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

Wang, J.

CHAPTER 5

Neutralization of IL-4 reverses the nonresponsiveness of $CD4^+$ T cells to regulatory T cell induction in non-responder mouse strains

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Abstract

It is well established that naive cells can be converted by TGF-β into $CD4^+CD25^+$ regulatory T (Treg) cells with therapeutic potentials. Likewise, it is shown that all-*trans* retinoic acid (ATRA) can greatly enhance TGF-β-induced Treg conversion, a phenomenon which has mainly been studied in C57BL/6 mice. Here we show that, although purified naive cells are highly susceptible to Treg generation, total $CD4^+$ T cell populations from different mouse strains display significantly different sensitivities to TGF-β/ATRA-induced Treg conversion. The resistance of "non-responder" strains is associated with an enhanced production of IL-4 by memory T cells as well as an increased sensitivity of naive T cells to the action of IL-4. Importantly, neutralization of IL-4 overcomes the differences, thereby enabling TGF-β/ATRA to generate large numbers of functional Treg cells from total $CD4^+$ T cells in a consistent manner across different mouse strains. Likewise, blockade of IL-4 significantly enhances TGF-β/ATRA induced Treg conversion from human naive T cells in the presence of memory cells. These results show that the inherent resistance of "non-responder" mouse strains to Treg conversion induced by TGF-β and ATRA can be reverted by neutralization of IL-4 and that inhibiting the action of IL-4 is beneficial or even necessary for consistent inducible Treg generation.

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Introduction

$CD4^+CD25^+$ regulatory T (Treg) cells are essential in maintaining tolerance to self antigens and preventing exuberant immune response to foreign antigens by dampening the function of many immune cells. Therefore, manipulating the number and/or function of Treg cells provides an attractive therapeutic approach to treat patients with immunological disorders. Indeed, adoptive transfer of $CD4^+CD25^+$ Treg cells has been successfully used in several animal models of graft rejection, inflammation and autoimmune diseases (Mottet *et al.*, 2003;Tang *et al.*, 2004;Morgan *et al.*, 2005;Golshayan *et al.*, 2007;van Mierlo *et al.*, 2008).

"Naturally occurring" Treg cells are generated in the thymus during T cell development (Fontenot *et al.*, 2003), while "adaptive/induced" Treg cells are thought to be generated in the periphery by the differentiation of $CD4^+Foxp3^-$ T cells. This can, for example, occur under conditions in which T cells are stimulated continuously by low dose antigen or in the presence of TGF-β (Apostolou and von Boehmer, 2004;Turner *et al.*, 2009;Selvaraj and Geiger, 2007). Recently, it has been shown that a specialized subset of dendritic cells in the gut contributes to the homeostasis of the intestinal immune system by promoting the generation of $Foxp3^+$ Treg cells *via* retinoic acid, the metabolite of vitamin A (Coombes *et al.*, 2007;Sun *et al.*, 2007;von Boehmer, 2007;Wang and Toes, 2008). *In vitro*, all-*trans* retinoic acid (ATRA) also greatly enhances TGF-β-induced conversion of naive effector cells into Treg cells (Mucida *et al.*, 2007;Schambach *et al.*, 2007;Benson *et al.*, 2007;Takaki *et al.*, 2008;Elias *et al.*, 2008;Xiao *et al.*, 2008a;Hill *et al.*, 2008;Nolting *et al.*, 2009;Kang *et al.*, 2009;Kang *et al.*, 2007). Importantly, these converted Treg cells are effective to suppress the pathogenic functions of effector T cells and to maintain mucosal integrity *in vivo* (Coombes *et al.*, 2007;Mucida *et al.*, 2007;Sun *et al.*, 2007;Kang *et al.*, 2009).

Notably, most of the studies performed in this area used C57BL/6 (B6) mice (Benson *et al.*, 2007;Elias *et al.*, 2008;Hill *et al.*, 2008;Mucida *et al.*, 2007;Nolting *et al.*, 2009;Sun *et al.*, 2007;Takaki *et al.*, 2008;Xiao *et al.*, 2008a). Since immune responses can vary substantially among hosts with different genetic backgrounds, we sought to investigate whether the effect of TGF-β and ATRA on the generation of Treg cells is consistently found in three commonly used mouse strains. Moreover, we wished to determine, in case differences are present, the underlying mechanism(s) responsible and whether these differences could be overcome by manipulation of the responsible pathway(s).

Our data show that, in contrast to T cells from B6 mice, DBA $CD4^+$ T cells are resistant to TGF-β/ATRA-induced Treg conversion. This is attributable to high levels of IL-4 produced by memory cells, as purified naive T cells are susceptible to Treg-conversion and neutralization of IL-4 during activation reverses this nonresponsiveness. This also allows the generation of high numbers of functional $Foxp3^+$ Treg cells from total $CD4^+$ T cell populations. Likewise, blockade of IL-4 largely reverses the inhibitory effect of memory cells and thereby greatly enhances Treg conversions in human naive T cells. Moreover, naive T cells from DBA mice, as compared to their B6 counterparts, are more sensitive to the inhibitory effect of IL-4 on TGF-β/ATRA-induced Foxp3 expression.

These results are relevant for the development of Treg-based immunotherapies, as they indicate that neutralization of IL-4 could facilitate, and even be necessary for inducible Treg generation in Th2-dominant diseases and/or IL-4 hypersensitive individuals.

Materials and Methods:

Mice. Female DBA/1J (DBA), C57BL/6 (B6) and BALB/c mice were obtained from Charles River Laboratory and housed in the animal facility of the Leiden University Medical Center and used at 6-12 wks of age. All experiments were performed in accordance with national legislation under the supervision of the University's animal experimental committee.

Culture medium. Cells were cultured in complete medium consisting of IMDM (Cambrex) supplemented with 10% heat-inactivated fetal calf serum (FCS, BD biosciences, the same batch in all experiments), 100 U/ml penicillin, 100 μg/ml streptomycin (Cambrex), 2 mM GlutaMAX and 20 μm β-mercaptoethanol (Invitrogen).

Cell isolation. Murine $CD4^+$ T cells were positively isolated from spleens by using Dynalbeads FlowComp Mouse CD4 isolation kit (Invitrogen). For FACS-sorting, purified $CD4^+$ T cells (pooled from 5-6 mice) were labeled with anti-$CD4^{Percp\text{-}cy5.5}$, anti-$CD25^{allophycocyanin}$, anti-$CD62L^{PE}$ and anti-$CD44^{FITC}$ for 30 min at 4°C. After washing, $CD4^+$, $CD4^+CD25^-CD62L^{high}CD44^{low}$ naive (Tn), $CD4^+CD25^-CD62L^{low}CD44^{high}$ memory (Tm) and $CD4^+CD25^+$ Treg cells were sorted *via* a FACS Aria cell sorter (BD Biosciences) with greater than 98% purity. Human peripheral blood was obtained from healthy blood bank donors after informed consent in accordance with procedures approved by the local human ethics committee. Human $CD4^+CD25^-CD45RA^+$ naive and $CD4^+CD25^-CD45RA^-$ memory effector cells were purified by FACS with greater than 95% purity as described previously (Wang *et al.*, 2009). The analysis and sorting gates were restricted to the small lymphocyte gate as determined by their characteristic forward and side scatter properties.

Induction of Treg cells in vitro. Purified murine total $CD4^+$, Tn or Tm cells were activated with 2 μg/ml plate-bound anti-CD3 (145-2C11), 0.1 μg/ml soluble anti-CD28 (37.51, both from BD Biosciences), and 50 IU/ml recombinant human IL-2 (PeproTech) in the absence/presence of 5 ng/ml recombinant human TGF-β (PeproTech), and/or 10 nM ATRA (Sigma) for 3 to 4 days. Human T cells were stimulated by 5 μg/ml plate-bound anti-CD3 (OKT-3, BD Biosciences) and 1 μg/ml soluble anti-CD28 (CLB-CD28/1, Sanquin) for 5 days as previously described (Wang *et al.*, 2009). TNF-α blockers (Etanercept, Wyeth) and neutralizing antibodies to murine/human IL-4 (11B11/MP4-25D2), IL-6 (MP5-20F3/MQ2-13A5), IFN-γ (R4-6A2/NIB42) were added at the final concentration of 10 μg/ml or otherwise indicated. Neutralizing abilities of these antagonists were confirmed by relevant bioassays (data not shown). After extensive washing, activated T cells were rested in medium supplemented with IL-2 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 with eBioscience intracellular staining buffer set, cells were incubated with allophycocyanin/PE-coupled antibody against murine (FJK-16s) or human (236A/E7, both from eBioscience) Foxp3. Cells were processed by a FACSCalibur and data were analyzed by CellQuest Pro software (BD Biosciences).

Cytokine detection. Cytokine levels in the supernatants collected were determined by ELISA.

Suppression assay. 5 x 10^4 $CD4^+$ responder T cells were plated in 96-well plates with/without titrated amounts of differently pre-conditioned total $CD4^+$ or Tn cells. Cells were activated with "Mouse T-Activator CD3/CD28" Dynalbeads (Invitrogen) at a ratio of 2 : 1 (cells : beads), and pulsed with ^{3}H-thymidine (0.5 μCi/well) on day 3. Proliferation was assessed 18 hours later using a liquid scintillation counter. In some cases, responder T cells were CFSE-labeled and cell proliferation was measured by FACS on day 4.

Statistical analysis. The Mann-Whitney U 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

Low induction of $Foxp3^+$ Treg cells by TGF-β/ATRA in $CD4^+$ T cell cultures from DBA mice

Recently, several groups have shown that ATRA can enhance TGF-β-induced $Foxp3^+$ Treg generation in mice (Mucida *et al.*, 2007;Coombes *et al.*, 2007;Benson *et al.*, 2007;Sun *et al.*, 2007;von Boehmer, 2007;Xiao *et al.*, 2008a;Elias *et al.*, 2008;Hill *et al.*, 2008;Nolting *et al.*, 2009). However, since most of the studies were performed with purified $CD4^+CD25^-$ cells on a B6 background, we sought to investigate whether TGF-β and ATRA could also be used to generate large numbers of Treg cells in two other commonly used mouse strains. To this end, we isolated total $CD4^+$ T cell populations from B6 and DBA mice, and subsequently exposed them to TGF-β and ATRA. 40% of cells from B6 mice were Foxp3 positive after a 4-day culture of total $CD4^+$ T cells with TGF-β only. This is further increased to 60% when ATRA was used simultaneously (Figure 1A, *middle panel*). In contrast, although there's no significant difference in the percentages of $Foxp3^+$ cells in the starting population, very few Foxp3-expressing cells were detected after 4 days' culture of DBA $CD4^+$ T cells with TGF-β. Likewise, the presence of ATRA did not further increase/induce the frequency of Foxp3-expressing cells (Figure 1A, *upper panel*). Similar results were observed with BALB/c $CD4^+$ T cells (Figure 1A, *bottom panel*).

To further study this intriguing observation, we next analyzed the expression of Foxp3 at different time points after activation. As shown in Figure 1B, the percentage of $Foxp3^+$ cells was rapidly increased in all cells 1 day after activation with TGF-β, and was further increased in the presence of ATRA. However, unlike B6 cells in which the percentage of Foxp3-expressing cells was continuously increased till day 3 and maintained at high levels on day 4 after stimulation, the frequency of $Foxp3^+$ cells in DBA and BALB/c cells peaked on day 2 and dramatically decreased thereafter (Figure 1B). As no significant differences in CFSE profiles and cell viabilities (determined by trypan blue exclusion) were observed among mouse strains during stimulation (data not shown), it is unlikely that the reduced generation of $Foxp3^+$ cells induced by TGF-β and ATRA is explained by the preferential expansion of $Foxp3^-$ effector and/or death of induced-$Foxp3^+$ cells in DBA and BALB/c $CD4^+$ cells. Moreover, low frequency of $Foxp3^+$ cells on day 4 was observed over a broad concentration range of exogenously added TGF-β (i.e., 0.1-20 ng/ml, data not shown). Together, these data suggest that $CD4^+$ T cells in DBA and BALB/c mice are less susceptible, compared to their B6 counterparts, to TGF-β/ATRA induced Foxp3 expression.

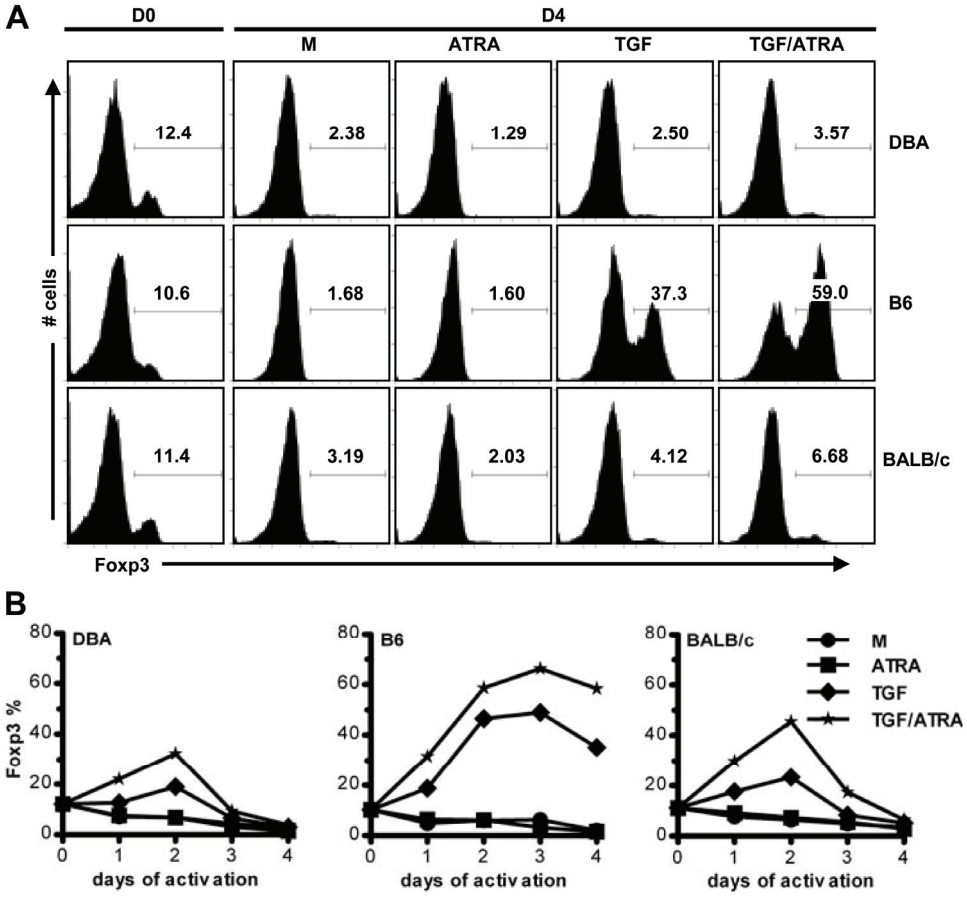

Figure 1. Low induction of Foxp3$^+$ cells by TGF-β/ATRA from DBA CD4$^+$ T cells. Murine CD4$^+$ T cells, purified from splenocytes, were activated with plate-bound anti-CD3, soluble anti-CD28 and IL-2, in the absence (M) or presence of ATRA (ATRA), TGF-β (TGF), or both (TGF/ATRA). Cells were collected to analyze Foxp3 expression by FACS at indicated times. **A**, representative histograms show Foxp3 expression in cells before (D0) or after 4 days' culture (D4). Numbers indicate the percentage of positive cells. **B**, line graphs show the dynamic of Foxp3 expression in DBA (*left panel*), B6 (*middle panel*) and BALB/c (*right panel*) cells in respective cultures. Results are representative for 4 (DBA & B6) or 2 (BALB/c) different experiments.

DBA CD4$^+$ cells inhibit TGF-β/ATRA-induced Foxp3 expression via soluble factors

Given the sharp decrease in the percentage of Foxp3-expressing cells between day 2 and day 3 in cell cultures from DBA mice (Figure 1B), we hypothesized the presence of a dominant negative-loop that reduces Foxp3 expression in an autocrine and/or paracrine manner. Therefore, we cultured B6 CD4$^+$ cells with TGF-β/ATRA in the absence/presence of DBA cells to analyze whether the latter cells can inhibit Foxp3 expression in the B6 population. Surface expression of H2-Kb was used to distinguish cells derived from these strains in the cocultures. Our data show that the percentage of Foxp3-expressing B6 cells on day 3 was dramatically decreased in the presence of cocultured DBA cells (16.4 % *vs.* 68.3%, Figure 2A). In line with previous results, this decrease became apparent mainly between day 2 and day 3, although the percentage of Foxp3$^+$ cells was already lower on day 2 (Figure 1B & Figure 2B). Moreover, the unaltered ratio of B6 to DBA cell numbers (1:1) in cocultures (Figure 2A, *middle panel*) indicates that cells from both strains respond in a comparable manner to the stimuli given. Thus, it is unlikely that different responses to stimulation or growth factors are responsible for the observed different levels of Treg conversion between DBA and B6 mice (Figure 1 & 2A).

Next, we wished to determine whether the inhibitory effect on Foxp3 expression was mediated by soluble factors. To this end, we transferred the supernatants from activated DBA or B6 $CD4^+$ T cells into B6 cell cultures. As shown in Figure 2C, addition of culture supernatants of activated DBA-, but not B6- cells strongly reduced the percentage of TGF-β/ATRA-induced Foxp3-expressing cells in B6 cell cultures. Notably, addition of DBA supernatants collected 2 days after activation already results in a potent reduction in the percentage of $Foxp3^+$ cells, albeit to a slightly less extent than supernatants collected on day 3 (Figure 2C).

Together, these data indicate that soluble factors produced by DBA $CD4^+$ cells during activation contribute to the low percentage of Foxp3-expressing cells induced by TGF-β and ATRA (Figure 1 & 2).

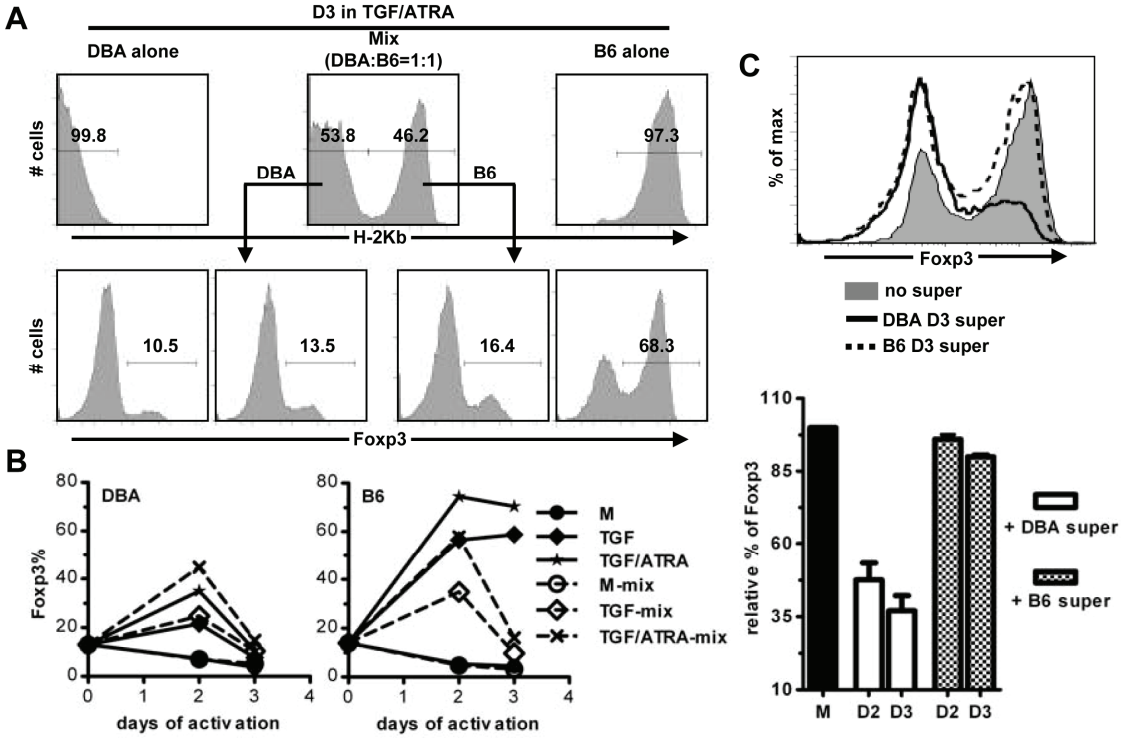

Figure 2. DBA $CD4^+$ T cells inhibit TGF-β/ATRA-induced Foxp3 expression in B6 cells *via* soluble factors. Isolated $CD4^+$ T cells from DBA or B6 mice, either separately or mixed at a ratio of 1:1 (mix), were activated as described in the legends to Figure 1. Cells were surface stained with anti-H-2Kb before intracellular Foxp3 staining. Foxp3 expression in DBA/B6 cells in cocultures was analyzed by gating H-2Kb negative/positive cells, respectively (**A & B**). **A**, representative histograms show Foxp3 expression on day 3 in DBA/B6 cells cultured in the presence of TGF-β and ATRA (TGF/ARTA). **B**, line graphs show the dynamics of Foxp3 expression in DBA (*left panel*) or B6 (*right panel*) cells in time as depicted. Cells were cultured either separately (solid lines) or mixed together (dotted lines) in the absence (M) /presence of TGF-β (TGF) or both TGF-β and ATRA (TGF/ATRA). **C**, $CD4^+$ T cells from B6 mice were activated with TGF-β and ATRA for 3 days in the absence or presence of supernatants from DBA or B6 $CD4^+$ cells stimulated for 2 (D2) or 3 (D3) days with plate-bound anti-CD3, soluble anti-CD28 and IL-2. Bar graph in the *lower panel* shows the relative percent of Foxp3 expression among different culture conditions in two different experiments. Percent of Foxp3 in cells cultured without transferred supernatant (M) was set as 100%. Results were representative of 4 (**A & B**) or 2 (**C**) independent experiments.

Neutralization of IL-4 generates functional $Foxp3^+$ Treg cells from DBA $CD4^+$ cells in the presence of TGF-β/ATRA

In B6 mice, a prominent role of IL-4, among other cytokines, has been implicated in the inhibition of TGF-β-induced Foxp3 expression (Takaki *et al.*, 2008;Hill *et al.*, 2008;Dardalhon *et al.*, 2008;Nolting *et al.*, 2009;Prochazkova *et al.*, 2009;Wei *et al.*,

2007;Mantel *et al.*, 2007). Therefore, we next investigated whether a similar pathway would be involved in other mouse strains as well. Our data show that IL-4 is the main factor that inhibits TGF-β/ATRA-induced Foxp3 in both DBA and BALB/c cells as addition of anti-IL-4 antibody almost completely abrogated the inhibitory effect of DBA supernatants and resulted in a high percentage of Foxp3$^+$ cells in total CD4$^+$ T cell culture from both mouse strains (Figure 3A & B). TNF-α, IFN-γ and IL-6 have also been implicated in the inhibition of Treg-conversion (Dominitzki *et al.*, 2007;Hill *et al.*, 2008;Valencia *et al.*, 2006;Xiao *et al.*, 2008b). However, no significant effect was observed when anti-TNF-α, anti-IFN-γ and anti-IL-6 antibodies were used separately, although they slightly enhanced the percentage of Foxp3$^+$ cells when used together with anti-IL-4 in DBA mice (Figure 3B). Moreover, depletion of CD25$^+$ T cells from total DBA CD4$^+$ T cells did not reduce the percentage of Foxp3-expressing cells after culture with TGF-β/ATRA in the absence/presence of anti-IL-4 (data not shown). These data indicate that the majority of, if not all, Foxp3$^+$ cells present on day 3 are derived from CD25$^-$Foxp3$^-$ T cells that have been converted into Treg cells by TGF-β/ATRA.

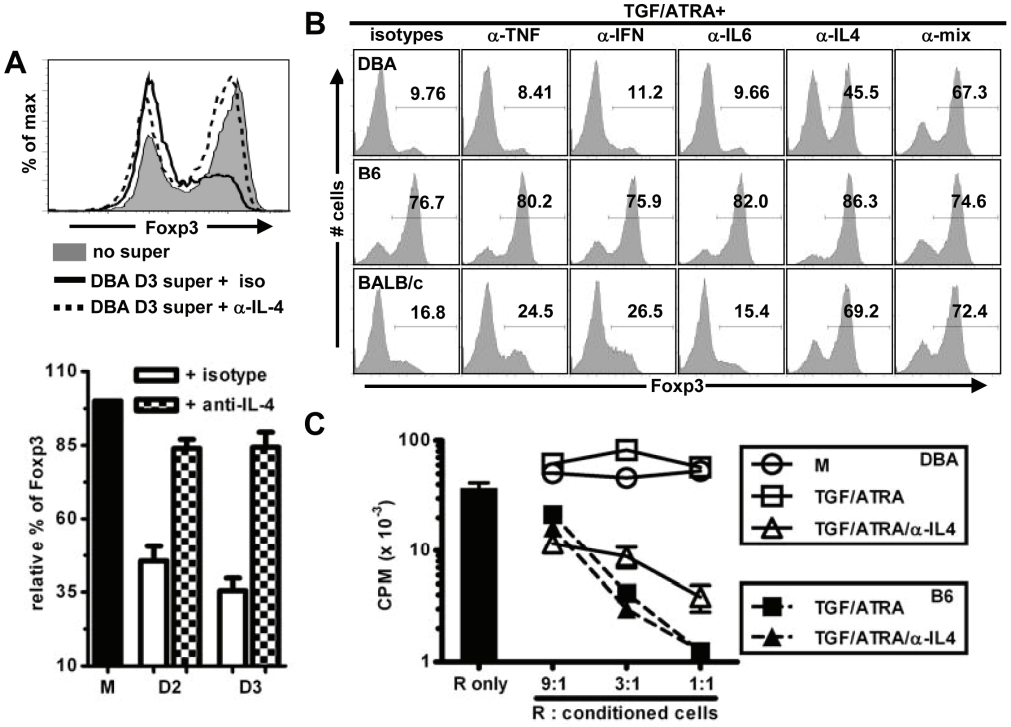

Figure 3. Neutralization of IL-4 enables TGF-β/ATRA to generate functional Foxp3$^+$ Treg cells from DBA CD4$^+$ cells. A, CD4$^+$ T cells from B6 mice were cultured as described in the legend to Figure 2C. Anti-IL-4 or a corresponding isotype control Ab was added to the culture as indicated. Foxp3 expression was analyzed on day 3. Bar graph in the *lower panel* shows the relative percent of Foxp3 expression among different culture conditions in two different experiments. Percent of Foxp3 in cells cultured without transferred supernatant (M) was set as 100%. **B & C**, CD4$^+$ T cells from indicated mouse strains were activated as described in the legends to Figure 1 in the absence (M) / presence of TGF-β and ATRA (TGF/ATRA) plus/minus antagonists to TNF-α, IFN-γ, IL-6, IL-4 or corresponding isotype controls. "Anti-mix" indicates a mix of anti-IFN-γ, TNF-α, IL-4, and IL-6 antibodies. Foxp3 expression was measured on day 3 (**B**). To analyze their suppressive abilities, responder CD4$^+$ T cells (R) from B6 (**C**) mice were activated with "mouse T-activator CD3/28" Dynalbeads (cells/beads = 2/1) in the absence or presence of titrated amounts of conditioned CD4$^+$ T cells from B6 (closed symbols) or DBA (open symbols) mice. Proliferation after 4 days was determined by ^{3}H-thymidine incorporation. DBA CD4$^+$ cells, only preconditioned with TGF/ATRA in the presence of anti-IL-4, inhibited the proliferation of responder cells. In contrast, anti-IL-4 is dispensable for TGF/ATRA to induce functional Treg cells from B6 CD4$^+$ cells (**C**). Results were expressed as means ± SEM of triplicate cultures and represent 2 (**A**), 3 (**C**) or 4 (**B**) independent experiments.

Consistent with the levels of Foxp3 expression, DBA $CD4^+$ cells, only pre-conditioned with TGF-β/ATRA in the presence of anti-IL-4, demonstrated potent suppressive capacity on the proliferation of responder cells isolated from B6 (Figure 3C) or DBA mice (data not shown). In contrast, anti-IL-4 treatment is dispensable for TGF-β/ATRA to induce functional $Foxp3^+$ Treg cells from total B6 $CD4^+$ cells (Figure 3B & C). Collectively, these data show that IL-4 is the dominant cytokine inhibiting Foxp3 induction upon TGF-β/ATRA exposure in the genetic backgrounds analyzed.

Memory $CD4^+$ T cells from DBA mice produce higher levels of IL-4 upon activation

The data so far indicate that IL-4 present in the DBA and BALB/c cell cultures inhibits TGF-β/ATRA-induced Treg conversions, as neutralization of IL-4 during activation reverses this nonresponsiveness (Figure 1-3). Moreover, given the intrinsic genetic differences among the strains analyzed, the data also indicate that IL-4 is the principle cytokine in mice responsible for the effects observed and that these strains differ in IL-4 production/sensitivity. To analyze whether differences in IL-4 production, sensitivity or both was at the basis of the inhibition of TGF-β/ATRA-induced Treg conversion, we first compared the IL-4 levels produced by T cells from DBA and B6 mice. As expected, DBA $CD4^+$ cells produce high levels of IL-4 upon activation, which is reduced by the addition of TGF-β or TGF-β and ATRA. In contrast, little amounts of IL-4 were secreted by activated $CD4^+$ T cells from B6 mice (Figure 4B, *upper panel*). It is unlikely that different IL-4 levels in cultures with TGF-β between DBA and B6 cells can be attributed to their different sensitivities to the action of TGF-β, as addition of TGF-β reduces the IL-4 production to a similar extent and cells from both strains express comparable levels of TGF-β receptor II (Figure 4B and data not shown).

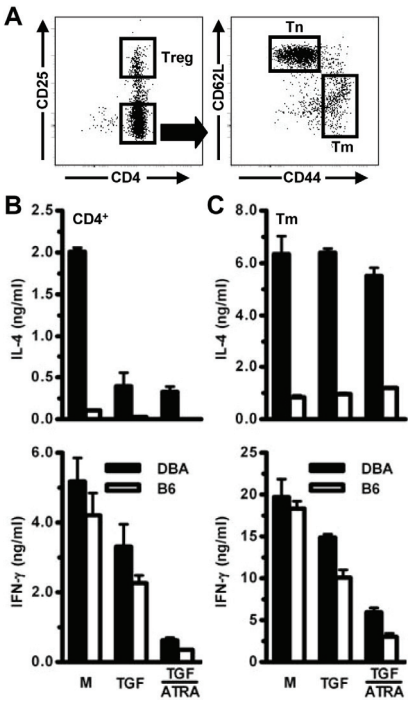

Figure 4. DBA memory cells produce higher amounts of IL-4 upon activation. $CD4^+$ T cells, pooled from 5 to 6 DBA or B6 mice, were stained with anti-CD4, anti-CD25, anti-CD44 and anti-CD62L for 30 mins at 4 ˚C. $CD4^+CD25^-CD62L^{high}CD44^{low}$ naive (Tn) and $CD4^+CD25^-CD62L^{low}CD44^{high}$ memory (Tm) T cells were sorted by first gating on $CD4^+CD25^-$ T cells (lower box in the *left panel* in **A**), then on CD62L and CD44 expression (*right panel* in **A**). Total $CD4^+$ were also sorted. Subsequently, cells were activated as described in the legends to Figure 1 in the absence (M) / presence of TGF-β (TGF) or both TGF-β and ATRA (TGF/ATRA). After 3 days, supernatants were collected, and cytokine levels were analyzed by ELISA. **A**, representative histogram demonstrates the gating strategy for different cell populations. **B & C**, levels of IL-4 (*upper panel*) and IFN-γ (*lower panel*) in supernatants of stimulated total $CD4^+$ (**B**) or Tm cells (**C**). No detectable levels of IL-4 and IFN-γ were observed in activated Tn cells (data not shown). Results were expressed at mean ± SEM from triplicate cultures and representative of 3 independent experiments.

As $CD4^+$ T cell populations comprise different T-cell populations, we further purified naive and memory T cells to track the source of IL-4. Only memory, not naive, cells produce detectable levels IL-4 upon activation in both B6 and DBA cultures (Figure 4C and data not shown). Nonetheless, when compared side by side, significantly higher amounts of IL-4 were observed in DBA memory cell cultures (Figure 4C, *upper panel*). In

keeping with these results, compared to B6 memory cells, addition of the same number of DBA memory cells results in a stronger reduction in the percentage of TGF-β/ATRA-induced Foxp3-expressing cells in naive B6 cell cultures (data not shown). Although DBA cells also produce more IFN-γ upon activation, the difference is less pronounced (Figure 4B & C).

The data described above indicate that memory cells from DBA mice produce relatively higher levels of IL-4, which inhibits Treg-conversion in naive cells (Figure 4 and data not shown). Nonetheless, they do not exclude the possibility that also naive T cells in DBA mice are more resistant to the conversion induced by TGF-β/ATRA. To analyze this possibility, we purified $CD4^+CD25^-CD62L^{high}CD44^{low}$ naive cells from DBA and B6 mice by FACS-sorting. These cells were subsequently activated by anti-CD3/28 and IL-2 in the absence or presence of TGF-β/ATRA and anti-IL-4. As shown in Figure 5, comparable percentages of $Foxp3^+$ cells were induced by TGF-β/ATRA from DBA and B6 naive cells. Accordingly, the proliferation of responder T cells was inhibited to a similar extent by the addition of TGF-β/ATRA induced $Foxp3^+$ cells from both strains (Figure 5A-C). Moreover, neutralization of IL-4 did not further increase the frequency of $Foxp3^+$ cells and regulatory function in naive cells from both mouse strains (Figure 5). These data show that IL-4 produced by naive T cells plays no or a very limited role in the inhibition of Treg conversion, and, more importantly, indicate that naive T cells from DBA mice do not harbor an intrinsic defect that would hamper such conversions.

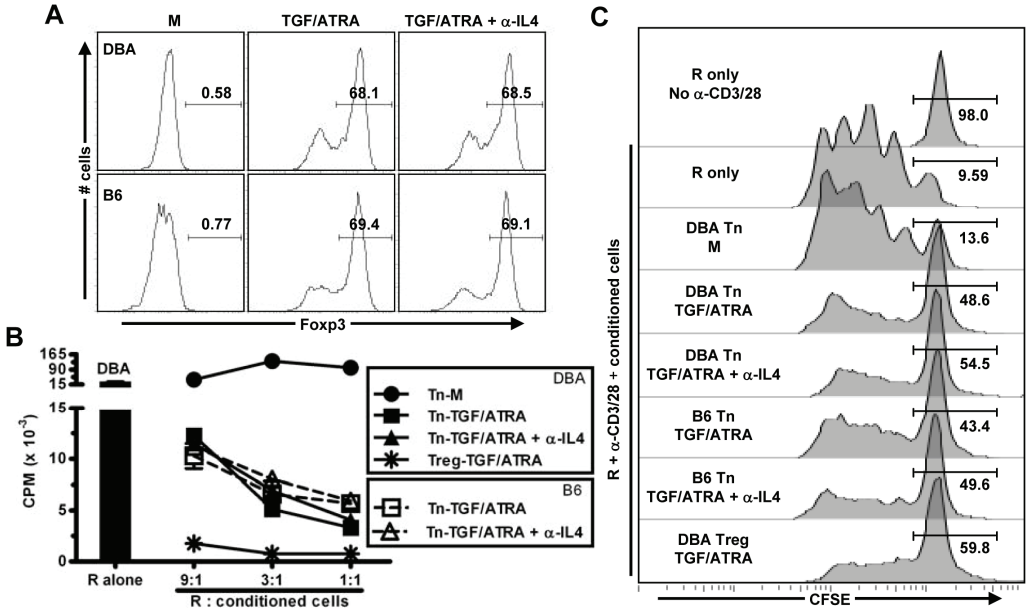

Figure 5. Purified DBA naive cells are susceptible to TGF-β/ATRA-induced Treg conversion. $CD4^+CD25^-CD62L^{high}CD44^{low}$ Tn and $CD4^+CD25^+$ Treg cells were sorted as depicted in Figure 4A. Cells were subsequently activated as described in the legends to Figure 1 in the absence (M) or presence of TGF-β and ATRA (TGF/ATRA) plus/minus blocking antibodies to IL-4. On day 4, cells were collected and analyzed for Foxp3 expression (**A**). After resting in IL-2 overnight, their suppressive ability was analyzed (**B & C**). **B**, proliferation was determined by ^{3}H-thymidine incorporation after 4 days. Results were expressed as means ± SEM of triplicate cultures. **C**, CFSE-profiles of responder $CD4^+$ T cells, activated with or without differently preconditioned cells at a ratio of 1:1, were analyzed by FACS on day 3. Purified $CD4^+CD25^+$ Treg cells from DBA mice were used as a positive control in the suppression assay. Results were representative of 4 (**A**) or 3 (**B & C**) independent experiments.

Based on these data, we conclude that high levels of IL-4 produced by memory cells result in the low responsiveness of total $CD4^+$ T cells from DBA mice to TGF-β/ATRA-induced Treg conversion, as 1) neutralization of IL-4 during activation induces

high numbers of Foxp3$^+$ Treg cells; 2) no detectable levels of IL-4 are observed from activated naive cells; and 3) purified naive cells are susceptible to Treg inductions in the absence of anti-IL-4 (Figure 1-5 and data not shown).

DBA naive cells are more susceptible to the inhibitory effect of IL-4 on TGF-β/ATRA-induced Foxp3 expression

As indicated above, an altered sensitivity of naive cells to the action of IL-4 could also contribute to the altered Treg induction upon exposure to TGF-β/ATRA between different mouse strains. To analyze this in more detail, we activated purified naive cells with TGF-β and ATRA in the absence or presence of titrated amounts of exogenous IL-4 for 3 days before the analyses of Foxp3 expression. IL-4 dose-dependently inhibited TGF-β-induced Foxp3 expression in both mouse strains (Figure 6B). However, addition of the same amount of IL-4 resulted in a greater reduction in the percentage of Foxp3$^+$ cells in DBA naive cells as compared to B6 cells (Figure 6A & B). Similar results were obtained with lower concentrations of TGF-β, regardless the presence of ATRA (Figure 6B and data not shown). Likewise, lower percentage of Foxp3-expressing cells was observed in DBA naive cells, as compared to their counterparts in B6 mice, when mixed with the same number of memory cells (data not shown).

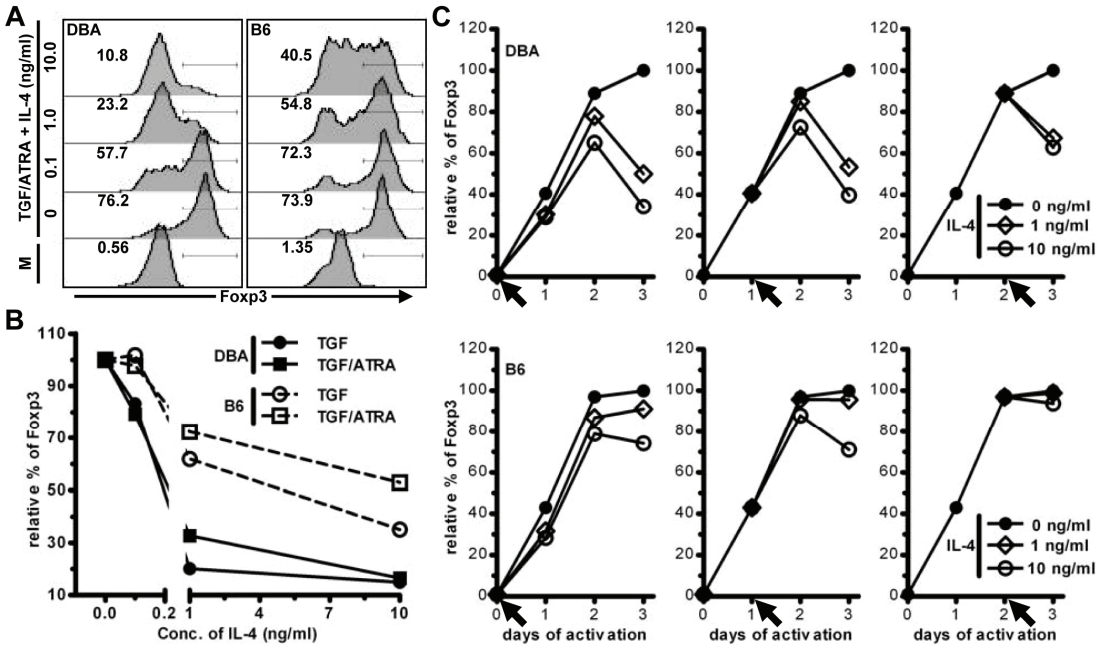

Figure 6. DBA naive cells are more susceptible to the inhibitory effect of IL-4 on TGF-β/ATRA-induced Foxp3 expression. FACS-purified CD4$^+$CD25$^-$CD62L^{high}CD44^{low} naive T (Tn) cells were activated as described in the legend to Figure 1 in the absence (M) / presence of 5 ng/ml TGF-β (TGF) and/or 10 nM ATRA (TGF/ATRA). Recombinant murine IL-4 was added to some wells on day 0 (**A & B**) or at indicated time points (**C**). **A**, representative histograms show the expression of Foxp3 in cultured DBA (*left panel*) or B6 cells (*right panel*). Numbers in upper left indicate the percentage of Foxp3$^+$ cells on day 3. **B**, line graph shows the relative percentages of Foxp3 expression in cells with titrated amounts of IL-4. The percentage of Foxp3$^+$ cells in cultures with TGF-β only (TGF, circles) or both TGF-β and ATRA (TGF/ATRA, squares) on day 3 in the absence of IL-4 was set as 100%. **C**, line graphs show the dynamic of relative Foxp3 expression in DBA (*upper panel*) and B6 (*lower panel*) cells in the absence or presence of IL-4. Cells were activated in the presence of TGF/ATRA and recombinant murine IL-4 was added on day 0, 1 or 2 as indicated by black arrows below X-axis. Cells were collected at indicated times and Foxp3 expression was analyzed. The percentage of Foxp3$^+$ cells on day 3 in cultures without additional IL-4 (black circles) was set as 100%. Results are representative of 3 (**A & B**) or 2 (**C**) independent experiments.

Different sensitivities to the action of IL-4 prompted us to investigate whether the expression of CD124 (IL-4 receptor α chain) also differs between naive DBA and B6 cells. As shown in Figure 7, higher levels of CD124 were observed on the surface of naive cells in DBA mice, both in terms of percentage (95.1 ± 0.30 % *vs.* 88.7 ± 0.79 %, $P < 0.01$) as well as the mean fluorescence intensity of positive cells (50.3 ± 0.98 % *vs.* 38.4 ± 0.65 %, $P < 0.01$). Similar results were obtained on memory or $CD4^+CD25^+$ Treg cells (data not shown). Thus, DBA naive T cells are more sensitive to the action of IL-4 in TGF-β/ATRA-induced Treg-conversion, which is associated with higher surface CD124 expression.

Together, our data indicate that both an enhanced production of IL-4 by memory cells and increased sensitivity to the action of IL-4 of naive cells contribute to the relative resistance of DBA cells to Treg conversion induced by TGF-β and ATRA.

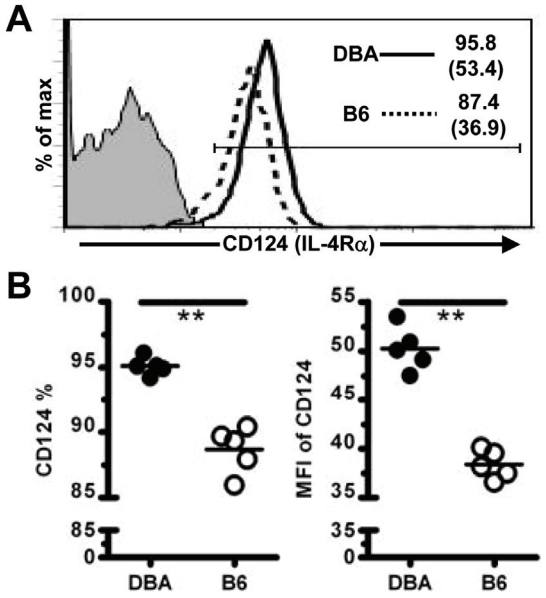

Figure 7. DBA naive T cells express higher levels of IL-4 receptor. A, representative overlay histogram shows CD124 (IL-4 receptor α chain) expression on gated naive $CD4^+CD25^-$ cells from DBA (solid line) *vs.* B6 (dotted line) mice. The quadrant was based on isotype control staining (filled gray). Numbers in the upper right corner indicate the percentage of positive cells, and numbers in brackets represent the mean fluorescence intensity (MFI) of $CD124^+$ cells. **B**, comparison of the percentage CD124-expressing naive $CD4^+CD25^-$ T cells (*left panel*) or MFI of $CD124^+$ cells (*right panel*) between DBA (filled circle) and B6 (open circle) mice. Each dot represents one mouse (n = 5) and the horizontal lines indicate the mean values for each group. **, $P < 0.01$.

Delayed effect of IL-4 on Treg differentiation

To gain more insights on the role of IL-4 in Treg development, we next examined the kinetic effect of IL-4 on TGF-β/ATRA-induced Foxp3 expression. To this end, exogenous IL-4 was added at different time points after the start of cultures and Foxp3 expression was analyzed on different days. Our results revealed that IL-4 does not significantly affect initial Treg development, as the percentage of Foxp3-expressing cells increases continuously in cells cultured with IL-4 in the first two days of culture, albeit to a slightly lower extent than that in control cells. In contrast, Foxp3 levels dropped remarkably between day 2 & 3 when IL-4 was added after 24 hrs, while increasing continuously in control cells (Figure 6C, *upper panel*). No significant cell death was observed in all culture conditions (data not shown). These data indicate that the action of IL-4 can still affect Foxp3-expression when added at later time points. Moreover, comparable levels of Foxp3 on day 3 were obtained in cells with exogenous IL-4 included on day 0 or day 1, indicating that the presence of IL-4 during the first 24 hrs of Treg induction has little effect on the subsequent Treg differentiation. Similar results were obtained with B6 cells, but, as expected, the difference in Foxp3 expression between cells cultured with or without IL-4 is less pronounced possibly due to the decreased sensitivity (Figure 6A-C).

Blockade of IL-4 enhances TGF-β/ATRA-induced human Treg conversion from naive T cells cocultured with memory cells

We have previously shown that human $CD4^+CD25^-CD45RA^+$ naive T cells are susceptible to TGF-β/ATRA-induced Treg conversion. Like their murine counterparts, human $CD4^+CD25^-CD45RA^-$ memory cells dampen this process mainly through the production of IL-4 as well (Wang *et al.*, 2009). Accordingly, blocking of IL-4, but not TNF-α, IFN-γ and IL-6, largely reversed this inhibition and thereby greatly enhanced the expression FOXP3 in human naive T cells. No effect of blocking antibodies was observed when naive T cells were cultured alone (Figure 8). Therefore, inherent differences in TGF-β/ATRA-induced Treg induction between subjects, both in mice and humans, can be overcome by neutralization of IL-4.

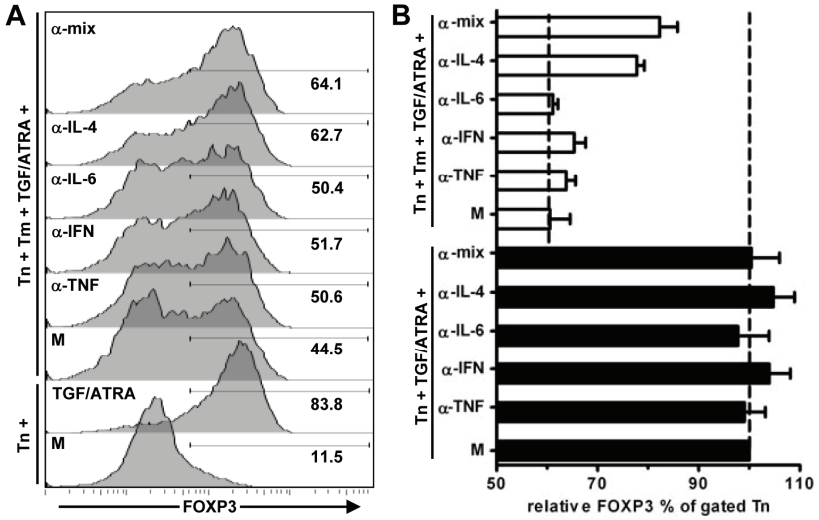

Figure 8. Blockade of IL-4 enhances TGF-β/ATRA-induced human Treg conversion from naive T cells cocultured with memory cells. $CD4^+CD25^-CD45RA^-$ memory (Tm) and $CD4^+CD25^-CD45RA^+$ naive effector T cells (Tn) were purified from adult peripheral blood by FACS-sorting. Tn were labeled with CFSE, mixed with Tm at the ratio of 1:0 or 1:1 (Tn : Tm, 1 x 10^5 cells/condition), and then stimulated with plate-bound anti-CD3, soluble anti-CD28 and IL-2 in the absence (M) or presence (TGF/ATRA) of exogenous TGF-β and ATRA. Neutralization Abs against TNF-α, IFN-γ, IL-6 and IL-4 were included at the concentration of 20 μg/ml. "Anti-mix" means the mixture of anti-TNF-α, IFN-γ, IL-6 and IL-6 (20 μg/ml of each). After 5 days, FOXP3 expression in activated Tn was analyzed by gating CFSE positive cells. **A**, representative histograms show FOXP3 expression in gated Tn among different culture conditions. **B,** Bar graph shows the relative percent of FOXP3 expression among different culture conditions in two different donors. Percent of FOXP3 expression in Tn cells stimulated with TGF-β/ATRA in the absence of Tm was set as 100%. No effect was observed with corresponding isotype Abs (data not shown).

Discussion

In this study, we describe that $CD4^+$ T cells from different mouse strains show different sensitivities to TGF-β-induced Treg conversion, in the absence or presence of ATRA. This is explained by different capabilities of memory cells to produce IL-4 and different sensitivities to the action of this cytokine by naive cells between different mouse strains. Importantly, neutralization of IL-4 during activation overcomes the strain-related difference, thereby enabling TGF-β/ATRA to generate functional Treg cells from total $CD4^+$ T cells in a consistent fashion. These data are relevant as they indicate that the intrinsic difference between "non-responder" and "responder" hosts can be overcome by

the inhibition of IL-4. Our observations also show that IL-4 signaling plays a dominant role in preventing Treg conversion induced by TGF-β in mice and humans and thereby extend the recent findings obtained in the B6 mouse system (Hill *et al.*, 2008;Nolting *et al.*, 2009;Takaki *et al.*, 2008;Dardalhon *et al.*, 2008;Prochazkova *et al.*, 2009). Moreover, our results indicate that neutralization of IL-4 may be necessary for inducible Treg generation in Th2-proned/dominated diseases and/or IL-4 sensitive hosts.

Given the great therapeutic potential of $CD4^+Foxp3^+$ Treg cells in the treatment of many immune-mediated disorders (Golshayan *et al.*, 2007;Morgan *et al.*, 2005;Mottet *et al.*, 2003;Tang *et al.*, 2004;van Mierlo *et al.*, 2008) and the limited availability of Foxp3-expressing "naturally occurring" Treg cells *in vivo*, the *de novo* generation of "adaptive or inducible" Treg cells by *in vitro* conversion of effector cells has been investigated intensively. Recent findings have shown that ATRA greatly enhances TGF-β-induced Treg conversion, providing a rational to generate functional Treg cells from total $CD4^+$ T cell populations. Indeed, high percentages of $Foxp3^+$ cells are induced by activation of total $CD4^+$ T cells in the presence of both TGF-β and ATRA in B6 mice (Figure 1). We now show that large numbers of $Foxp3^+$ cells can also be obtained from DBA and BALB/c $CD4^+$ T cells when IL-4 is neutralized during activation (Figure 1 & 3). Since no further purification of naive cells is required, our findings are relevant for the generation of Treg cells across different mouse stains. More importantly, it is conceivable that our findings are also relevant for the human settings where often donor-to-donor variations are present. Indeed, Treg cell generation by TGF-β/ATRA is also feasible in humans, but subjected to a donor-to-donor variability (Kang *et al.*, 2007;Wang *et al.*, 2009). Likewise, in these cases, the production of IL-4 by memory cells seems to play a prominent role (Wang *et al.*, 2009), indicating that human Treg generations by TGF-β/ATRA will be improved consistently by inhibiting the action of IL-4 as well (Figure 8).

ATRA has been shown, in the B6 mouse system, to enhance TGF-β-induced Treg generation through multiple mechanisms, either directly on naive cells or indirectly *via* memory cells (Hill *et al.*, 2008;Mucida *et al.*, 2007;Nolting *et al.*, 2009;Xiao *et al.*, 2008a). The direct effect is partially explained by an enhanced TGF-β-signaling pathway, whereas the indirect effect is mainly mediated by affecting cytokine secretions of memory T cells (Hill *et al.*, 2008;Nolting *et al.*, 2009;Xiao *et al.*, 2008a). Here we extend these findings by showing that even the combination of TGF-β and ATRA is clearly not sufficient to induce high numbers of Treg cells in mice with memory cells that produce high levels of IL-4 (Figure 1 & 4). Our data on the role of IL-4 in Treg-conversion across strains are compatible with the findings described above, as they show that the action of IL-4 is more pronounced in DBA cultures both through an (direct) increased sensitivity of naive cells as well as an (indirect) enhanced production of IL-4 by bystander memory T cells (Figure 4 & 6).

Although IL-4 was reported to increase the number of human $FOXP3^+$ T cells independent of TGF-β or IL-10 (Skapenko *et al.*, 2005), recent studies have shown that IL-4 can inhibit TGF-β-induced Foxp3 expression in naive B6 cells through the induction of Stat 6 (Takaki *et al.*, 2008) and/or the Th2 lineage-specific transcription factor GATA-3 (Dardalhon *et al.*, 2008;Wei *et al.*, 2007;Mantel *et al.*, 2007). In this way, the inhibition is mediated by the direct binding of Stat-6 and GATA-3 to the Foxp3 promoter region and thereby prevents its transcription (Takaki *et al.*, 2008;Mantel *et al.*, 2007). Our experiments on the kinetic effect of IL-4 indicate that the presence of IL-4 is not required within the first 24 hrs after stimulation as addition of IL-4 on day 1 still resulted in maximum suppression of Foxp3 expression. Likewise, the expression of Foxp3 is only significantly reduced by IL-4 after 48 hrs of stimulation. These observations are also in line with the Foxp3 expression pattern depicted in Figure 1B, where the relatively high

level of IL-4 in the supernatants present on day 1 (IL-4 levels were 367.2 ± 21.44, 514.2 ± 17.03 & 573.4 ± 37.4 pg/ml on day 1, 2 and 3, respectively) is only followed by a reduction in Foxp3 levels on day 2. The latter data thus suggest that IL-4 affects the maintenance of Foxp3 expression at later time points rather than preventing its expression shortly after T-cell activation (Figure 6C).

The data described above are interesting as they might add to our understandings on the number of FOXP3-expressing cells under inflammatory conditions. Several groups have described a reduced Foxp3 expression during inflammation (Zhou *et al.*, 2009;Prochazkova *et al.*, 2009). In keeping with our *in vitro* data, mice with disrupted IL-4 signaling harbor more $Foxp3^+$ Treg cells during inflammation (Nagase *et al.*, 2007;Cardoso *et al.*, 2009). Nonetheless, we consider it unlikely that induced Treg cells contribute significantly to the peripheral pool of Treg cells in naive animals because of the comparable expression of Foxp3 in spleens among different mouse strains (Figure 1) and the low levels of homeostatic conversion of Treg cells *in vivo* (Coombes *et al.*, 2007;Sun *et al.*, 2007).

Intriguingly, not only differences in IL-4 production by memory T cells, but also differences in the sensitivity to IL-4 by naive T cells (Figure 4-6) were observed between different mouse strains. Although the latter could possibly be explained by different expressions of IL-4 receptor CD124 (Figure 6 & 7), more in depth analyses are required to support the latter notion in a more convincing manner.

In conclusion, we have demonstrated that $CD4^+$ T cells from different mouse strains respond differently to TGF-β/ATRA-induced Treg conversion because of different IL-4-producing abilities of memory cells and different sensitivities to IL-4 of naive cells. Neutralization of IL-4 could facilitate the development of Treg-based immunotherapies, as it overcomes the strain-related difference and enables TGF-β/ATRA to consistently generate Treg cells from total $CD4^+$ T cells.

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Abbreviations

Treg, T regulatory; ATRA, all-*trans* retinoic acid; B6, C57BL/6; DBA, DBA/1J; Tn: $CD4^+CD25^-CD62L^{high}CD44^{low}$ naive T cells; Tm: $CD4^+CD25^-CD62L^{low}CD44^{high}$ memory T cells.

References

1. Apostolou,I., von Boehmer,H., 2004. *In vivo* instruction of suppressor commitment in naive T cells. J. Exp. Med. 199**,** 1401-1408.
2. Benson,M.J., Pino-Lagos,K., Rosemblatt,M., Noelle,R.J., 2007. All-*trans* retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. Journal of Experimental Medicine 204**,** 1765-1774.
3. Cardoso,C.R., Provinciatto,P.R., Godoi,D.F., Ferreira,B.R., Teixeira,G., Rossi,M.A., Cunha,F.Q., Silva,J.S., 2009. IL-4 regulates susceptibility to intestinal inflammation in murine food allergy. Am. J. Physiol Gastrointest. Liver Physiol 296**,** G593-G600.
4. Coombes,J.L., Siddiqui,K.R.R., Arancibia-Carcamo,C.V., Hall,J., Sun,C.M., Belkaid,Y., Powrie,F., 2007. A functionally specialized population of mucosal CD103(+) DCs induces Foxp3(+) regulatory T cells *via* a TGF-beta- and retinoic acid-dependent mechanism. Journal of Experimental Medicine 204**,** 1757-1764.
5. Dardalhon,V., Awasthi,A., Kwon,H., Galileos,G., Gao,W., Sobel,R.A., Mitsdoerffer,M., Strom,T.B., Elyaman,W., Ho,I.C., Khoury,S., Oukka,M., Kuchroo,V.K., 2008. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. Nat. Immunol. 9**,** 1347-1355.
6. Dominitzki,S., Fantini,M.C., Neufert,C., Nikolaev,A., Galle,P.R., Scheller,J., Monteleone,G., Rose-John,S., Neurath,M.F., Becker,C., 2007. Cutting edge: trans-signaling *via* the soluble IL-6R abrogates the induction of FoxP3 in naive CD4+CD25 T cells. J. Immunol. 179**,** 2041-2045.
7. Elias,K.M., Laurence,A., Davidson,T.S., Stephens,G., Kanno,Y., Shevach,E.M., O'Shea,J.J., 2008. Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat-3/Stat-5 independent signaling pathway. Blood 111**,** 1013-1020.
8. Fontenot,J.D., Gavin,M.A., Rudensky,A.Y., 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat. Immunol. 4**,** 330-336.
9. Golshayan,D., Jiang,S., Tsang,J., Garin,M.I., Mottet,C., Lechler,R.I., 2007. *In vitro*-expanded donor alloantigen-specific CD4+CD25+ regulatory T cells promote experimental transplantation tolerance. Blood 109**,** 827-835.
10. Hill,J.A., Hall,J.A., Sun,C.M., Cai,Q., Ghyselinck,N., Chambon,P., Belkaid,Y., Mathis,D., Benoist,C., 2008. Retinoic Acid Enhances Foxp3 Induction Indirectly by Relieving Inhibition from CD4(+)CD44(hi) Cells. Immunity 29**,** 758-770.
11. Kang,S.G., Lim,H.W., Andrisani,O.M., Broxmeyer,H.E., Kim,C.H., 2007. Vitamin A metabolites induce gut-homing FoxP3+ regulatory T cells. J. Immunol. 179**,** 3724-3733.
12. Kang,S.G., Wang,C., Matsumoto,S., Kim,C.H., 2009. High and low vitamin A therapies induce distinct FoxP3+ T-cell subsets and effectively control intestinal inflammation. Gastroenterology 137**,** 1391-1402.
13. Mantel,P.Y., Kuipers,H., Boyman,O., Rhyner,C., Ouaked,N., Ruckert,B., Karagiannidis,C., Lambrecht,B.N., Hendriks,R.W., Crameri,R., Akdis,C.A., Blaser,K., Schmidt-Weber,C.B., 2007. GATA3-driven Th2 responses inhibit TGF-beta1-induced FOXP3 expression and the formation of regulatory T cells. PLoS. Biol. 5**,** e329.
14. Morgan,M.E., Flierman,R., van Duivenvoorde,L.M., Witteveen,H.J., van Ewijk,W., van Laar,J.M., de Vries,R.R., Toes,R.E., 2005. Effective treatment of collagen-induced arthritis by adoptive transfer of CD25+ regulatory T cells. Arthritis Rheum. 52**,** 2212-2221.
15. Mottet,C., Uhlig,H.H., Powrie,F., 2003. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. J. Immunol. 170**,** 3939-3943.
16. Mucida,D., Park,Y., Kim,G., Turovskaya,O., Scott,I., Kronenberg,M., Cheroutre,H., 2007. Reciprocal T(H)17 and regulatory T cell differentiation mediated by retinoic acid. Science 317**,** 256-260.
17. Nagase,H., Jones,K.M., Anderson,C.F., Noben-Trauth,N., 2007. Despite increased CD4+Foxp3+ cells within the infection site, BALB/c IL-4 receptor-deficient mice reveal CD4+Foxp3-negative T cells as a source of IL-10 in Leishmania major susceptibility. J. Immunol. 179**,** 2435-2444.
18. Nolting,J., Daniel,C., Reuter,S., Stuelten,C., Li,P., Sucov,H., Kim,B.G., Letterio,J.J., Kretschmer,K., Kim,H.J., von Boehmer,H., 2009. Retinoic acid can enhance conversion of naive into regulatory T cells independently of secreted cytokines. J. Exp. Med. 206**,** 2131-2139.

19. Prochazkova,J., Fric,J., Pokorna,K., Neuwirth,A., Krulova,M., Zajicova,A., Holan,V., 2009. Distinct regulatory roles of transforming growth factor-beta and interleukin-4 in the development and maintenance of natural and induced CD4+ CD25+ Foxp3+ regulatory T cells. Immunology 128**,** e670-e678.

20. Schambach,F., Schupp,M., Lazar,M.A., Reiner,S.L., 2007. Activation of retinoic acid receptor-alpha favours regulatory T cell induction at the expense of IL-17-secreting T helper cell differentiation. Eur. J. Immunol. 37**,** 2396-2399.

21. Selvaraj,R.K., Geiger,T.L., 2007. A kinetic and dynamic analysis of Foxp3 induced in T cells by TGF-beta. J. Immunol. 178**,** 7667-7677.

22. Skapenko,A., Kalden,J.R., Lipsky,P.E., Schulze-Koops,H., 2005. The IL-4 receptor alpha-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25+CD4+ regulatory T cells from CD25-CD4+ precursors. J. Immunol. 175**,** 6107-6116.

23. Sun,C.M., Hall,J.A., Blank,R.B., Bouladoux,N., Oukka,M., Mora,J.R., Belkaid,Y., 2007. Small intestine lamina propria dendritic cells promote *de novo* generation of Foxp3 T reg cells *via* retinoic acid. Journal of Experimental Medicine 204**,** 1775-1785.

24. Takaki,H., Ichiyama,K., Koga,K., Chinen,T., Takaesu,G., Sugiyama,Y., Kato,S., Yoshimura,A., Kobayashi,T., 2008. STAT6 inhibits TGF-beta 1-mediated Foxp3 induction through direct binding to the Foxp3 promoter, which is reverted by retinoic acid receptor. Journal of Biological Chemistry 283**,** 14955-14962.

25. Tang,Q., Henriksen,K.J., Bi,M., Finger,E.B., Szot,G., Ye,J., Masteller,E.L., McDevitt,H., Bonyhadi,M., Bluestone,J.A., 2004. *In vitro*-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. J. Exp. Med. 199**,** 1455-1465.

26. Turner,M.S., Kane,L.P., Morel,P.A., 2009. Dominant role of antigen dose in CD4+Foxp3+ regulatory T cell induction and expansion. J. Immunol. 183**,** 4895-4903.

27. Valencia,X., Stephens,G., Goldbach-Mansky,R., Wilson,M., Shevach,E.M., Lipsky,P.E., 2006. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. Blood 108**,** 253-261.

28. van Mierlo,G.J., Scherer,H.U., Hameetman,M., Morgan,M.E., Flierman,R., Huizinga,T.W., Toes,R.E., 2008. Cutting edge: TNFR-shedding by CD4+CD25+ regulatory T cells inhibits the induction of inflammatory mediators. J. Immunol. 180**,** 2747-2751.

29. von Boehmer,H., 2007. Oral tolerance: is it all retinoic acid? Journal of Experimental Medicine 204**,** 1737-1739.

30. Wang,J., Huizinga,T.W., Toes,R.E., 2009. *De novo* generation and enhanced suppression of human CD4+CD25+ regulatory T cells by retinoic acid. J. Immunol. 183**,** 4119-4126.

31. Wang,J., Toes,R.E., 2008. Mechanisms of oral tolerance revisited. Arthritis Res. Ther. 10**,** 108.

32. Wei,J., Duramad,O., Perng,O.A., Reiner,S.L., Liu,Y.J., Qin,F.X., 2007. Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells. Proc. Natl. Acad. Sci. U. S. A 104**,** 18169-18174.

33. Xiao,S., Jin,H., Korn,T., Liu,S.M., Oukka,M., Lim,B., Kuchroo,V.K., 2008a. Retinoic acid increases Foxp3(+) regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. Journal of Immunology 181**,** 2277-2284.

34. Xiao,X., Kroemer,A., Gao,W., Ishii,N., Demirci,G., Li,X.C., 2008b. OX40/OX40L costimulation affects induction of Foxp3+ regulatory T cells in part by expanding memory T cells *in vivo*. J. Immunol. 181**,** 3193-3201.

35. Zhou,X., Bailey-Bucktrout,S.L., Jeker,L.T., Penaranda,C., Martinez-Llordella,M., Ashby,M., Nakayama,M., Rosenthal,W., Bluestone,J.A., 2009. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells *in vivo*. Nat. Immunol. 10**,** 1000-1007.