Regulatory T cells induced by rAAV carrying the forkhead box P3 gene prevent autoimmune thyroiditis in mice

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Abstract. $CD4^+CD25^+$ regulatory T (Treg) cells are immunosuppressive and help maintain peripheral immune tolerance. The *forkhead*/winged helix family member, Foxp3, has been shown to be a critical regulator of $CD4^+CD25^+$ Treg cells. We demonstrate by quantitative real-time PCR that $CD4^+CD25^+$ T cells express higher levels of Foxp3 mRNA than other T cell populations. Recombinant adeno-associated virus vector carrying mouse Foxp3 gene under the control of the CMV promoter was generated and used to transduce $CD4^+CD25^-$ T cells. These Foxp3-transduced T cells are similar to naturally occurring $CD4^+CD25^+$ regulatory T cells which show anergy and immunosuppressive activity *in vitro*. Furthermore, these Foxp3-transduced T cells prevent autoimmune thyroiditis transferred by pathogenic T cells *in vivo*. Our data indicates that Foxp3-transduced $CD4^+CD25^-$ T cells may open new therapeutic strategies for immune disorders.

Introduction

Experimental autoimmune thyroiditis (EAT) is a chronic inflammatory organ-specific autoimmune disease, and represents a good model for human Hashimoto's thyroiditis, which is the gradual destruction of the thyroid gland by infiltrating mononuclear cells (1). EAT can be induced in susceptible $H\text{-}2^k$ mice after challenge with thyroglobulin in adjuvant or adoptive transfer by pathogenic T cells. There is strong evidence that thyroglobulin-specific $CD4^+$ T cells, especially IL-12-dependent Th1 cells, are both sufficient and necessary in EAT induction. Several reports have suggested that the elimination of $CD4^+CD25^+$ T cells in mice resulted in the development of multiorgan autoimmune diseases, including thyroiditis, whereas the reconstitution of $CD4^+CD25^+$ T cells inhibited the development of autoimmunity (2,3).

$CD4^+CD25^+$ regulatory T (Treg) cells are a subset of T cells involved in regulating immune response through the suppression of effector $CD4^+$ T cells or cytotoxic $CD8^+$ T cells. Treg cells are often characterized by their constitutive expression of membrane molecules such as CD25, CTLA-4, GITR (glucocorticoid-induced tumor necrosis factor) and CD103, which are also expressed in activated T cells (4-8). Although TGF-ß and CTLA-4 have been implicated in Treg cell functions, data show that Treg cells are immunosuppressive in the absence of these molecules (9,10). The mechanisms by which Treg cells are generated and function remain unclear.

Mutation in the X-linked Foxp3 (forkhead box P3) gene in *scurfy* (*sf*) mice and human patients with IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome) leads to an autoimmune disease characterized by multiorgan lymphocytic infiltration and inflammatory diseases as a result of uncontrolled activation and expansion of $CD4^+$ T cells (11-15). Foxp3 is specifically expressed in $CD4^+CD25^+$ Treg cells and is required for the thymic development of Treg cells. Foxp3-deficient mice lack Treg cells, whereas mice that overexpress Foxp3 display an increased number of Treg cells. In transgenic mice overexpressing Foxp3, $CD4^+CD25^-$ T cells express GITR and show suppressive activity (16-18). Thus, Foxp3 may be a 'master control gene' and a critical regulator of $CD4^+CD25^+$ Treg cells in their development and function.

Adeno-associated virus (AAV) is a nonpathogenic parvovirus that contains a single-stranded DNA as its genome and requires coinfection with a helper virus, usually an adenovirus, for its optimal replication. In the absence of coinfection with the helper virus, the wild-type AAV establishes a latent infection and the viral genome integrates into chromosomal DNA in a site-specific manner. The non-pathogenicity of AAV and the remarkable site specificity of its integration have led to the development of recombinant AAV vectors for gene transfer and gene therapy. To date, of the eight recognized AAV serotypes, the biology of AAV2 has been most extensively studied and this serotype has been widely used. Although recombinant AAV genomes do not appear to integrate site specifically, AAV vectors have been

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successfully used to deliver genes to a wide variety of cells and tissues *in vitro* and *in vivo*. AAV vectors have also been used in phase I clinical trials for gene therapy of cystic fibrosis (19,20).

In this study, recombinant AAV (rAAV) vector containing the mouse Foxp3 cDNA under the control of the CMV promoter was constructed. We next determined whether the expression of Foxp3 in naïve $CD4^+CD25^-$ T cells converted these cells toward a regulatory T cell phenotype by rAAV-Foxp3 transduction *in vitro* and whether these transduced cells prevented autoimmune thyroiditis by adoptive transfer *in vivo*. Our results support the potential application of rAAV-based vectors in Foxp3 gene-modified T cells for immunotherapy.

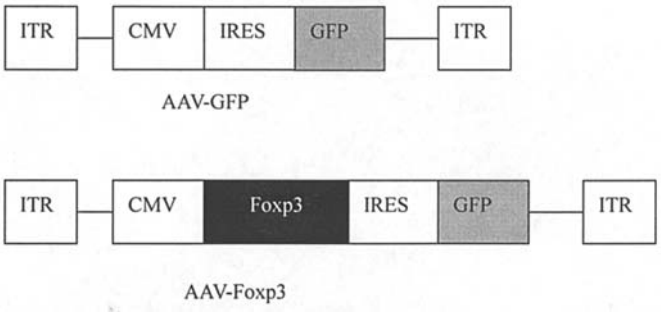

Figure 1. Schematic representation of the genomic organization of the recombinant AAV vector.

Materials and methods

Materials. A mouse $CD4^+$ T cell isolation kit and anti-FITC microbeads were purchased from Miltenyi Biotech. TRIzol® reagent and Superscript first-stand synthesis system for RT-PCR kits were purchased from Invitrogen. pMD18-T vector, DNA restriction enzymes, ExTaqDNA polymerase and DNA marker were the products of TaKaRa Biotech. T4 DNA ligase was purchased from MBI. SYBR-Green I was purchased from Molecular Probes Inc. PE-conjugated anti-CD4 mAb and FITC-conjugated anti-CD25 mAb were purchased from BD Pharmingen. Anti-Foxp3 mAb and HRP-conjugated rabbit anti-mouse IgG were purchased from eBioscience Co. An ECL advanced Western blotting detection kit was purchased from Amersham Bioscience Ltd. The plasmids pZac2.1 (AAV2 vector plasmid containing CMV promoter, MCS, and SV40 polyA flanked by AAV2 ITRs), P5E18 (AAV2 packaging plasmid containing AAV2 rep and cap gene) and pAd Delta F6 (adenovirus helper plasmid) were kindly provided by Dr Sarah A.E. Haecker and L.L. Wang (University of Pennsylvania, Philadelphia, USA). *E. coli* DH5α, was kept in our laboratory. All other chemicals were local products of analytic grade.

Isolation of $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells. Mononuclear cell suspensions were prepared from the splenocytes by Ficoll-Hypaque density gradient centrifugation. $CD4^+$ T cells were purified by MACS with a mouse $CD4^+$ T cell isolation kit according to the manufacturer's instructions. After detaching, $CD4^+$ T cells were washed once in PBS plus 0.5% BSA plus 3 mM EDTA, and stained with 1 μg of FITC-conjugated anti-CD25 mAb for 15 min on ice, washed two times, and incubated for an additional 15 min with anti-FITC microbeads, $CD25^+$ T cells and $CD25^-$ T cells were sorted via MACS, respectively. Immunofluorescent staining was performed after washing the cells twice with PBS-0.5% BSA. For staining, 10^5 cells were suspended in 100 μl PBS and were incubated with 1 μg of PE-conjugated anti-CD4 mAb for 30 min on ice. Cells were washed three times, and were analyzed by flow cytometry (FACScalibur™, CellQuest software; Becton Dickinson).

Detection of Foxp3 mRNA expression in $CD4^+CD25^+$ T cells. Total cellular RNA was extracted from $5x10^5$ $CD4^+CD25^+$ T cells using TRIzol reagent, and the total RNA was reverse transcribed using a superscript first-stand synthesis system for RT-PCR kits according to the manufacturer's instructions, respectively. First-strand cDNA PCR conditions were: a 2.5 min at 94˚C denaturation step followed by 35 cycles of 30 sec at 94˚C denaturation, 30 sec at 60˚C annealing, a 90 sec at 72˚C extension and a 10 min at 72˚C extension. The reactions were carried out in a total volume of 25 μl containing 1.25 U ExTaqDNA polymerase, 10 pmol sense primer and 10 pmol antisense primer, 2 mmol/l $MgCl_2$, 0.2 mmol/l dNTP in 1x buffer on a Mastercycler thermo-cycler (Eppendorf). The primer sequences were as follows: 5'-GCT CCC GGC CTG GTC TGC TC-3' (forward primer) and 5'-AGG TGG CGG GGT GGT TTC TGA-3' (reverse primer).

The Foxp3 mRNA levels in $CD4^+CD25^+$ T cells were quantified by quantitative real-time PCR (QPCR) using the Rotor-Gene 2000 system (Corbett Research Inc). Analyses were performed by using specific primers to Foxp3 or ß $_2$mG by fluorescent SYBR-Green I method. Foxp3 primers were: 5'-GCT CCC GGC CTG GTC TGC TC-3' (forward primer) and 5'-AGG TGG CGG GGT GGT TTC TGA-3' (reverse primer). ß $_2$mG was used as an endogenous reference with primers: 5'-TCA CTG ACC GGC CTG TAT GCT ATC-3' (forward primer) and 5'-GTG AGG CGG GTG GAA CTG TGT (reverse primer). Amplification was carried out in a total volume of 25 μl containing 0.4 pmol primers and 1:10000 SYBR-Green I, and consisted of a 2.5 min at 94˚C denaturation step followed by 40 to 45 cycles of 15 sec at 94˚C denaturation, 30 sec at 60˚C annealing, and 30 sec at 72˚C extension. Samples were run in triplicate, and their relative expression was determined by normalizing the expression of each target to ß $_2$mG, and then comparing this normalized value to the normalized expression in a reference sample to calculate a fold-change value.

Cloning and construction of mouse Foxp3 gene. The PCR-amplified full-length Foxp3 cDNA was inserted into pMD 18-T vector according to the manufacturer's instructions. Cloning vectors containing inserted fragments were sequenced by Bioasia Ltd. (Shanghai, P.R. China).

Subsequently, the full-length Foxp3 cDNA was isolated from pMD 18-T vector by *Eco*RI and *Sal*I digestion and inserted into the *Eco*R I/*Sal* I restricted AAV2 vector plasmid pZAC2.1 containing IRES and GFP genes. Fig. 1 schematically represents the AAV2 vector plasmids AAV-GFP (as control plasmid) and AAV-Foxp3.

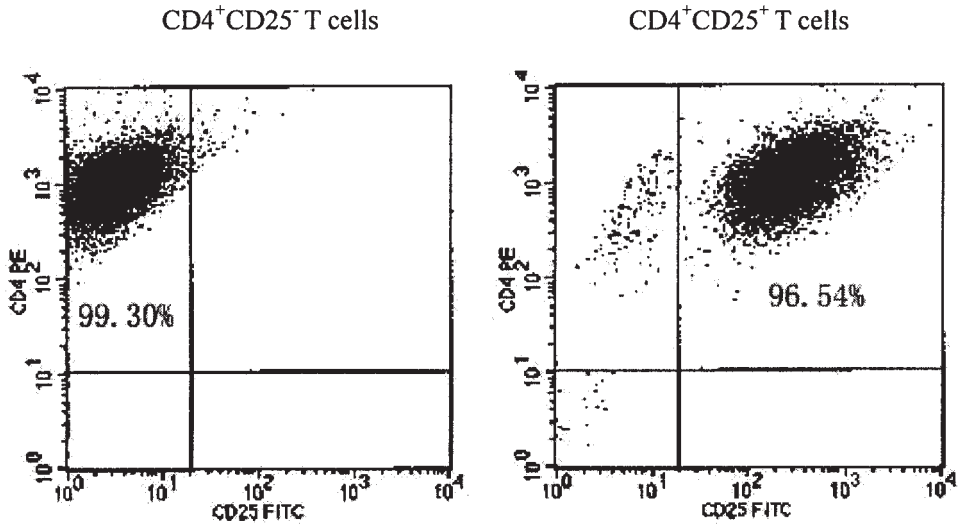

Figure 2. Purity analysis of freshly isolated CD4+CD25- T cells and CD4+CD25+ T cells by flow cytometry. This is a representative experiment which has been replicated.

Recombinant AAV production and titration. Recombinant AAV (rAAV) expressing Foxp3 containing the CMV promoter-controlled Foxp3 expression cassette was generated by the calcium phosphate precipitation-based cotransfection method (21). Briefly, the 293 cell density was 70-80% and the cells were maintained in DMEM medium supplemented with 10% fetal calf serum at 37˚C in a humidified atmosphere containing 5% CO_2. The transfection cocktail containing AAV-Foxp3 vector (650 μg), P5E18 (650 μg) and pAd Delta F6 (1300 μg) plasmids was prepared. Transfection was carried out using the standard calcium phosphate coprecipitation method. After 72 h of posttransfection, the cells were harvested as rAAV primary stocks. The lysate of cells was clarified by centrifugation and the virus-containing supernatant was further purified using heparin affinity chromatography. The genome titers (vector genomes/ml) of the vector preparations were determined by quantitative real-time PCR (22). rAAV expressing GFP were packaged as described above.

Detection of Foxp3 mRNA transcription and protein expression in transduced T cells. Primary CD4+CD25- T cells were infected with rAAV-Foxp3 or rAAV-GFP ($2x10^4$ genomes/cell) in DMEM for 4 h at 37˚C with 5% CO_2. The cells were expanded and maintained in medium supplemented with 100 U/ml IL-2 until day 7 of postinfection. The cells were harvested by gentle scraping and washed twice with 2 ml of cold PBS.

Total cellular RNA was extracted from transduced T cells and Foxp3 mRNA levels were quantified by QPCR as described above.

The Foxp3 protein was detected by immunoblotting assays. Briefly, transduced T cells were added to 0.25 ml cold lysing buffer (0.5 M NaCl, 1% Triton X-100, 0.2% Tween-20, 50 mM HEPES, pH 7.0). Lysates were transferred to Eppendorf tubes and homogenized by repeated pipetting on ice, and the supernatants were collected. Protein samples in a loading buffer were heated at 70˚C for 10 min and loaded onto 10% SDS-PAGE gels. After electrophoresis, the proteins were electro-transferred onto PDVF membranes and blocked with 5% nonfat dry milk in TBS buffer for 1 h. The membranes were probed with anti-mouse Foxp3 mAb for 1 h at 37˚C.

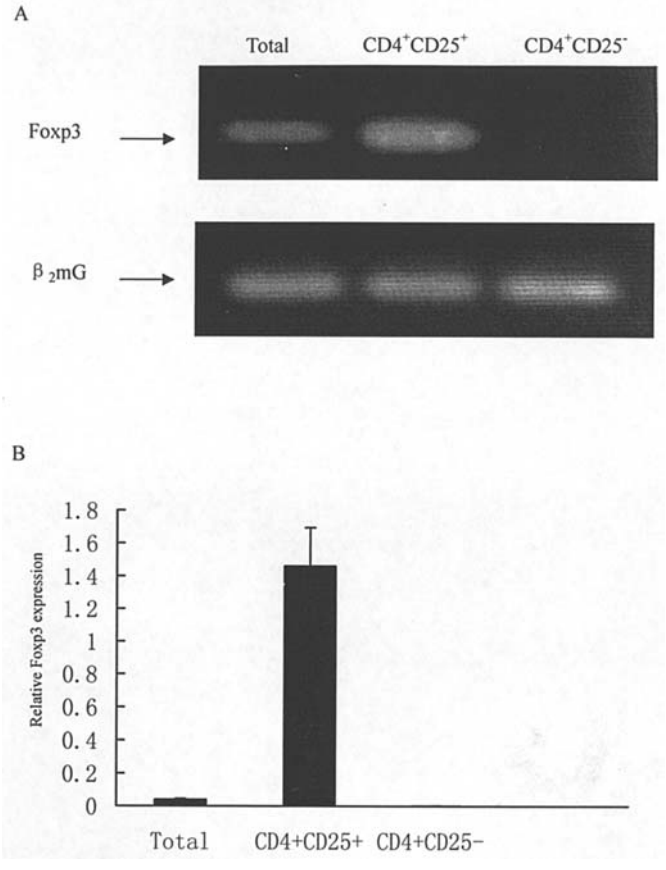

Figure 3. Foxp3 mRNA expression in T cell populations. (A) Foxp3 mRNA expression from total CD4+ T cells, CD4+CD25- T cells and CD4+CD25+ T cells by RT-PCR using Foxp3 or ß 2mG primers. (B) Quantitative real-time PCR for T cell populations. The relative quantity of Foxp3 in each sample was normalized to the relative quantity of ß 2mG. Data are representative of three independent experiments.

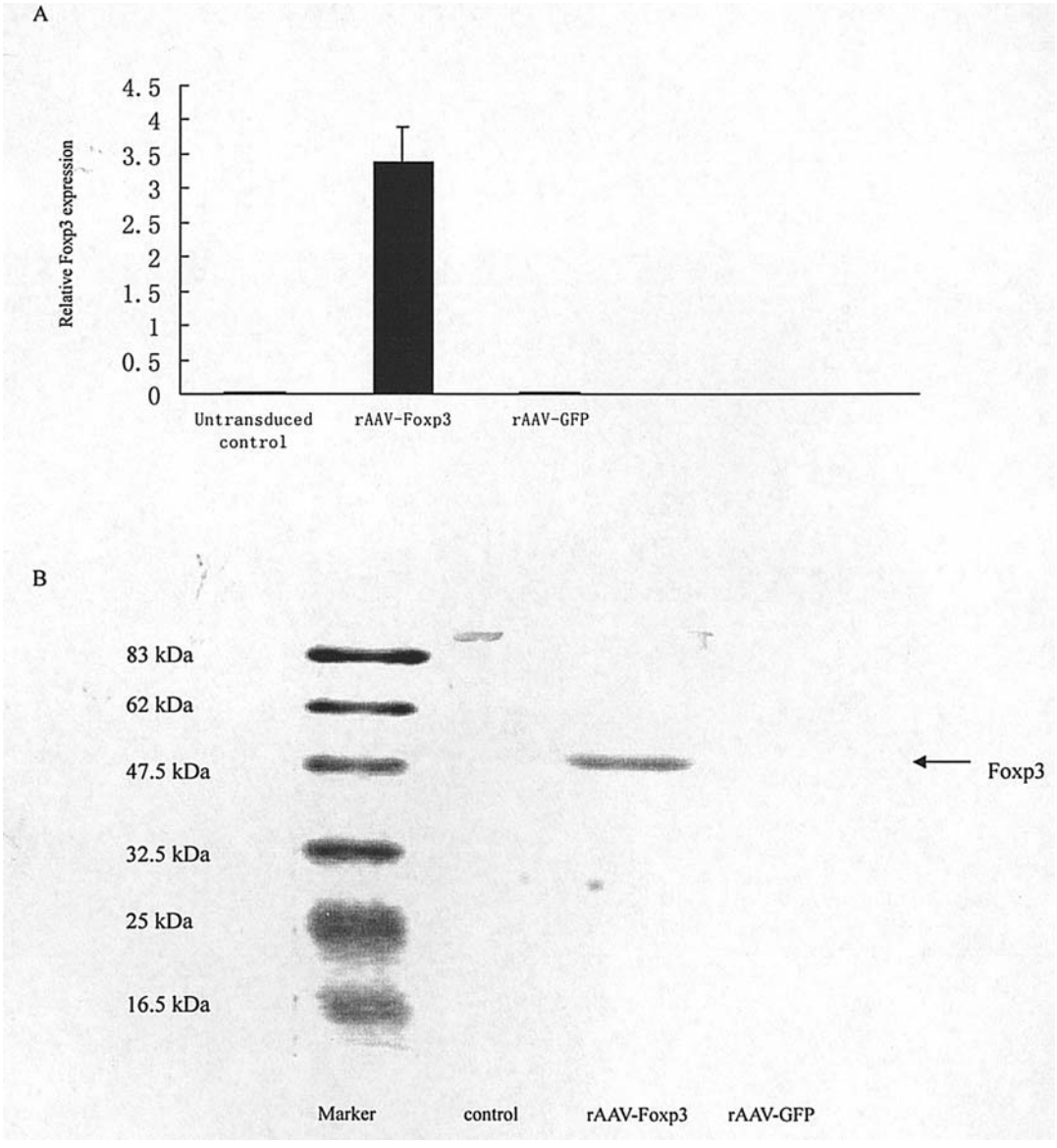

Figure 4. Detection of Foxp3 transcription and protein expression in transduced $CD4^+CD25^-$ T cells by rAAV-Foxp3 or by rAAV-GFP vectors. (A) Foxp3 mRNA levels were quantified by QPCR. (B) Foxp3 protein was detected by immunoblotting assay.

Detection was performed using goat anti-mouse IgG conjugated with HRP and visualized with an ECL advanced Western blotting detection kit in accordance with the manufacturer's recommendations.

Cell proliferation and suppression assays. Cell proliferation and suppression assays were performed as described. Briefly, for proliferation assay, $CD4^+CD25^-$ T cells, and transduced T cells ($2x10^4$ cells/well) were primarily activated with anti-CD3 mAb (5 μg/ml) in the presence of $1x10^5$ irradiated syngeneic spleen mononuclear cells in 96-well plates. T cell proliferation was measured after 72 h of incubation and an additional 12-h pulse with [^{3}H]Tdr (37 kBq/well) using a liquid scintillation counter (Beckman LS6500). A suppression assay was performed under the same conditions using $2x10^4$ $CD4^+CD25^-$ T cells as responders, $1x10^5$ irradiated syngeneic spleen mononuclear cells and 1:1 titration of the indicated transduced T cell populations at a starting concentration of $2x10^4$ cells/well in the presence of anti-CD3 mAb. All data are shown as mean [^{3}H]Tdr incorporation in triplicate cultures.

Induction and histological assessment of EAT. For direct induction of EAT, mice were challenged *s.c.* with thyroglobulin in Freund complete adjuvant emulsion and were boosted 14 days with the same emulsion. EAT was assessed by histological examinations of the thyroids, 4 weeks after the initial challenge. Thyroid glands were collected, fixed by formalin, and embedded in methacrylate. The approximately sections were obtained, and then were stained with hematoxylin and eosin and were scored according to the presence of mononuclear cell infiltration index (I.I.) as follows: 1, interstitial accumulation of cells between two or three follicles; 2, one or two foci of cells at least the size of one follicle; 3, extensive infiltration, 10-40% of the total area; 4, extensive infiltration, 40-80% of the total area; and 5, extensive infiltration, >80% of the total area. The highest infiltration index observed per gland was assigned to each mouse.

Table I. Adoptive transfer of thyroiditis and cell cotransfer.

Transfer cell type	I.I.				No. of mice with EAT	I.I. mean	p value
	0	1	2	3			
10^7 $CD4^+CD25^-$ T cells from EAT	1	1	1	3	5/6	2.00	
10^7 $CD4^+CD25^-$ T cells from EAT +2x10^6 transduced T cells by rAAV-Foxp3	5	1	0	0	1/6	0.17	0.016
10^7 $CD4^+CD25^-$ T cells from EAT +2x10^6 transduced T cells by rAAV-GFP	0	2	3	1	6/6	1.83	0.616

Thyroids collected upon termination of experiments on day 28 were evaluated. The mononuclear cell I.I. was scored, as described in Materials and methods. Statistical analysis was performed using the nonparametric Mann-Whitney U test. Values of p were calculated by comparing 10^7 $CD4^+CD25^-$ T cells from EAT-treated mice.

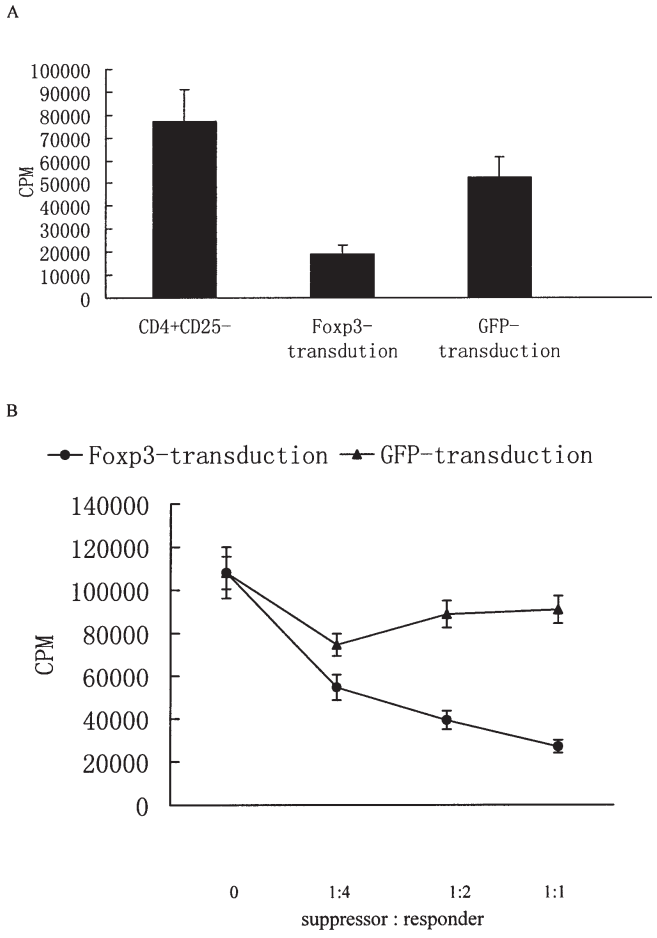

Figure 5. Proliferation and suppression of rAAV-transduced $CD4^+CD25^-$ T cells (A) CD3 mAb-stimulated proliferation of fresly isolated $CD4^+CD25^-$ T cells, and rAAV-Foxp3- or rAAV-GFP-infected $CD4^+CD25^-$ T cells. (B) Suppressive activity of Foxp3-transduced $CD4^+CD25^-$ T cells. [^{3}H]Tdr incorporation was measured as an indicator of cell proliferation. Data are representative of two independent experiments.

Adoptive transfer of thyroiditis and cell cotransfer. Adoptive transfer of thyroiditis was performed as previously described. Briefly, $CD4^+CD25^-$ T cells from EAT mice were cultured for 72 h in the presence of 20 μg/ml thyroglobulin and 3 μg/ml ConA. The cells were then harvested, and after washing three times, 1x10^7 cells in 200 μl of PBS were injected i.v. into syngeneic recipients. Transduced T cells (2x10^6) by rAAV-Foxp3 or rAAV-GFP were mixed with 1x10^7 $CD4^+CD25^-$ T cells as indicated, and were injected i.v. into CBA mice (6 mice/ group). The I.I. of each group was assessed as described above.

Statistical analyses. Mean, SD, and statistical significance were calculated using an SPSS application. Statistical analysis was performed using the nonparametric Mann-Whitney U test. A value of $p \leq 0.05$ was considered significant.

Results

Foxp3 is preferentially expressed in mouse $CD4^+CD25^+$ T cells. To analyze the expression of Foxp3 in mouse T cells, spleen mononuclear cells were isolated and specific subsets were purified on the basis of cell-surface phenotype. $CD4^+CD25^+$ T cells were positively selected from isolated $CD4^+$ T cells. Both populations ($CD4^+CD25^+$ T cells or $CD4^+CD25^-$ T cells) showed a purity of >90% (Fig. 2).

Foxp3 mRNA expression was first ascertained by RT-PCR. Total $CD4^+$ T cells transcribed by the Foxp3 gene, and the $CD25^+$ subset, which constituted 5-10% of $CD4^+$ T cells, exhibited predominant transcription (Fig. 3A). QPCR analysis revealed that the Foxp3 mRNA levels were increased 30-fold in $CD4^+CD25^+$ T cells compared to $CD4^+CD25^-$ T cells (Fig. 3B).

Foxp3 gene expression mediated by rAAV. rAAV vectors carrying either the Foxp3 or the GFP expression cassette were successfully generated. Foxp3 mRNA levels of rAAV-Foxp3-transduced $CD4^+CD25^-$ were determined by QPCR. As shown in Fig. 4A, rAAV-Foxp3 significantly increased the Foxp3 expression levels in transduced $CD4^+CD25^-$ T cells, while rAAV-GFP-transduced $CD4^+CD25^-$ T cells and control T cells showed no increase in Foxp3 expression levels.

Because protein levels are biologically more relevant to their function, the Foxp3 protein expression was examined by immunoblotting assays. Immunoblotting assays revealed that a 48-kDa protein was detectable in AAV-Foxp3-

transduced T cells and was virtually undetectable in rAAV-GFP-transduced T cells and control T cells (Fig. 4B).

Assessment of rAAV-Foxp3-transduced T cell function in vitro. $CD4^+CD25^-$ T cells transduced by rAAV-Foxp3 demonstrated a significantly reduced proliferative response towards CD3 mAb-induced stimulation, whereas rAAV-GFP-transduced $CD4^+CD25^-$ T cells (or untreated, freshly isolated $CD4^+CD25^-$ T cells) showed normal proliferation rates (Fig. 5A). rAAV-Foxp3-transduced T cells (suppressors) and untreated $CD4^+CD25^-$ T cells (responders) were co-cultured in order to determine the anti-proliferative effect of Foxp3 on the proliferation of naïve $CD4^+CD25^-$ T cells in the presence of CD3 mAb. As shown in Fig. 5B, rAAV-Foxp3-transduced T cells significantly reduced the proliferation of $CD4^+CD25^-$ T cells. Furthermore, increasing the ratio of rAAV-Foxp3-transduced T cells (suppressors) to untreated $CD4^+CD25^-$ T cells (responders) increased the anti-proliferative effect of Foxp3.

rAAV-Foxp3-transduced T cells prevent thyroiditis development. To evaluate the role of AAV-Foxp3-transduced T cells in controlling thyroiditis development, we cotransferred them, together with $CD4^+CD25^-$ T cells from early thyroiditis mice, into recipient mice. As shown in Table I, transfer of $1x10^7$ $CD4^+CD25^-$ T cells from EAT mice induced significant thyroiditis with a mean I.I. reaching 2.0. With a cotransfer of $1x10^7$ $CD4^+CD25^-$ T cells from EAT mice and $2x10^6$ rAAV-Foxp3-transduced T cells, only one of six animals developed thyroiditis, but a cotransfer with rAAV-GFP-transduced T cells did not prevent thyroiditis development. These results indicate that transduced T cells by rAAV-Foxp3 may control thyroiditis development *in vivo*.

Discussion

Increasing interest in regulatory T cells has emerged after the observation of Sakaguchi and colleagues that naturally occurring $CD4^+CD25^+$ T cells are capable of preventing autoimmune disease (2). Treg cells have been found and studied in humans and rodents. These cells have been shown to develop in the thymus and represent a minor (5-10%) portion of $CD4^+$ T cells but possess potent immuno-suppressive activity (4-7). Initial studies indicate that *scurfy* mice are hyperresponsive to immune stimulation and develop a lymphoproliferative disease similar to that seen in animals deficient in either CTLA-4 or TGF-ß (23-25). The disease-associated gene was recently identified as a novel member of the *forkhead*/winged helix family of transcriptional regulators and was designated as Foxp3. In addition to the forkhead domain, the protein also contains a single C_2H_2 zinc finger and a sequence that resembles the leucine-zipper motif of the N-myc protein. Foxp3 is thought to act as a transcriptional repressor and the targets are composite nuclear factors of activated T cells/activator protein1 sites in the 5' regulatory sequences of cytokine genes, such as IL-2 (26,27). Deficiency or mutation in Foxp3 leads to a fatal T cell-mediated autoimmune disease in both mice and humans (13,16,28). Our data demonstrate that $CD4^+CD25^+$ T cells express higher levels of Foxp3 mRNA than other T cell populations. These findings suggest that Foxp3 is a specific marker of $CD4^+CD25^+$ Treg cells.

Regulatory T cells can be delineated into two broad subsets: naturally occurring cells with an innate immuno-suppressive activity and those that act immunosuppressively upon stimulation. Evidence exist for the possibility to generate *in vitro* regulatory T cells derived from mice and humans (6,29,30). rAAV vectors carrying the mouse Foxp3 gene under the control of the CMV promoter were used to transduce naïve $CD4^+CD25^-$ T cells. Subsequently, Foxp3 overexpressing $CD4^+CD25^-$ T cells were highly resistant to a CD3 mAb-induced proliferation stimulus. In addition, these Foxp3-transduced T cells were able to specifically reduce the proliferation of freshly prepared $CD4^+CD25^-$ effector T cells in the presence of CD3 mAb *in vitro*. These data demonstrate that Foxp3-transduced $CD4^+CD25^-$ T cells are similar to naturally occurring $CD4^+CD25^+$ regulatory T cells which show weak proliferative capacity and suppressive activity.

It has been shown that $CD4^+CD25^+$ regulatory T cells are immunosuppressive and that this immunosuppressive property is indeed beneficial during autoimmunity and transplantation rejection in mice (31-33). A number of strategies to expand Treg cells using cytokines such as TGF-ß, IL-2 and IL-15 have been proposed (34,35). As a 'master control gene', the foxp3 gene is the inducer of specific regulation. In the present study, we have determined that pathogenic T cells induce autoimmune thyroiditis. rAAV-Foxp3-transduced $CD4^+CD25^-$ T cells, but not rAAV-GFP, inhibit autoimmune thyroiditis development when cotransferred with pathogenic T cells into recipients. This raises the possibility of Foxp3-transduced $CD4^+CD25^-$ T cells as a therapeutic strategy for immune disorders.

Acknowledgements

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