary Figure 4B), and, more importantly, preserves and enhances their suppressive capacity (Figure 7A, B & D).

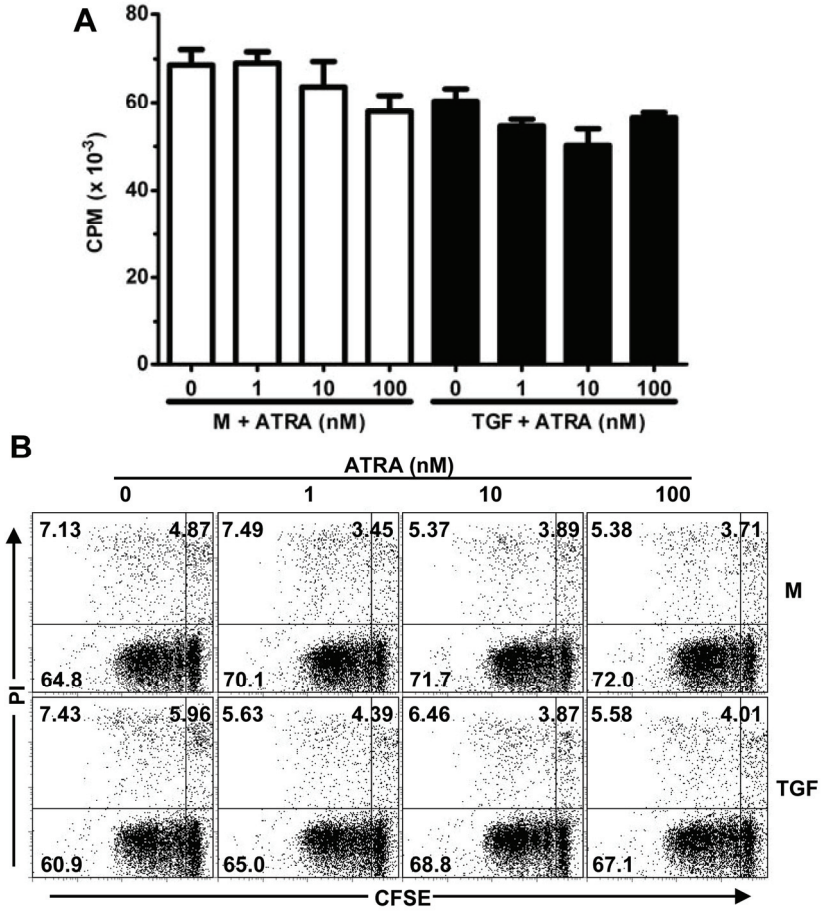
CTLA-4 has recently been shown to control the *in vivo* function of murine $CD4^+Foxp3^+$ Treg cells (48, 49) and to correlate with the acquisition of regulatory function of activated human T cells (41). However, TGF-β/ATRA-cultured naive or $CD4^+CD25^{++}$ T cells do not express higher levels of CTLA-4 as compared with those cultured in other conditions (Figure 6A). As Treg cells induced by ectopic expression of FOXP3 also express low levels of CTLA-4, these results indicate that CTLA-4 is dispensable for the *in vitro* suppressive function of human Treg cells (8).

In conclusion, we have demonstrated that in the presence of TGF-β, ATRA consistently induces the *de novo* generation of $CD4^+FOXP3^+$ Treg cells with potent and stable suppressive function from adult peripheral blood $CD4^+CD25^-CD45RA^+$ naive T cells. Moreover, $CD4^+CD25^-CD45RA^-$ memory cells are not only resistant to Treg conversion but also actively inhibit the induction of Treg cells from cocultured naive cells. The latter can be partially reverted by anti-IL-4. Furthermore, addition of TGF-β and ATRA during the activation of isolated $CD4^+CD25^{++}$ T cells preserves and enhances their repressor capacity during *in vitro* expansion. However, it will be intriguing to know

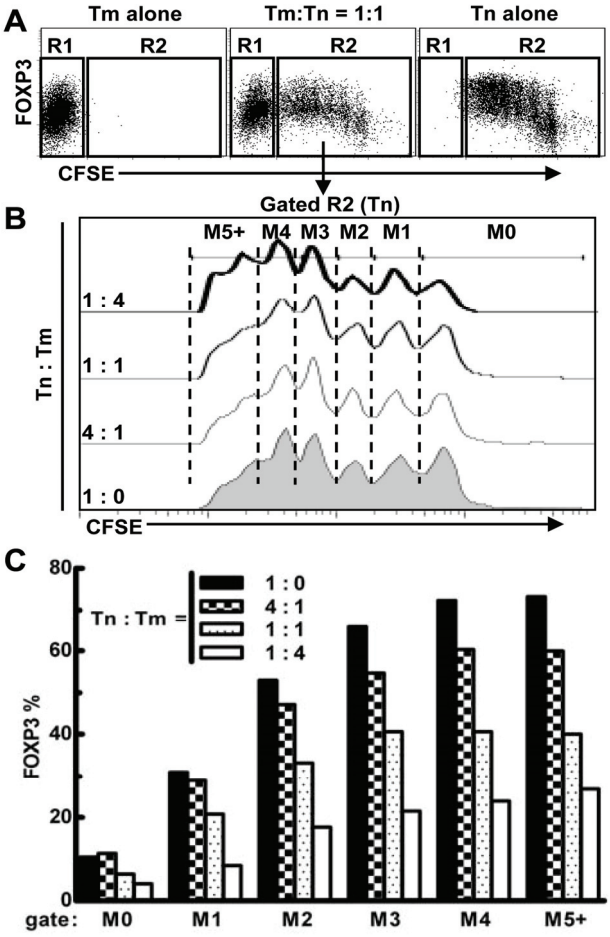
whether such Treg cell populations are effective *in vivo* to combat unwanted immune reactions such as in patients with transplant rejection and autoimmunity.



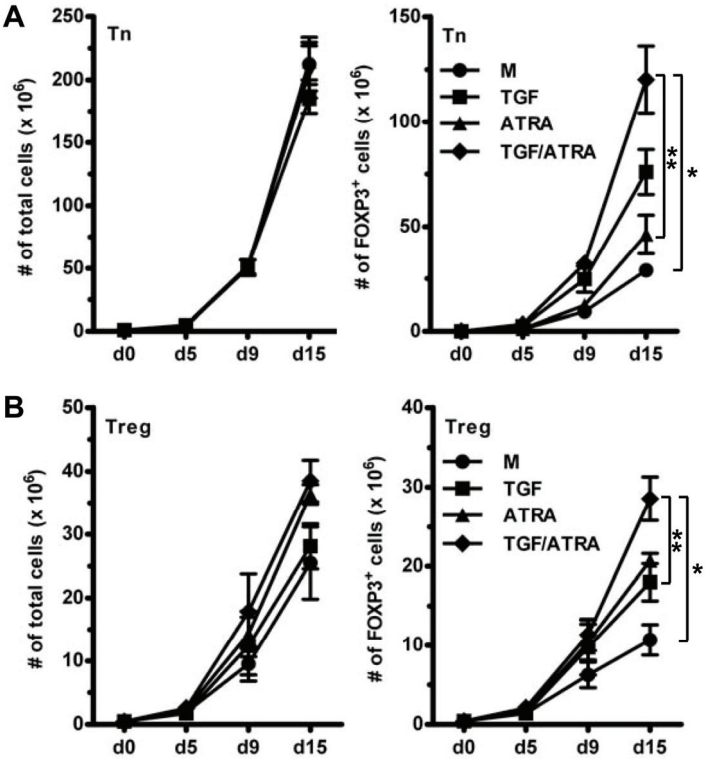
Supplementary Figure 1. Expression of FOXP3 in human activated effector cells. FACS-isolated $CD4^+CD25^-CD45RA^+$ naive cells were stimulated with plate-bound anti-CD3, soluble anti-CD28, and IL-2, in the absence (dotted line) or presence of TGF-β (solid line) for 5 days. The expression of FOXP3 was analyzed by staining the cells with anti-FOXP3 (PCH101) and the corresponding isotype control Abs (filled gray). M1 and M2 indicate the $FOXP3^{low}$ and $FOXP3^{high}$ cells, respectively. Although identical results were obtained with other anti-FOXP3 Abs (236A/E7, data not shown), only $FOXP3^{high}$ cells were designated as $FOXP3^+$ cells in this study as it has been debated whether these $FOXP3^{low}$ cells are genuine $FOXP3^+$ cells.



Supplementary Figure 2. No effect of ATRA on the proliferation/survival of human $CD4^+$ T cells in primary culture. $CD4^+$ T cells, purified by Dynal-beads from adult human peripheral blood, were labeled with CFSE and activated with plate-bound anti-CD3, soluble anti-CD28, and different concentrations of ATRA in the absence (M) or presence of 5 ng/ml TGF-β (TGF). **A**, cell proliferation was determined by ^{3}H-thymidine incorporation 5 days later. Results were expressed as means ± SEM of triplicate cultures. **B**, proliferation and viability were detected based on CFSE dilution and propidium iodide (PI) staining by FACS. Numbers indicated the percentage of cells in each quadrant. One out of two experiments is shown.



Supplementary Figure 3. Memory cells inhibit ATRA/TGF-β induced FOXP3 conversion in cocultured naive cells. $CD4^+CD25^-CD45RA^-$ memory (Tm) and $CD4^+CD25^-CD45RA^+$ naive effector T cells (Tn) were purified from adult peripheral blood by FACS-sorting. Tn were labeled with CFSE, mixed with Tm at different ratios, and then stimulated with plate-bound anti-CD3, soluble anti-CD28, and IL-2, in the presence of exogenous TGF-β and ATRA. FOXP3 expression in activated Tn was analyzed by gating CFSE positive cells on day 5 (R2). **A,** representative dot plots show CFSE *vs.* FOXP3 staining in cultured cells. **B,** histograms show CFSE profiles of gated Tn (gate R2 in **A**) in different culture conditions as depicted. M0 ~ M5+ indicates daughter generations of divided Tn. No effect of Tm on the division of Tn was observed. **C,** percent FOXP3 expression in gated daughter generations of differently cultured Tn. Similar results were obtained in three different experiments with different donors.



Supplementary Figure 4. Addition of TGF-β and ATRA results in more $FOXP3^+$ cells. FACS-sorted $CD4^+CD25^-CD45RA^+$ naive effector T cells (Tn, 1 x 10^6 cells/condition) or $CD4^+CD25^{++}$ Treg cells (Treg, 5 x 10^5 cells/condition) were stimulated with plate-bound anti-CD3, soluble anti-CD28, and IL-2, in the absence (M) or presence of TGF-β (TGF), ATRA (ATRA), or both (TGF/ATRA) for 5 days. Hereafter the cells were rested in medium supplemented with IL-2 only. Cells were collected and counted at indicated times. Absolute numbers of total cells (*left panel*) and $FOXP3^+$ cells (*right panel*) were shown for cultured Tn (**A**) and Treg (**B**). No significant difference in cell viability (by trypan blue exclusion) was observed (data not shown). Results were expressed as means ± SEM of 3 different individuals. *, $P < 0.05$; **, $P < 0.01$.

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Abbreviations

Treg, T regulatory; **ATRA**, all-*trans* retinoic acid.

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