N-Ras or K-Ras inhibition increases the number and enhances the function of Foxp3 regulatory T cells

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Naturally occurring regulatory T cells (Treg) driven by their transcriptional controller Foxp3 are compromised in immune-mediated disorders and confer protection when adoptively transferred. We examined the Ras-inhibitory effect on functional determinants of Treg in vivo and in vitro. Ras was inhibited in Jurkat T cells by transfection with a dominant-negative form of Ras, or by shRNA for N-Ras, K-Ras, and H-Ras, or by farnesylthiosalycylic acid, a small-molecule inhibitor. Except for H-Ras transduction with shRNA, each inhibitory mode increased expression of Foxp3 and nuclear factor of activated T cell proteins, and surface expression of CD25. Ras inhibition in PBMC and spleen-derived lymphocytes reproduced these findings. The heightened Foxp3 expression reflected both increased basal cellular protein and peripheral conversion of non-Treg to Treg. Ras inhibition enhanced Treg-induced suppression; thus, when adoptively transferred to mice, Ras-inhibited Treg reduced the incidence of diabetes. Inhibition of Foxp3 by respective siRNA reversed the enhancement. Thus, inhibition of the N- or K-Ras isoform triggers an anti-inflammatory effect by up-regulating, via Foxp3 elevation, the numbers and functional suppressive properties of Treg.

Key words: Autoimmunity · Diabetes mellitus · Foxp3 · Ras · Regulatory T cells

Introduction

Regulatory CD4+CD25+ T cells (Treg) that originate in the thymus are termed 'naturally occurring' Treg. This population is distinct from another population of Treg, which are induced in the periphery in response to different antigens and are called 'adaptive' Treg [1, 2]. Patients with various autoimmune disorders possess naturally occurring CD4+CD25+ Treg that have diminished functional properties and whose numbers in the circulation are relatively small [3–5]. These observations are corroborated by findings of essentially similar defects in Treg cell homeostasis in animal studies of experimental immune-mediated disorders [3–5].

The Foxp3 protein is the master transcriptional regulator of naturally occurring Treg. It belongs to the forkhead protein family and is principally viewed as a repressor of genes that promote inflammation [6]. Full-length Foxp3 is encoded by 11 exons and contains a forkhead DNA-binding domain at the C terminus that acts by forming a repressor complex with nuclear factor of activated T cells (NFAT) [7]. This complex can bind in turn to the IL-2 promoter and repress transcription of IL-2 mRNA. In addition to the DNA-binding domain, which is also important for the protein's nuclear location, Foxp3 encodes zinc-finger and leucine-zipper domains that permit homo- or heterodimerization with other forkhead family members or other DNA-binding cofactors. The protein's N terminus also possesses essential sites for its repressor activity [8, 9].

The Ras genes (N-Ras, K-Ras, and H-Ras) encode members of the superfamily of small GTP-binding proteins, which have diverse intracellular signaling functions including the control of cell differentiation, proliferation, growth, and apoptosis [10]. Activating mutations in Ras occur in up to 30% of human malignancies [11]. Ras proteins are signal-switch molecules that modulate cell...
fate by cycling between inactive GDP-bound and active GTP-bound conformations (Ras-GDP and Ras-GTP). Ras receives extracellular signals from membrane receptors and recruits various effectors to the plasma membrane [11]. Raf, an important Ras effector, then phosphorylates Mek, which in turn phosphorylates the Erk/MAPK kinases that relay information to multiple cytoplasmic and nuclear effector targets [12].

Several lines of evidence support a role for Ras signaling in T cell activation. Those data come from studies showing that mutations in distinct forms of Ras result, both in experimental animals and in humans, in a robust proliferative autoimmune phenotype [13]. In addition, recent data indicate that Ras inhibitors are capable of suppressing experimental autoimmune disorders in animals and favor a T helper (Th)1-dominated phenotype in lymphocytes [14, 15].

In view of the involvement of Ras in immune-mediated disorders, we put forward the hypothesis that the immune suppression, which accompanies Ras inhibition, might be mediated by up-regulation of Treg cell number and function via an increase in Foxp3.

Results

Ras inhibition in vitro increases the amount of Foxp3 and CD25 expression

To test the hypothesis that Ras inhibition results in up-regulation of Treg, we transfected Jurkat T cells with dominant-negative (DN) green fluorescent protein-labeled Ras (GFP-Ras) or a GFP-labeled control plasmid, and assayed the total Ras protein, Ras-GTP, Foxp3, and NFAT. The results clearly showed an increase in the amounts of both Foxp3 and NFAT and a decrease in Ras-GTP (Fig. 1A), suggesting that active Ras inhibition might positively regulate the expression of Foxp3. To determine which of the three prominent Ras isoforms participate in this negative regulation we retrovirally transduced specific short-hairpin RNA (shN-Ras, shK-Ras, and shH-Ras RNA) into Jurkat T cells. Whereas all three shRNA forms significantly inhibited Ras protein expression, only N-Ras and K-Ras were associated with up-regulation of Foxp3 (Fig. 1B). Thus, up-regulation of Foxp3 appears to be exerted by N-Ras and by K-Ras isoforms, but not by H-Ras.

To verify the effect of Ras inhibition on Foxp3 levels in human cell types other than Jurkat T cells, we employed the small-molecule Ras inhibitor, farnesylthiosalycylic acid (FTS, Salirasib). First, we examined whether FTS mimics the previously observed effects of DN-Ras and of the shRNA on Foxp3 levels in the Jurkat T cells. Ras inhibition by FTS in these cells was associated with an increase in Foxp3 protein, which was already detectable 24 h after the onset of FTS treatment and persisted for at least 72 h (Fig. 1C). We then examined the effect of FTS on the Foxp3 content of PBMC obtained from healthy donors and incubated for 48 h with the drug. During that period, we observed a dose-dependent increase in the Foxp3 content of the treated PBMC (Fig. 1D). Flow cytometric analysis showed that this FTS-induced increase in Foxp3 levels was associated with a dose-dependent increase in the expression of CD25 in CD4+ cells (Fig. 1D). In separate experiments, FTS treatment of Jurkat T cells was accompanied by significant up-regulation of Foxp3 mRNA levels, with a peak at 24 h (Fig. 1E) and a lack of effect observed at later time points. These findings indicate that Ras inhibition by FTS (Fig. 1C) led to an increase in mRNA Foxp3 expression (Fig. 1E).

Inhibition of Ras in mouse splenocytes up-regulates nuclear Foxp3 expression

We next examined the effects of Ras inhibition on the Foxp3 content of murine cells. We incubated splenocytes obtained from BALB/c mice with various concentrations of FTS and then measured their contents of CD4+ and Foxp3+. As shown in Fig. 2A, FTS treatment was accompanied by a dose-dependent increase in the number of CD4+ cells expressing Foxp3. We then examined whether the up-regulation of Foxp3 induced by the Ras inhibitor was occurring in the cytoplasm or in the nucleus of the splenocytes. This was done by repeating the above experiment in which mouse splenocytes were incubated with FTS, then performing nuclear fractionation and subjecting the obtained proteins to western blotting. The results showed that the up-regulatory effect of FTS on Foxp3 and NFAT was confined to the nucleus (Fig. 2B).

FTS up-regulates Foxp3 and induces conversion of effector T cells (Teff) to Treg

Up-regulation of Foxp3 could potentially occur in two ways: via an increase in basal expression of protein per cell, or by conversion of CD4+CD25- cells to CD4+CD25+ cells acquiring Foxp3 expression, and thereby potentially equipping them with the capacity for regulation. To examine the first possibility, we used a cell sorter (see Materials and methods) to isolate CD4+CD25− T cells from mouse splenocytes treated in vivo with FTS (20 mg/kg, daily for 3 weeks), as well as the corresponding CD4+CD25+ cells from the splenocytes of control mice treated with PBS. Immunoblot analysis of the two groups of Treg disclosed that the Foxp3 protein content was significantly higher in the cells derived from FTS-treated mice, pointing to an increase in Foxp3 levels per cell (Fig. 3A). We also found, however, that CD4+CD25− cells (Teff) lacking Foxp3, which were also isolated from murine splenocytes by the cell sorter, showed an increase in both CD25 and Foxp3 after FTS treatment in vitro (Fig. 3B). Taken together, these results suggest that Ras inhibition both increased Foxp3 levels in pre-existing Treg and induced conversion of effector (CD4+CD25− T cells) to Foxp3 Treg (CD4+CD25+).
Figure 1. Ras inhibition in vitro increases the Foxp3 content of Jurkat and Treg. (A) Jurkat T cells were transfected with DN-Ras-GFP or GFP plasmid (GFP) or empty (pc) plasmid, and the levels of Foxp3, Ras-GTP, Ras, NFAT and tubulin were then determined by immunoblotting as described in Materials and methods. Left: representative immunoblots. Right: densitometry; *p<0.05, n=3. (B) shRNA for H-Ras (H), K-Ras (K), N-Ras (N) or a scrambled derivative (-) were retrovirally introduced into Jurkat T cells, and the expression of each Ras isoform and of Foxp3 was then determined by immunoblotting. Left: representative blots. Right: densitometry; *p<0.05 n=3. (C) Jurkat T cells were incubated in the absence or presence of FTS (50 μM) for 24 or 72 h and then assayed by immunoblotting for Foxp3, Ras, Ras-GTP, and tubulin. Representative blots (left panel) and densitometry (right panel, n=3) are shown; *p<0.05. (D) Human PBMC were incubated with or without concentrations of FTS for 48 h, and were assayed for Foxp3 and tubulin by immunoblotting or by flow cytometry as described in Materials and methods. Left: representative immunoblots. Right: densitometry, *p<0.05, n=3. Lower panel: Flow cytometric analysis demonstrating the dose-dependent increase in CD4+CD25+ Treg. (E) Jurkat T cells were incubated with or without FTS (50 μM) for the indicated periods and were then assayed for Foxp3 mRNA and GAPDH mRNA by RT–PCR. Representative gels (left panel) and densitometry (right panel, n=3) are shown; *p<0.05.
Ras inhibition increases Foxp3 and NFAT expression in vivo

We then examined the effects of Ras inhibition in vivo on the numbers of functionally active Treg. Hydrodynamic injection of DN-GFP-Ras or a control GFP plasmid into the tail veins of wild-type BALB/c mice resulted in a significant decrease in Ras-GTP levels in the splenocytes of mice treated with the DN-GFP-Ras (data not shown), as well as a significant increase in the number of CD25⁺Foxp3⁺ cells (Fig. 4A). To confirm these in vivo findings, we treated mice with FTS or PBS as described above and then assayed their splenocytes for Foxp3 and NFAT expression by immunoblotting and counted the Treg by flow cytometry. Consistently with the outcome of the hydrodynamic DN-Ras injections, in vivo FTS treatment significantly increased both the expression of Foxp3 and NFAT in the splenocytes (Fig. 4B) and the numbers of CD4⁺Foxp3⁺ (as well as of CD25⁺Foxp3⁺ cells) in the spleens (Fig. 4C).

Ras inhibition enhances the suppressive properties of Treg in a Foxp3-dependent manner

Next, we examined the possibility that Ras inhibition was associated not only with an increase in the number of Foxp3 Treg but also with enhancement of their suppressive properties, as reflected in their ability to diminish the proliferation of effector T cells. We treated BALB/c mice with FTS (20 mg/kg, i.p., daily for 3 weeks) or with PBS, then isolated the CD4⁺CD25⁺ Treg from their splenocytes and assessed their suppressive properties by incubating them with Teff (CD4⁺CD25⁻) from untreated mice, at Treg to responder ratios of 1:1 to 1:8 (Fig. 5A). The suppressive activity of Treg from the FTS-treated mice was significantly higher than that of Treg from the PBS-treated mice (Fig. 5A). Overall, these findings thus indicate that Ras inhibition not only increased Treg cell numbers and their Foxp3 content, but also enhanced their suppressive capacity.

To determine whether the enhanced suppressive activity of Treg from the FTS-treated mice is Foxp3 dependent, we carried out a suppression experiment similar to the one described above except that the expression of Foxp3 was blunted. To achieve this effect, we designed short interfering (si)RNA oligos, which, unlike a control siRNA, effectively reduce Foxp3 expression in murine splenocytes (Fig. 5B). We then subjected Treg obtained from the splenocytes of FTS-treated mice to transfection with the control or the Foxp3 siRNA, and examined the effects of these transfectants on the proliferation of effector CD4⁺CD25⁺ T cells obtained from naive mice. The partial deletion of Foxp3 expression (Fig. 5B) strongly attenuated the suppressive activity of the Treg obtained from the FTS-treated mice (Fig. 5C). This finding indicated that Foxp3 plays an essential role in mediating the increased suppressive capacity of Treg induced by Ras inhibition.

To examine whether the enhanced suppressive activity of Treg induced in mice by FTS has functional consequences in vivo, we...
used an animal model of diabetes in which Treg have been shown to play a protective role [16]. After isolating Treg from FTS-treated and from PBS-treated BALB/c mice as before, we adoptively transferred Treg from each group into mice with cyclophosphamide-induced diabetes. The incidence of diabetes was then assessed in the two recipient groups (Fig. 5D). Transfer of Treg from the FTS-treated mice induced a markedly lower incidence of diabetes than that recorded in a control group of mice injected with PBS only (70 and 50%, respectively, on days 9 through 13 and on days 14 through 21 after cell transfer; Fig. 5D). The transfer of Treg from PBS-treated mice had only a minor effect on the incidence of diabetic mice (20% lower than in the control group; Fig. 5D).

**Discussion**

Activated Ras binds to and activates a number of signaling molecules that trigger T cell proliferation, differentiation, and functional programs [10, 17]. In line with those observations are reported findings that inhibition of Ras activation results in the development of anti-inflammatory responses [15] and attenuation of experimental autoimmune disorders [14, 18, 19].

The novel finding of the present study is that the effects of active Ras on the immune system are mediated, at least in part, by control of the homeostasis of naturally occurring Treg. We showed, first, that inhibition of Ras activation by transfection with a DN-Ras, or by infection with shRNA for N-Ras or K-Ras, or by the activity of the small-molecule Ras inhibitor FTS was capable of up-regulating Foxp3, the master transcriptional factor for Treg. Similar and consistent findings were obtained when we treated murine lymphocytes with FTS. Taken together, these experiments strongly suggest that N-Ras and K-Ras signaling play an important role in the control of Foxp3+ Treg.
Although post-transcriptional effector mechanisms of Foxp3 remain elusive, it was recently suggested that Foxp3-related anti-inflammatory programs rely on the formation of complexes with NFAT, which imposes a repressive signal on the IL-2 promoter [7]. Accordingly, we chose to assay NFAT in most assays involving Ras inhibition. We found that the patterns of NFAT expression essentially mimicked those observed for Foxp3, suggesting either that cross-talk mechanisms govern their regulation or, alternatively, that their induction is controlled by common factors.

The suppressive effect of Treg on the proliferation of effector T cells appears to play an even more detrimental role than their numbers in the circulation. Disease activity in various autoimmune disorders is indeed found to be associated with defective suppressive properties [3, 5]. We showed here that the suppressive effect of Treg from mice treated with the Ras inhibitor is more robust than that from untreated controls. We then demonstrated that the effect of Ras inhibition on Foxp3 expression is twofold: it increases Foxp3 protein levels in lymphocytes that express this protein, and it promotes conversion of CD4⁺CD25⁻Foxp3⁻ cells (effector T cells) to CD4⁺CD25⁺Foxp3⁺ Treg. We found that the enhancement of Treg-induced suppressive properties that is caused by Ras inhibition was mediated via Foxp3 up-regulation, as inhibition of the latter by the relevant siRNA led to attenuation of the suppressive effect of FTS. These cumulative findings prompted us to assay the effects of FTS-induced Treg in an in vivo model of experimental diabetes, an immune-mediated disorder.

Diabetes mellitus is an autoimmune disease in which Treg appear to play an important role [16, 20]. Thus, for example, in diabetic patients both the numbers and the function of Treg are compromised [21], and in experimental animal models of diabetes the adoptive transfer of Treg results in amelioration of the disorder [16]. We therefore considered diabetes to be a suitable animal model for studying the efficacy of Ras inhibition on Treg cell function in vivo. We found that adoptive transfer of Treg from FTS-

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Figure 4. FTS and DN-Ras increase the Foxp3 and the NFAT content in vivo. (A) BALB/c mice were hydrodynamically injected (i.v.) with DN-GFP-Ras or with GFP-encoding plasmids, as described in Materials and methods. Expression of CD25 and of Foxp3 in splenocytes was assayed by flow cytometry 48 h after injection. The figure represents typical flow cytometry data obtained from four independent experiments (two left panels) and mean + SEM from four separate experiments (right panel; *p<0.05). (B) BALB/c mice were treated i.p., daily for 3 weeks, with FTS (20 mg/kg; n = 4) or with PBS (n = 4) and the expression levels of Foxp3, Ras and NFAT proteins in their splenocytes were then assayed by western blotting. Densitometry is shown in the right. *p<0.05. (C) BALB/c mice were treated with FTS or PBS as in (B). Their splenocytes were then stained for CD25 and Foxp3, or for CD4 and Foxp3, and analyzed by flow cytometry. n = 4 in each experiment. *p<0.05.
treated mice into mice with cyclophosphamide-induced hyperglycemia indeed resulted in a significantly more substantial decrease in the incidence of diabetes than that observed in recipients of Treg from untreated mice. These findings support the assumption that Ras inhibition conferred by FTS treatment provides partial protection against diabetes by promoting the suppressive functions of Treg.

In conclusion, we found that inhibition of Ras signaling in T cells enhances the expression of Foxp3 and NFAT and consequently increases the numbers of functionally active, naturally occurring Treg. These findings might shed light on the mechanisms controlling Treg cell induction and extend the use of Ras inhibitors to immune-mediated disorders.

**Materials and methods**

**Cell culture and reagents**

Jurkat T cells, HEK 293T cells, and splenocytes were cultured in complete RPMI medium or DMEM medium supplemented with 10% FBS (Biological Industries, Kibbutz Beit haEmek, Israel) for the indicated periods. FTS and cell treatment conditions were described earlier [22]. All peripheral blood samples were obtained from healthy subjects. PBMC were isolated from freshly human blood by Ficoll gradient.
Western blotting and Ras-GTPase pull-down assays

Lysates containing 50–100 µg protein were subjected to SDS-PAGE followed by western blotting, as described [22], with one of the following antibodies: pan-Ras Ab (1:2500, Ab-3; Calbiochem, San Diego, CA), anti-phosphorylated ERK Ab (1:2500), anti-phosphorylated Akt Ab (1:1000), anti-tubulin Ab (1:500), anti-Foxp3 Ab (1:1000, eBioScience, San Diego, CA) and anti-NFATc2 Ab (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). The immunoblots were then exposed to peroxidase goat anti-mouse IgG (1:5000), peroxidase goat anti-rabbit IgG (1:5000), or peroxidase donkey anti-goat IgG (1:5000). Protein bands were visualized by enhanced chemiluminescence and quantified by densitometry with Image Master VDS-CL (Amersham, Arlington Heights, IL) using TINA 2.0 software (Ray Tests). Lysates containing 500 µg protein were used to determine Ras-GTP by the glutathione S-transferase (GST)–Ras-binding domain pull-down assay, and this was followed by western blotting with anti pan-Ras Ab as previously described [22].

Cell separation and flow cytometry

PBMC, splenocytes, and Jurkat cells were stained with combinations of the following mAb: FITC-labeled anti-CD4 (RPA-T4), phycoerythrin (PE)-labeled anti-CD25 (BC96), FITC-labeled or PE-labeled mouse IgG1 isotype control (P3) for nonspecific staining (all from eBioscience). Staining of cells was analyzed with a FACSCalibur (Becton Dickinson, NJ). Human Foxp3 was stained using the anti-Foxp3 staining kit (eBioscience), according to the manufacturer’s instructions. CD4+CD25− T cells (effector T cells) and CD4+CD25+ (Treg) cell populations were isolated from 1 × 10^6 splenocytes by FACS sorting (FACSAria, Becton Dickinson) after staining with FITC-labeled anti-CD4 and PE-labeled anti-CD25 Ab for 30 min.

Nuclear extracts

Jurkat cells (1 × 10^6 cells) were seeded and treated with 50 mM FTS. The cells were then washed with PBS and resuspended in 400 µL of hypotonic buffer A (10 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA 10 mM KCl, 1 mM DTT, and protease inhibitors). The cells were gently resuspended, Nonidet P-40 was added (10%), and the pellet was vortexed and kept on ice for 30 min. Lysates were spun down at 14 000 rpm at 4°C for 1 min. The supernatant was removed (cytoplasmic extract) and the nuclear pellet was resuspended in 30 µL of buffer B (20 mM Tris-HCl pH 7.9, 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitors) with frequent vortexing for 15 min at 4°C. Finally, the nuclear extract was spun at 14 000 rpm for 5 min and analyzed by western blotting as described above.

Transfection of DN-Ras and siRas constructs

Jurkat T cells (2 × 10^7 cells per sample) were electroporated with 50 µg of dominant-negative DN-GFP-Ras (17N), enhanced green fluorescent protein plasmid (pEGFP), or empty plasmid (pDNA3) at 310 V for 10 ms in an ElectroSquarePorator (ECM 830; BTX, San Diego, CA). The cells were harvested 48 h after transfection and analyzed by western blotting and flow cytometry. Splenocytes were transfected with 25 nM small interfering RNA (siRNA) for Foxp3 or a scrambled nonsilencing control oligo (msRNA), using TransIT-TKO siRNA Transfection Reagent (Mirus Bio Corporation, Madison, WI) according to the manufacturer's instructions. Knockdown efficiency was assessed 3 days later by immunoblotting. The oligonucleotide sequence for Foxp3 was 5’ACACCCAGGAAAGACAGCAACCUUU3’.

Infections

The shRNA for H-Ras, K-Ras, N-Ras, and a control scrambled derivative were purchased from Open Biosystems (Huntsville, AL). The sequences of these oligos were as follows: K-Ras, 5’GCATTATTTACATGTACTA3’; H-Ras, 5’CAGTACAGGGAGCATC3’; N-Ras, 5’CAGATGTCTAATATTGTA3’. Using the calcium-phosphate transfection method, subconfluent HEK 293T cells (30–40%) were co-transfected with 6 µg of transfer plasmid containing the shRNA (H/K/N Ras or shRNA control), 3 µg of packaging plasmid (pCGP), and 3 µg of envelope vector (pMD2G). After 24 h, the medium was changed and 24 h later the supernatants were harvested and filtered through 45-µm pore membranes. Jurkat cells (1 × 10^6 cells) were seeded in 60-mm plates and transduced with the filtered virus in the presence of 2 µg/mL polyberne. The medium was changed 24 h after infection, and 72 h after infection the cells were harvested and analyzed by immunoblotting using anti K-Ras, anti-H-Ras, and anti-N-Ras Ab (1:50; Calbiochem).

Functional suppression assays

For assay of function, 96-well plates (CoStar, Corning, New York) were coated at 4°C overnight with 1 µg/mL anti-CD3 mAb (17A2, eBioscience) and then washed. CD4+CD25− cells (Teff) and CD4+CD25+ (Treg) were cultured (1 × 10^4 cells/well) in RPMI medium supplemented with 10% FBS, at different ratios of responder to suppressor T cells (1:1, 1:2, 1:4, and 1:8). All cells were cultured, in a final volume of 200 µL, in the presence of 2.5 µg/mL anti-CD28 mAb (37.51, eBioscience). After 72 h, [3H]thymidine (1 µCi/well) was added for 16 h, and proliferation was then assayed by a scintillation beta counter. Percent inhibition of proliferation was determined from the following formula: 1–(median [3H]thymidine uptake of 1:1 CD4+CD25−:CD4+CD25+ (1:1 Treg/Teff) coculture/median [3H]thymidine uptake of CD4+CD25− cells).
In vivo studies: Adaptive transfer and hydrodynamic delivery

The study was approved by the Institutional Ethics Committee at the Tel Aviv Medical Center.

To study the effect of FTS on Treg, we injected 8-week-old female BALB/c mice with FTS (20 mg/kg body weight; i.p., n = 10) or a control vehicle (n = 10) six times a week for 3 weeks. The mice were then killed, their spleens were removed, and expression levels of CD4, CD25, and Foxp3 in splenocytes were assayed by western blotting and by flow cytometry.

To accelerate diabetes development in BALB/c mice, cyclophosphamide was prepared in 0.9% normal saline (200 mg/mL) and injected i.p. on day 0.

Purified populations of T cells from FTS-treated mice or PBS-treated controls were obtained by FACS sorting, as described. Purified Treg (0.5 × 10^6 cells) from each group were transferred i.v. into 8-week-old female BALB/c mice 24 h after cyclophosphamide injection. Glucose levels in all recipient mice were then assayed.

BALB/c mice (n = 8) received hydrodynamic DNA injections which involves rapid, high-pressure injection of a DNA solution into the tail vein. The mice were injected with 100 μg pDN-Ras-GFP in 2 mL of PBS, i.v. into the tail vein within 5–8 s. Control mice were injected with pGFP (n = 8) or pcDNA3 (n = 6). Seventy-two hours after injection, mice were sacrificed and expression levels of Ras, Foxp3, CD4, and CD25 in splenocytes were assayed.

Statistical analysis

Data are presented as mean + SEM. Comparison between the groups was performed employing the Student’s t-test. Diabetes incidence data were analyzed by comparison of survival curves with Kaplan-Meier statistics. The p value <0.05 was considered significant.

Acknowledgements: This work was supported in part by a grant by the Israel Science Foundation (JG; Grant no. 832/06) and by in part by The Wolfson Foundation (YK). Yoel Kloog is the incumbent of The Jack H. Skirball Chair for Applied Neurobiology. The work done by Adi Mor partially fulfills the Ph.D. degree requirements at the Sackler Faculty of Medicine, Tel Aviv University, Israel. We thank Shirley R. Smith for editorial assistance. All four authors took part in the design of the study and in the writing process. Most studies were conducted by A. M.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References


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Abbreviations: **DN**: dominant negative  
**FTS**: farnesylthiosalicylic acid  
**Teff**: effector T cells

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Received: 28/2/08  
Revised: 3/3/08  
Accepted: 18/3/08

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