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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 ($T_{\alpha CD3}$) 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 ($T_{\alpha CD3/CD28}$). $T_{\alpha CD3}$ 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 $T_{\alpha CD3/CD28}$. There were no differences in the expression of CD25 or CTLA4 on $T_{\alpha CD3}$ as compared to $T_{control}$ or $T_{\alpha CD3/CD28}$, and $CD4^+$ $CD25^-$ $T_{\alpha CD3}$ cells were identical to $CD4^+$ $CD25^+$ $T_{\alpha CD3}$ cells in their *in vitro* suppressive properties. Recombinant IL-2 *in vitro* abrogated the suppressive effect of $T_{\alpha CD3}$. The suppressive effect was not related to apoptosis, was independent of HLA since $T_{\alpha CD3}$ 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 $T_{\alpha CD3}$ 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.

Keywords: Anti-CD3; Regulatory T cells; Autoimmunity

1. 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].

Clinical trials in humans have 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

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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 downregulation 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.

2. Materials and methods

2.1. Cell separation and culture

Peripheral blood samples were obtained from healthy donors. Peripheral blood mononuclear cells (PBMCs) 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 PBMCs 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 μg/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 μg/ml of mouse anti-human CD3 mAb (BD Bioscience #555336) or with its isotype control (1 μg/ml mouse IgG_{2a}) for 5 days, in some cases soluble mouse anti-human CD28 mAb was added (10–50 μg/ml) (all from PharMingen, San Diego, CA). After 5 days, the cells were transferred into new un-coated plates and recombinant human IL-2 (PharMingen) was added to the culture at a final concentration of 10 U/ml. New complete culture medium was added every other day. After the $CD4^+$ T cells had been cultured for 10–13 days and were in the 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).

2.2. 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 PBMCs in 96-well round-bottom plates (Corning Coster, Cambridge, MA) and stimulated with 1 μg/ml of soluble anti-CD3 mAb. In some experiments neutralizing monoclonal antibodies against IL-10 or TGF-b were added to the co-culture. After 48 h of culture, ^{3}H-thymidine (1 μCi/well) was added for the last 12 h of culture, cells were then harvested and the incorporation of thymidine was measured using the LKB Betaplate liquid scintillation counter. In some experiments, 1 μg/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.

2.3. 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, USA). Intracytoplasmic staining of T cells was 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 at room temperature. The PBMCs were then added to the culture and 48–72 h later flow cytometric analysis was performed with a FACSort flow cytometer (Becton Dickinson) according to the standard procedure.

Dendritic cells (DC) 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).

2.4. Cytokine assay

$CD4^+$ conditioned T cells were incubated in 96-well plates that were pre-coated with 1 μg/ml anti-CD3 mAb (PharMingen). Supernatants were taken from cultures at 24–48 h 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 $T_{\alpha CD3}$ and PBMCs.

2.5. Apoptosis

PBMCs were stained with CFSE as described, before co-culture with induced $CD4^+$ T cells. 24–48 h after co-culture cells were stained with PE conjugated Annexin-V (R&D