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***In vitro* induction of regulatory T cells by anti-CD3 antibody in humans**

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Abstract

Therapy with anti-CD3 antibody is effective in controlling models of autoimmune diseases and can reverse or prevent rejection of grafts. We studied the *in vitro* immunomodulatory effect of anti-CD3 treated human T cells. CD4⁺ T cells were stimulated with plate bound anti-CD3 and cultured for 12 days after which they were cultured with autologous peripheral blood mononuclear cells (PBMCs) and stimulated with soluble anti-CD3. We found that CD4⁺ T cells that were stimulated with anti-CD3 (T_{αCD3}) markedly suppressed the proliferation and cytokine production of autologous PBMCs. These regulatory T cells were not induced by incubation with isotype control (T_{Control}) antibody or when anti-CD3 was combined with high doses of anti-CD28 (T_{αCD3/CD28}). T_{αCD3} regulatory cells were anergic and produced lower levels of IFN-γ, TNF-α and IL-2, and higher levels of TGF-β than T_{Control} or T_{αCD3/CD28}. There were no differences in the expression of CD25, CD45RB or CTLA-4 on T_{αCD3} as compared to T_{Control} or T_{αCD3/CD28} and CD4⁺ CD25[−] T_{αCD3} cells were identical to CD4⁺ CD25⁺ T_{αCD3} cells in their *in vitro* suppressive properties. Recombinant IL-2 *in vitro* abrogated the suppressive effect of T_{αCD3}. The suppressive effect was not related to apoptosis, was independent of HLA since T_{αCD3} also suppressed allogeneic PBMCs, and was not related to soluble factors. Finally, no suppression was observed when non T cells were removed from culture or when cultures were stimulated with plate bound anti-CD3, consistent with the ability of T_{αCD3} to downregulate CD80 on dendritic cells in co-culture experiments. Thus, we have identified human T cells with strong *in vitro* regulatory properties induced *in vitro* by anti-CD3 which appear to act in a non-HLA restricted fashion by affecting antigen presenting cells.

Introduction

Treatment with anti-CD3 antibody has been shown to be effective in several models of immune mediated disease. It reverses the rejection of renal (1,2), heart and liver transplantations, prevents and reverses virus-induced autoimmune diabetes and recent onset spontaneous autoimmune insulin-dependent diabetes of NOD mice (3). Treatment with anti-CD3 antibody was also found to reverse established EAE (4,5).

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Clinical trials in humans has also shown efficacy of anti-CD3 antibody. It prevents the rejection of renal, liver (6) and cardiac grafts (7); in recent-onset type1 diabetes, treatment with anti-CD3 antibody improved insulin production (8-11); in psoriatic arthritis anti-CD3 antibodies were found to improve the number of inflamed joints and the pain scale (12). Anti-CD3 treatment also was found to prolong the survival of allogeneic islet allografts implanted in recipients with long-standing type1 diabetes (13).

Different mechanisms have been proposed to explain the therapeutic effect of anti-CD3 antibody. One mechanism is via depletion of T cells. Depletion may occur by induction of apoptosis (particularly on activated T cells) as shown in vitro (14,15), by complement mediated depletion, or by antibody-dependent cellular cytotoxicity (ADCC) (16,17). Another mechanism that has been proposed is down regulation of the T cell receptor (TCR) complex after internalization. Finally, it has been shown in the mouse model of autoimmune diabetes that IV anti-CD3 therapy induces CD4+CD25+regulatory T cells that act in a TGF- β dependent fashion (3,18) and we have found the induction of CD4+CD25-LAP+ TGF- β dependent regulatory T cells following oral administration of anti-CD3(19).

In the present study we investigated the effect of in vitro anti-CD3 on T cell function in humans and found that anti-CD3 induced T regulatory cells that were anergic and appeared to function by affecting antigen presenting cells.

Materials and Methods

Cell Separation and Culture

Peripheral blood samples were obtained from healthy donors. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation (Pharmacia LKB Biotechnology, Piscataway, NJ) and CD4⁺ T cells were positively selected by using Dynabeads M-450 CD4 beads (DynaL Biotech ASA, Oslo, Norway). Some PBMC were frozen in 10% DMSO for use in a culture with antibody treated autologous CD4⁺ T cells. The CD4⁺ T cells were re-suspended (10^6 cells/ml) in complete culture media consisting of RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 25 mM HEPES buffer, 50 u/ml penicillin and 50 μ g/ml streptomycin (all from Whittaker Bioproducts, Walkersville, MD). The cells were incubated in 96-well plates (Coster, Corning, NY) that were pre-coated with 1 μ g/ml of mouse anti-human CD3 mAb (BD Bioscience #555336) or with its isotype control (1 μ g/ml mouse IgG_{2a}) for 5 days, in some cases soluble mouse anti-human CD28 mAb was added (10-50 μ g/ml) (all from PharMingen, San Diego, CA). After 5 days cells were removed to new un-coated plates and recombinant human IL-2 (PharMingen) was added to the culture in final concentration of 10 U/ml. New complete culture media was added every other day. After the CD4⁺ T cells had been cultured for 10-13 days and were in resting phase, they were collected and viable cells were isolated by Ficoll-Hypaque density gradient centrifugation. CD4+CD25⁺ and CD4+CD25⁻ T cells were isolated from PBMCs by sorting using FACSVantage SE cell sorter (BD Biosciences, Franklin Lakes, NJ) after staining with Cy-Chrome-conjugated mouse mAb directed at human CD4 and PE-conjugated mouse mAb directed at CD25 (both from PharMingen).

Proliferation Assays

Different numbers of conditioned CD4⁺ T cells ($5 - 50 \times 10^3$ cells/well) were co-cultured with 70×10^3 autologous or non-autologous PBMC in 96-well round-bottom plates (Corning Coster, Cambridge, MA) and stimulated with 1 μ g/ml of soluble anti-CD3 mAb. After 48h of culture, ³H-thymidine (1 μ Ci/well) was added for the last 12h of culture, cells were then harvested and the incorporation of thymidine was measured using the LKB Betaplate liquid scintillation counter. In some experiments 1 μ g/ml of plate-bound anti-CD3 was used for

stimulation. The proliferation of the conditioned CD4⁺ T cells was studied after stimulation with 1 µg/ml of plate-bound anti-CD3 mAb with or without addition of 1 µg/ml soluble anti-CD28 mAb or 20 U/ml of recombinant IL-2.

Flow Cytometry

T cells were detected for the expression of CD4, CD25, and CTLA4 using fluorochrome conjugated monoclonal antibodies (PharMingen). Intracytoplasmic staining of cytokines was done after incubation overnight in complete culture media with 3 µM monensin (PharMingen). After washing cells were stained and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA, U.S.A). Intracytoplasmic staining of T cells were done by PE-conjugated directed to human IL-10, TNF- α , IL-4 and IFN- γ in a Perm/Wash buffer (PharMingen). Proliferation was also measured by flow cytometry using 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, Oregon). $1-5 \times 10^6$ PBMCs/ml were incubated before culture with 2 µM CFSE in room temperature. The PBMC then were added to the culture and 48-72h later flow cytometric analysis was performed with FACSsort flow cytometer (Becton Dickinson) according to standard procedure.

Dendritic cells were studied by a cocktail of FITC-conjugated mouse mAb directed at human CD3, CD14, CD16, CD19, CD20, CD56 (lin 1) or appropriate isotype control antibodies and PE-conjugated mouse mAb directed at human CD11c or isotype control (all from BD Biosciences). Surface molecules such as HLA-DR, CD80, CD86, CD40 and their isotype controls were stained by Cy-Chrome-conjugated mouse mAb directed at these molecules (PharMingen, San Diego, CA).

Cytokine Assay

CD4⁺ conditioned T cells were incubated in a 96-well plates that were pre-coated with 1 µg/ml anti-CD3 mAb (PharMingen). Supernatants were taken from cultures at 24h-48h to determine the secreted levels of IL-2, IL-10, IL-5, TNF- α , IL-4, IL-13, IFN- γ and active TGF- β . All these cytokine levels were studied by an array-based ELISA assay (Pierce Biotechnology, Waburn, MA). Supernatants for cytokine were also taken from the co-culture of T _{α CD3} and PBMCs.

Apoptosis

PBMCs were stained with CFSE as described, before co-culture with induced CD4⁺ T cells. 24-48h after co-culture cells were stained with PE-conjugated Annexin-V (R&D System, Minneapolis, MN) and 7-Aminoactinomycin D (7-AAD) (Sigma, St. Louis, MO) for 15 minutes at RT. Apoptotic effect in the co-culture was study by flow cytometer.

Results

Stimulation of CD4 T cells in vitro with anti-CD3 induces T cells that suppress the proliferation of autologous PBMC

We investigated the effect of anti-CD3 stimulation of CD4⁺ T cells in vitro using a two step cell culture protocol as depicted in Figure 1. In the induction phase, CD4⁺ T cells prepared by positive selection with beads were cultured with plate bound anti-CD3 for 5 days in 96 U well plates (200,000/well). After 5 days the cells were harvested, combined, and ficolled. These cells that had been stimulated (conditioned) in vitro with anti-CD3 are referred to as T _{α CD3}. T _{α CD3} were then tested for their ability to suppress autologous T cell responses by mixing 70,000 autologous PBMC with 5,000, 25,000 or 50,000 T _{α CD3} cells for 72 hours in U-shaped 96 well plates with soluble anti-CD3. Proliferation was measured by adding thymidine at 48 hours of culture. 1 µg/ml was found to be an optimal dose for anti-CD3 in the induction culture (Fig 2A) and was used throughout the described experiments. As shown in Fig 2B, T _{α CD3}

significantly suppressed autologous PBMC proliferation as compared to T cells that had been cultured with an isotype control antibody in the induction culture (T_{control}). ($p < 0.0001$, $T_{\alpha\text{CD}3}$ vs. T_{control} in culture with 25 or 50×10^3 $T_{\alpha\text{CD}3}$ cells). As shown in Fig 2C, the suppressive effect of $T_{\alpha\text{CD}3}$ was observed for proliferation of both CD4 and CD8 T cells as measured by CFSE staining of responder cells. ($p < 0.001$). CFSE responder cells were also stained for intracytoplasmic cytokines after culture with $T_{\alpha\text{CD}3}$. As shown in Fig2D the percentages of autologous CD4⁺ T cells producing IFN- γ , TNF- α , IL-10 and IL-4 and of autologous CD8 cells producing IFN- γ was significantly reduced by culture with $T_{\alpha\text{CD}3}$ cells ($p < 0.01$). These results demonstrate that stimulation of CD4⁺ T cells by plate bound anti-CD3 induces T cells with profound regulatory properties in vitro ($T_{\alpha\text{CD}3}$ regulatory cells). It has been shown that high doses of exogenous IL-2 or anti-CD28 antibody can abrogate the suppressive activity of CD4⁺CD25⁺ regulatory T cells (20) and we found the same to be true for the induction of $T_{\alpha\text{CD}3}$ regulatory cells in our system when we added either 10-50ug or 20u/ml of IL-2 to the induction culture. Of note, we were also able to induce $T_{\alpha\text{CD}3}$ regulatory T cells using soluble anti-CD3 in the induction culture though the effect was markedly less (data not shown)

Induction of $T_{\alpha\text{CD}3}$ regulatory cells from both CD25⁺ and CD25⁻ T cells

In the murine model of diabetes in vivo treatment with parenteral anti-CD3 induced CD4⁺CD25⁺ Treg cells that could adoptively transfer protection (3). In order to investigate the relationship between the $T_{\alpha\text{CD}3}$ regulatory cells in our system to CD4⁺CD25⁺ Treg cells we measured the expression of CD25 and CTLA4 on $T_{\alpha\text{CD}3}$ cells and T cells induced with both anti-CD3 and anti-CD28. As shown in Fig. 3A there was no increased expression of CD25 or CTLA4 in $T_{\alpha\text{CD}3}$ cells compared to T_{control} although we did observe an increase when T cells were given a second signal with anti-CD28, cells which we have shown above do not have suppressive properties. We then asked whether the induction of $T_{\alpha\text{CD}3}$ regulatory cells by anti-CD3 was due to their preferential stimulation of CD25^{hi} T regulatory cells vs. CD25⁻ T responder cells. In order to address this, we sorted PBMCs into CD4⁺CD25⁻ and CD4⁺CD25⁺ populations and tested them in induction cultures with anti-CD3. As shown in Figure 3B, anti-CD3 induced $T_{\alpha\text{CD}3}$ regulatory T cells equally as well from CD25⁻ and from CD25⁺ populations.

$T_{\alpha\text{CD}3}$ regulatory cells are anergic and secrete increased amounts of TGF- β

In order to determine if $T_{\alpha\text{CD}3}$ regulatory T cells were anergic, we checked the proliferative responses of $T_{\alpha\text{CD}3}$ cells. As shown in Figure 3C, $T_{\alpha\text{CD}3}$ cells did not proliferate to either anti-CD3 or anti-CD3 plus anti-CD28. To further characterize the $T_{\alpha\text{CD}3}$ regulatory T cells we checked their cytokine profile after stimulation with anti-CD3 or with anti-CD3 plus anti-CD28. As shown in Table 1, as compared to T control, $T_{\alpha\text{CD}3}$ cells secreted less IL-2, IFN- γ , TNF- α , IL-10, IL-4 and IL-13. The only cytokine that was increased in $T_{\alpha\text{CD}3}$ regulatory T cells was TGF- β . We also measured the expression of Foxp3 (21) in $T_{\alpha\text{CD}3}$ cells and found a slight increase relative to T control that was comparable to levels in CD25^{int} effector cells but markedly less than in CD25^{hi} T regulatory cells (Fig 3D).

Suppression by $T_{\alpha\text{CD}3}$ regulatory T cells is dependent on non-T cells

To investigate whether suppression by $T_{\alpha\text{CD}3}$ regulatory T cells is dependent on the presence of non-T cells, we tested the ability of $T_{\alpha\text{CD}3}$ regulatory T cells to suppress the proliferation of T cells in the absence of non-T cells. As shown in Figure 4A, no suppression was observed when $T_{\alpha\text{CD}3}$ regulatory T cells were cultured with CD4⁺ cells alone whereas $T_{\alpha\text{CD}3}$ regulatory T cells suppressed whole PBMCs. Furthermore, $T_{\alpha\text{CD}3}$ regulatory T cells failed to suppress the proliferation of PBMC when the co-culture was stimulated with plate bound anti-CD3 antibody. $T_{\alpha\text{CD}3}$ regulatory T cells suppress the proliferation of allogeneic PBMC equally as

well as they suppress autologous PBMC. Thus, the suppressive effect is not HLA restricted. These results suggest that the suppression is mediated by interaction between the T_{aCD3} cells and non-T cells. We did not find that suppression by T_{aCD3} regulatory T cells was related to apoptosis of the target cell or secretion of soluble factors.

T_{aCD3} regulatory cells downregulate CD80 expression on dendritic cells

In order to investigate whether T_{aCD3} regulatory T cells affect surface characteristics or stimulatory capacity of DCs, dendritic cells were studied 24-48h after co-culture of conditional CD4⁺ T cells with autologous PBMC at a 1:1 ratio. DCs were studied by a cocktail of FITC-conjugated mouse mAb directed at humans CD3, CD14, CD16, CD19, CD20, CD56 (lin 1) or appropriate isotype control antibodies and PE-conjugated mouse mAb directed at human CD11c or isotype control (BD Biosciences). Surface molecules HLA-DR, CD80, CD86, CD40 and their isotype controls were stained by Cy-Chrome-conjugated mouse mAb directed at them molecules (PharMingen, San Diego, CA). As shown in Fig. 4B and 4C, there was a significant decrease in the expression of CD80 on DC activated in the presence of T_{aCD3} as compared to T control. There was no effect on the expression of HLA-DR, CD86, CD83 or CD40.

Discussion

There is increasing evidence that therapy with monoclonal antibody against CD3 is beneficial against T cell mediated immune diseases. Several mechanisms have been proposed for the action of anti-CD3 antibody. Inducing depletion of effector T cells, down regulation of TCR, and immune deviation of the pathogenic T cells toward Th2 cells reviewed in (22). The induction of regulatory T cells by anti-CD3 antibody has also been demonstrated in the animal model for diabetes, in which there was induction of CD4⁺CD25⁺ regulatory T cells that differ from the naturally occurring CD4⁺CD25⁺ regulatory T cells. The induced immune regulation was dependent on TGF- β , and raise the possibility that the induced T cells exert their regulatory function via TGF- β (3,18,23). We observed an increase of CD4⁺CD25⁺LAP⁺ T cells after oral anti-CD3 in mice (19). We were not able to differentiate LAP expression in the current human *in vitro* studies of anti-CD3 due to different expression patterns of LAP in humans vs. mice.

We have studied the *in vitro* effect of anti-CD3 antibody on human CD4⁺ T cells and found that it induces regulatory cells. CD4⁺ T cells that were treated with anti-CD3 antibody were able to suppress proliferation of autologous PBMCs, including both CD4⁺ and CD8⁺ T cells, and to suppress cytokine production from these T cells. These anti-CD3 induced regulatory T cells were found to be anergic.

The regulatory effect of T_{aCD3} did not appear to be due to soluble factors, and was not reversed by addition of neutralizing anti-IL-10 and anti-TGF- β . Furthermore, we found that the regulatory properties of T_{aCD3} required the presence of APC, as the response of purified T cells was not inhibited by T_{aCD3} . T_{aCD3} regulatory cells did not suppress the proliferation of PBMC that were stimulated by plate-bound anti CD3, but only those that were stimulated with soluble anti-CD3. We believe this relates to the fact that plate bound anti-CD3 directly stimulates T cells and thus the attachment of anti-CD3 to APCs that occurs. With soluble anti CD3 does not happen and APCs are required for the suppressive activity of T_{aCD3} . Consistent with this, analysis of dendritic cells (DC) after co-culture with T_{aCD3} showed a decrease in the percentages of DC expressing MHC class II and CD40, and no increase in the percentages of DC expressing CD80 and CD86. Similar effects of anergic T cells on DC have been described previously (24,25). Our observation that T_{aCD3} could not suppress the response of PBMCs stimulated with both anti-CD3 and anti-CD28 may be explained by the ability of anti-CD28 to overcome the down-modulatory effects of T_{aCD3} on CD80 and CD86 costimulatory molecules. CD25 was not found to be a good marker for T_{aCD3} T regulatory cells since there

was no correlation between expression of CD25 and the suppressive function of T_aCD3⁺ T reg cells(26). Furthermore, T_aCD3⁺ cells could be induced from either CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells.

The mechanism of action of T_aCD3 regulatory cells we have described is dependent on cell contact since we could not find a suppressive effect of supernatants from the co-culture of T_aCD3 with autologous PBMCs by either transferring the supernatant from the co-culture to a culture of PBMCs alone or by a transwell assay. This mechanism is consistent with reports that regulatory T cells function by modulating dendritic cell function (24,25,27). This fact, together with the profile of the secreted cytokines of T_aCD3, suggests that T_aCD3 is not a Tr1 type of regulatory cell or a Th3 cell. The cell-cell interactions are not restricted by HLA since T_aCD3 could suppress the proliferation of allogeneic PBMCs. We also found that the immune suppression mediated by T_aCD3 does not induce increased programmed cell death or apoptosis. Taken together our results suggest that the T_aCD3 regulatory T cells we induced in vitro function by inhibiting antigen presenting cell stimulatory capacity via a cell contact-dependent mechanism. Table 2 summarize characteristics that distinguishes between T_aCD3 CD4⁺CD25⁺ Treg, Tr-1 and Th3 cells. We note that this manuscript is part of a special series of papers on the mosaic of autoimmunity (28-38).

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Induction of $T_{\alpha CD3}$

Assay for $T_{\alpha CD3}$ suppression

Figure 1. The induction of regulatory T cells by anti-CD3 monoclonal antibody

CD4 T cells were positively selected by Dynabeads from peripheral blood mononuclear cells (PBMC). In the induction phase, CD4 T cells ($1 \times 10^6/\text{ml}$) were stimulated in vitro with plate-bound anti-CD3 mAb ($1 \mu\text{g}/\text{ml}$) for 5 days. After 5 days IL-2 was added ($10 \text{ u}/\text{ml}$) and fresh complete culture media was added every other day. After 12 days in the culture the cells were harvested and viable cells were selected by ficoll and centrifugation. These cells are referred to as $T_{\alpha CD3}$. Thereafter, $T_{\alpha CD3}$ were co-cultured with autologous PBMC for 72h and their ability to suppress proliferation was tested by incorporation of thymidine after stimulation with anti CD3 mAb and detection of the irradiation with β -counter.

Figure 2. The induction conditions of anti-CD3 stimulated regulatory CD4⁺ T cells

T _{α CD3} were tested for their ability to suppress autologous T cell responses by co-culture with PBMC. Proliferation was measured by adding thymidine at 48 hours of culture. A) Different doses of plate-bound anti-CD3 mAb (0.01, 0.1, 1 and 10 μ g/ml) were tested in the induction culture as described in Figure 1 for their ability to induce regulatory T cells. The ability to suppress the proliferation of autologous PBMC which is expressed as percent of suppression, increased in a dose dependent manner and reached its maximum effect at 1-10 μ g/ml of anti-CD3 mAb. B) Autologous PBMC (70×10^3 cells/ well) were co-cultured with different amount (5, 25 or 50×10^3 cells/well) of either T _{α CD3}, T_{Control} which consist of CD4⁺ T cells that were stimulated with an isotype control antibody in the induction culture, or with unconditioned CD4⁺ T cells in the presence of soluble 1 μ g/ml anti-CD3 for 72h. Only in the presence of T _{α CD3} was there a suppressive effect on PBMC proliferation, which increased depending on the number of T _{α CD3} cells. Proliferation was measured by H³-thymidine incorporation. C) PBMCs were stained with CFSE before co-culture with T _{α CD3} in the same cell proportion as above. 48h after co-culture cells were stained with either PE conjugated CD4 or PE conjugated CD8 mAb and the proliferation of the stained cells was measured by flow cytometry. T _{α CD3} suppressed the proliferation of both CD4⁺ and CD8⁺ T cells in the PBMC. D) CD4 and CD8 cells were stained for intracytoplasmic cytokines using APC-conjugated anti-IFN- γ , or anti-TNF- α , anti- IL-10 or anti-IL-4 mAb. Single cell cytokine production by flow cytometry showed suppression of IFN- γ , TNF- α , IL-10 and IL-4 CD4⁺ producing T cells and IFN- γ producing CD8⁺ T cells. There was no detection of IFN- γ , TNF- α , IL-10 and IL-4 in the CD8⁺ T cells.

Figure 3. Characteristics of anti-CD3 induced regulatory CD4⁺ T cells

A) The expression of CD25 and CTLA4 on $T_{\alpha CD3}$, $T_{control}$ and $T_{\alpha CD3/\alpha CD28}$ that were induced by anti-CD3 and high dose anti-CD28 (50 μ g/ml) was measured by flow cytometry after staining with FITC labeled CD4, PE labeled CD25 or PE labeled CTLA4. B) Sorted CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were stimulated with plate-bound anti-CD3 to induce regulatory T cells as described above in figure 1. After the induction phase, conditioned CD4⁺ T cells (5, 25 or 50 $\times 10^3$ cells/well) were co-cultured with autologous PBMC (70 $\times 10^3$ cell/well) and stimulated with 1 μ g/ml anti-CD3 mAb for 72h for proliferation assay. Both cell types yielded $T_{\alpha CD3}$ regulatory cells with no differences in their suppressive capacity. C) Proliferation responses after 72h of $T_{\alpha CD3}$ and $T_{control}$ to polyclonal stimulation with plate-bound anti-CD3 (1 μ g/ml) with or without soluble anti-CD28 (1 μ g/ml) or recombinant IL-2 (20 u/ml) D) Relative levels of FOXP3 mRNA were determined by real-time PCR.

Figure 4. The suppressive activity of T_αCD3 suppress is HLA restricted and is dependent on the presence of APC

A) Induced T_αCD3, as described above were co-cultured with allogeneic PBMC (70×10^3 cells/well) in the presence of soluble or plate bound anti-CD3 (1 μ g/ml) for 72h. Only PBMC that were stimulated with soluble anti CD3 mAb were suppressed by T_αCD3 cells. B) Expression of CD80 on CD11c+ dendritic cells that were activated in the presence of T_αCD3.

Table 1Cytokine profile of T_αCD3 regulatory T cells

	T _α CD3	T _α CD3/ αCD28	T _{control}
IL-2	2	30	22
IFN-γ	128	707	441
TNF-α	103	546	343
IL-4	3	9	5
IL-13	26	32	88
IL-10	0	4	6
TGF-β	1972	1072	1103

T_αCD3 regulatory T cells, T_{control} and CD4 T cells induced by anti-CD3 and a high dose of soluble anti-CD28 (50 µg/ml), were incubated in a 96-well plates that were pre-coated with 1 µg/ml anti-CD3 mAb. Supernatants were taken from cultures at 24h-48h and levels of IL-2, IL-10, IL-5, TNF-α, IL-4, IL-13, IFN-γ and TGF-β were measured by an array-based ELISA assay.

Table 2

Characteristics of regulatory T cells

	CD4+CD25+	Tr1	Th3	T_hCD3
Induction	Emerge directly from the thymus	Differentiate in the presence of IL-10	Low doses of orally administered antigen	In vitro antiCD3 mAb
Expression of CD25^{hi}	Yes	None	Unknown	None
FOXP3 Expression	High	None	Intermediate	Intermediate
IL-10 Secretion	Yes	Yes	Yes	None
TGF-β Secretion	Yes	Yes	Yes	Yes
Cytokine-mediated Suppression	In some systems	Yes	Yes	No
Cell Contact Dependent	Yes	None	None	Yes