

Essential roles of TGF- β in anti-CD3 antibody therapy: reversal of diabetes in nonobese diabetic mice independent of Foxp3⁺CD4⁺ regulatory T cells

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Abstract: Anti-CD3 mAb have potentials to treat overt autoimmunity as reported recently. However, the underlying mechanisms remain unclear. In this report, using an animal model of type 1 diabetes, we found that TGF- β 1, an important immunoregulatory cytokine, plays a critical role in anti-CD3-mediated diabetes reversion and immune tolerance. Anti-CD3 treatment increased the TGF- β 1 production, lasting for a long period of time, which contributed to maintaining peripheral tolerance by controlling pathogenic cells. Furthermore, we found that anti-CD3 treatment did not increase the forkhead box p3+ (Foxp3⁺)CD4⁺ regulatory T cells (Tregs). When fractionated from anti-CD3-treated, remitting mice and cotransferred with splenic cells from diabetic NOD mice, these Tregs failed to inhibit diabetes development in NOD.scid mice. Moreover, we found that the depletion of these Tregs did not affect an anti-CD3-mediated, therapeutic effect and the level of TGF- β 1 production, which suggested that an increased level of TGF- β 1 may not derive from these Tregs. Thus, our data showed a dispensable role of Foxp3⁺CD4⁺ Tregs in anti-CD3 antibody-reversed diabetes in NOD mice. These findings may have an important implication for understanding the involved mechanisms responsible for immunomodulatory function of anti-CD3 antibody on autoimmune diseases. *J. Leukoc. Biol.* 83: 000–000; 2008.

Key Words: autoimmunity · immunotherapy · cytokine · regulatory lymphocyte

INTRODUCTION

TGF- β is a potent regulatory cytokine, produced by virtually all cell types, with diverse effects on hemopoietic cells [1]. The pivotal function of TGF- β 1, the predominant isoform of the TGF- β superfamily in the immune system [2], is to maintain tolerance via the regulation of lymphocytes proliferation, differentiation, and survival, as well as chemotaxis [3]. The utmost importance of TGF- β 1 in the immune system is under-

scored by the finding that TGF- β 1-deficient mice developed a multifocal inflammatory disease that led to their early demise at 3–4 weeks of age [4, 5].

T cells are key targets of TGF- β 1 in vivo, revealed by the studies that specific attenuation or blockade of TGF- β signaling in mice leads to an autoimmune phenotype associated with T cell activation and differentiation [6–8]. In peripheral tissue, TGF- β inhibits T cell proliferation, T cell activation, and effector T cell differentiation and maintains regulatory T cells (Tregs) [9]. This dual effect of TGF- β on effector T cells and Tregs contributes to its regulation of peripheral T cell tolerance.

A critical function of TGF- β 1 in regulating leukocyte functions in autoimmune diseases including type 1 diabetes (T1D) has been emphasized recently [10]. T1D is an autoimmune disease with T cell-mediated destruction of insulin-producing β cells in the pancreatic islets. Constitutive expression of TGF- β 1 in β cells under insulin promoters inhibits the development of T1D, which is associated with immune deviation from Th1 to Th2 responses [11]. Endogenous production of TGF- β 1 by a tolerogenic agent administration also regulates autoimmune T1D, likely involving CD4⁺CD25⁺ Tregs [12]. These cells, in some cases, produce TGF- β 1 for maintenance of peripheral tolerance [13]. However, the virtual source of TGF- β 1 is controversial, given that TGF- β 1 secreted by other cell type also contributes to immune homeostasis in several models [14–16].

In a previous study, anti-CD3 antibody administration has been shown effective for treating newly established autoimmune diabetes in NOD mice [17]. In this study, a role of TGF- β 1 in anti-CD3-mediated diabetes reversion is investigated. TGF- β 1 production is enhanced significantly in anti-CD3-treated, remitting mice, and its protective function is highlighted by preventing diabetogenic T cell-mediated diabetes in a cotransfer model. Notably, our findings reveal a dis-

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pensable role of forkhead box p3⁺ (Foxp3⁺)CD4⁺ Tregs in anti-CD3-mediated diabetes reversion. The up-regulated TGF- β 1 does not derive from Foxp3⁺CD4⁺ Tregs, indicating a critical function of other cell type-derived TGF- β 1, independent of Foxp3⁺CD4⁺ Tregs.

MATERIALS AND METHODS

Mice and glycemia screening

NOD, NOD.scid, and C57BL/6 mice were obtained originally from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in our facilities under specific pathogen-free conditions. Care, use, and treatment of mice in this study were in strict agreement with the guidelines in the care and use of laboratory animals set forth by the Institute of Basic Medical Sciences. The incidence of diabetes in these mice is 80–90% by 30 weeks of age. At 10 weeks of age, NOD mice were monitored for fasting blood glucose weekly. Diabetes was defined as ≥ 11.3 mmol/L on two consecutive measurements.

Preparation of anti-CD3 F(ab')₂ antibodies

Hamster anti-murine CD3 mAb (145 2C11) was stored in our lab. Anti-CD3 fragments were obtained by pepsin digestion. Briefly, anti-CD3 mAb was dialyzed against citrate buffer (pH=3.5), and then 0.1 mg/ml pepsin was dissolved in citrate and was used at an early:late (atrial) ratio of 1:20, after incubation at 37°C for 3.5 h, which was optimized. The reaction was stopped by adding 1/10 vol 3 M Tris base (pH=8.8). The pepsin-digested mAb preparation was dialyzed immediately against PBS (pH=8.0, 4°C). A protein A-sepharose CL-4B column was prepared, and the mixture was concentrated and loaded onto a prepared Sephacryl S-200 superfine column. Fractions, corresponding to a molecular weight of 110 kDa, were collected. The purity of anti-CD3 mAb F(ab')₂ has been verified by SDS-PAGE and stored in PBS at 4°C for use.

Antibody treatment

The diabetic NOD mice, within 7 days of the onset of overt diabetes, were treated i.v. with anti-CD3 F(ab')₂ (40 μ g/mouse) for 5 consecutive days. The untreated, diabetic littermates were regarded as controls. The percentage of remission of diabetes was calculated as follows: Remission of diabetes (%) = number of mice with normoglycemia at a certain time-point after antibody treatment/total number of mice in the group \times 100%.

Skin grafting

C57BL/6 mice were used as skin donors. Tail skin grafts (0.5 cm \times 1 cm) were transplanted onto the lateral thoracic wall of the recipient and then covered with gauze for the first few days. Graft survival was evaluated daily starting on the 4th day.

In vitro proliferation assay

Lymphocytes from pancreatic draining lymph nodes were isolated from the anti-CD3-treated mice and the control counterparts 3 weeks after antibody therapy. Proliferation assay was performed as described previously [12]. Briefly, lymphocytes were suspended with RPMI-1640 culture medium supplement with 10% FCS and were seeded in 96-well plates in triplicate (4 \times 10⁵ cells per well) with glutamic acid decarboxylase (GAD)65 (10 μ g/ml), 500–585 part of GAD65 (GAD_{500–585}; 10 μ g/ml, all prepared in our lab), porcine insulin (20 μ g/ml), OVA (15 μ g/ml), or Con A (2 μ g/ml, all from Sigma Chemical Co., St. Louis, MO, USA), respectively. On Day 3, cultures were pulsed with 0.5 μ Ci/well [³H] thymidine for the last 16 h, and the cells were harvested and counted by standard liquid scintillation.

Flow cytometry

Antibodies used for flow cytometry analysis are as follows: FITC-labeled anti-murine CD4 (GK1.5) was purchased from BD PharmMingen (San Diego, CA, USA). PE-labeled anti-murine CD25 (PC61) and Foxp3 (FJK-16S) were purchased from eBioscience (San Diego, CA, USA). Cell were stained in PBS

with 2% heat-inactivated FCS and 0.2% sodium azide and fixed using PBS with 1% paraformaldehyde. For intracellular staining, cells were first stained with antibodies specific to CD4 for 30 min and then fixed for 20 min with 1 ml fixation buffer (Fix and Perm cell permeabilization kit, eBioscience). After washing, the fixed cells were incubated with anti-mouse-Foxp3 antibodies for 30 min. Data collection and analysis were performed on a FACSCalibur flow cytometry using CellQuest software (Becton Dickinson, San Jose, CA, USA).

ELISA of TGF- β production

Splenocytes (5 \times 10⁵) were incubated in 96-well flat-bottom microtiter plates in the presence of 20 μ g/ml recombinant (r)GAD65 protein (prepared in our lab). Supernatants were harvested after 48 h. The levels of TGF- β were determined in triplicate in 0.1 ml supernatant by sandwich ELISA. The ELISA kits used in this study were purchased from R&D Systems (Minneapolis, MN, USA).

Antibody depletion

Depletion of CD4⁺CD25⁺ immunoregulatory cells was performed by i.p. injection of 250 μ g/mouse anti-CD25 mAb (PC61, BD PharmMingen). The interval between the first and second injection was 3 days; thereafter, injection was once every 5 days until mice were killed. The isotype controls were administered with the same dosage and periodicity. About 90% of CD4⁺CD25⁺ and 80% of CD4⁺Foxp3⁺ cells were eliminated and confirmed by flow cytometry.

Cell purification and adoptive transfer of diabetes

Purification of CD4⁺CD25⁺ T cells by MACS (Miltenyi Biotec, Germany) was as described previously [12]. In brief, CD4⁺ T cells were isolated by depletion of magnetically labeled non-CD4⁺ T cells. CD4⁺ T cells labeled with PE-conjugated anti-CD25 were then magnetically labeled with anti-PE microbeads. The magnetically labeled cells were passed through a column placed in the magnetic field of a MACS separator, and CD4⁺CD25⁺ T cells were pooled through positive separation (purity >96%).

CD4⁺CD25⁺ T cells from spleen of anti-CD3-treated or control mice were mixed with splenocytes (1 \times 10⁷ cells) from untreated, acutely diabetic NOD mice and given i.v. into the tail veins of 4- to 8-week-old NOD.scid mice. Age-matched NOD.scid mice receiving only 1 \times 10⁷ diabetic splenocytes or spleen cells of anti-CD3-treated, remitting mice were used as controls. In some cases, NOD.scid mice i.p. received TGF- β 1 cytokines (Peprotech, Rocky Hill, NJ, USA) or the serum from anti-CD3-treated or control mice twice daily at the indicated dose simultaneously. Recipients were tested every week for diabetes and diagnosed as described above.

Histopathology

NOD mice were killed by cervical dislocation, and the pancreas was removed immediately. Each pancreas was fixed with 10% buffered formalin, embedded in paraffin, sectioned at 4.5 μ m, and stained with H&E.

Statistical analysis

The Kaplan-Meier method was used to compare diabetes remission. Student's *t*-test was used to compare mean values. Values of *P* < 0.05 were considered significant.

RESULTS

Anti-CD3 F(ab')₂ administration reverses overt diabetes in NOD mice through reconstituting self-tolerance

First, to test the efficacy of anti-CD3 F(ab')₂ antibody on treating new-onset diabetes in an animal model of T1D, freshly diabetic NOD mice were randomly divided into two groups and received a low dose anti-CD3 F(ab')₂ antibody administration i.v. on 5 consecutive days or not. As a result, over 80% of mice treated with anti-CD3 F(ab')₂ antibody returned to normal level

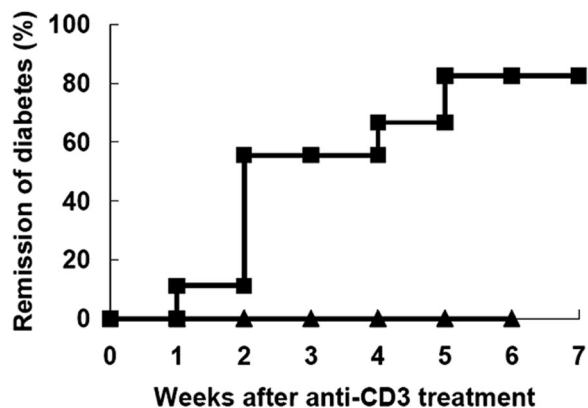


Fig. 1. Anti-CD3 F(ab')₂ antibody administration efficiently reverses new-onset diabetes in NOD mice. Antibodies preparation and injection were described in Materials and Methods. New-onset diabetic NOD mice were injected with anti-CD3 F(ab')₂. The diabetic, untreated littermates were regarded as controls. Mice were screened for glucose levels every week after antibody treatment, and complete remission was defined as a return to normal glycemia. Results were analyzed statistically between the control group (▲) and the antibody-treated group (■). Each group consists of 15–30 mice.

of blood glucose and exhibited diabetes-free survival by 7 weeks post-treatment (**Fig. 1**). In stark contrast, all untreated control mice showed a progressively heightened glycemia and almost died 6 weeks after diagnosed as diabetic. This effect was associated with a collapse of aggressive infiltration in pancreatic islets, confirmed by the presence of intact islets (**Fig. 2, C and D**), comparable with normal islets from prediabetic mice (**Fig. 2A**).

The anti-CD3 therapeutic effect appears to be attributed to restoring self-tolerance instead of immunosuppression. To address this issue further, first, an *ex vivo*-proliferative recall response to antigens of pancreatic draining lymph node lymphocytes from anti-CD3-treated or control mice was examined. Two candidates of islet β cell autoantigens, GAD, and insulin [18], as well as some unrelated antigens such as OVA and Con A, were used as stimulators. In addition, GAD_{500–585}, a peptide that consists of dominant epitopes of GAD65 [12, 19], was used in proliferation T cell assay. The treatment with anti-CD3 F(ab')₂ antibodies resulted in significant hyporesponsiveness of lymphocytes to GAD65, GAD_{500–585}, and insulin compared with the control group (*, $P < 0.05$; **, $P < 0.01$). However, no statistical differences on the proliferative responses to Con A or

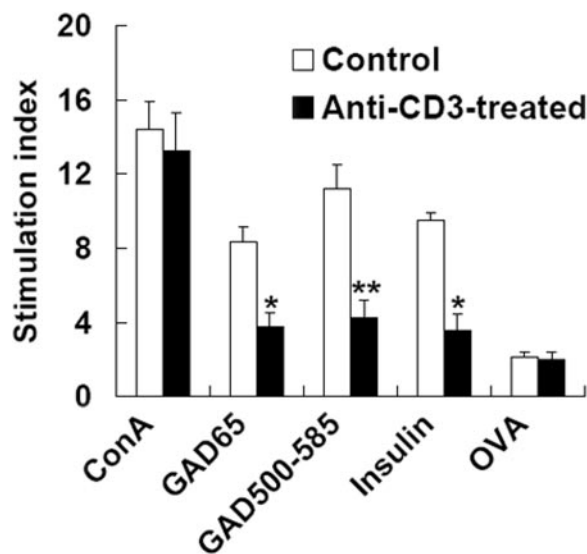


Fig. 3. Anti-CD3 F(ab')₂ administration diminished T cell responses in NOD mice. Lymphocytes isolated from pancreatic draining lymph nodes of anti-CD3 F(ab')₂-treated NOD mice or the controls 3 weeks after antibody treatment were reacted with mouse rGAD65 protein (GAD65), GAD_{500–585}, porcine insulin, Con A, or OVA, and the cells were incubated with 0.5 μ Ci [³H] thymidine. Proliferation was determined by [³H] thymidine uptake. Data are expressed as stimulation indices \pm SD of the mean from six mice with the background of 1249–2414 cpm, tested in triplicate. *, $P < 0.05$; **, $P < 0.01$, as compared with the control counterparts.

OVA between two groups were observed ($P > 0.05$; **Fig. 3**). Second, skin allograft was performed for determining immune response of anti-CD3-treated, remitting mice to heterogeneous, cutaneous-associated antigens. The results showed that rejection of allografted skin in anti-CD3-treated mice was similar to that in control, diabetic mice, which was \sim 10 days (**Table 1**). These data indicated anti-CD3 administration effectively reversed overt diabetes through re-establishing tolerance to islet β cells with full response to unrelated antigens such as cutaneous-associated antigens.

CD4⁺ Tregs are dispensable for anti-CD3 treatment-induced immune tolerance

The studies about immune tolerance restoration and maintenance reveal an important role of CD4⁺CD25⁺ Tregs in this process [20]. Recently, Foxp3 is identified as a master regu-

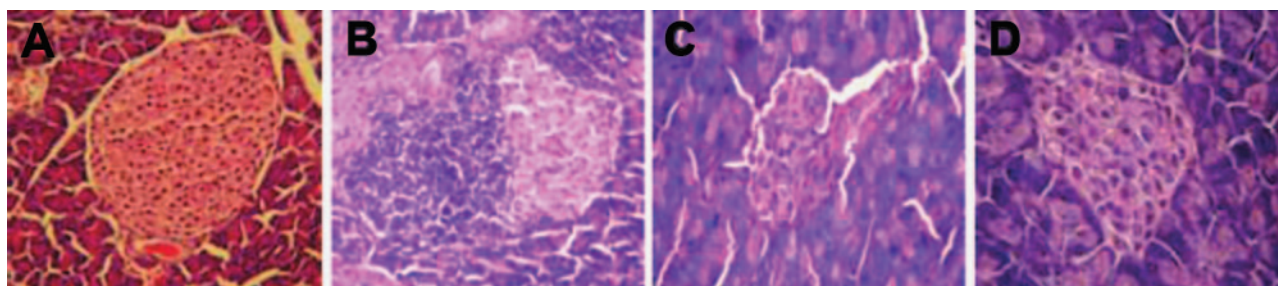


Fig. 2. Anti-CD3 F(ab')₂ treatment diminishes insulinitis in NOD mice. Pancreas was isolated, conventionally processed, and stained with H&E. (A) Normal islet without infiltrates from prediabetic NOD mice. (B) The islet from control, diabetic mice with infiltration of numerous lymphocytes. (C) The islets from mice 5 weeks after anti-CD3 F(ab')₂ treatment. (D) The islets from mice 10 weeks after anti-CD3 F(ab')₂ treatment. Original magnification: 200 \times .

TABLE 1. Skin Allograft Rejection in Anti-CD3-Treated and Control Mice

Mice	Mean \pm SD survival time (d)
Anti-CD3-treated ($n = 7$)	10.4 \pm 1.3
Controls ($n = 5$)	10.2 \pm 0.5

lators for CD4⁺CD25⁺ Treg development, and Foxp3⁺CD4⁺ T cells are considered as a Treg population [21, 22]. Thus, we examined whether Foxp3⁺CD4⁺ T cells were involved in anti-CD3 efficacy. The abundance of these cells was not increased significantly in spleen (Fig. 4A) or pancreatic lymph nodes (Fig. 4B) after anti-CD3 treatment. The similar results were obtained from mesenteric lymph nodes and thymus (data not shown). As anti-CD3 therapy leads to depletion of T cells transiently [23], to determine whether no increase of Foxp3⁺CD4⁺ T cells was a result of a general decrease of CD4⁺ cells following anti-CD3 therapy, we also examined the percentage of Foxp3⁺ cells gated on CD4⁺ cells and did not find augmented expression of Foxp3 in the CD4 T cell population (Fig. 4, A and B).

Next, to further determine CD4⁺ Treg function in anti-CD3-mediated diabetes reversion and tolerance restoration, using neutralizing anti-CD25 mAb, we depleted this Treg population in remitting NOD mice. The results indicated that depletion of these T cells failed to influence an anti-CD3 therapeutic effect

(Fig. 4C), suggesting a dispensable role of CD4⁺ Tregs in this process.

Finally, using the adoptive transfer model, the protective role of CD4⁺CD25⁺ T cells was addressed. Purified CD4⁺CD25⁺ T cells from spleen of remitting mice or control diabetic mice were transfused into immune-compromised recipients (NOD.scid mice) with diabetic spleen cells, and the onset of diabetes was examined. As high as 3×10^6 CD4⁺CD25⁺ T cells from anti-CD3-treated mice could not protect the recipients from diabetes transfer, a pattern similar to that observed in the counterparts receiving CD4⁺CD25⁺ T cells from control mice or diabetic splenocytes alone (Fig. 5). These mice all developed overt diabetes by 5 weeks after transfer. Meanwhile, diabetogenic potentials of splenocytes from remitting mice were examined. We found that the recipients receiving these cells exhibited diabetes by 3 weeks post-transfer (Fig. 5). These data indicated a protective population of Tregs and did not generate after treatment, and splenocytes from tolerated mice retained pathogenic potentials.

TGF- β 1 plays critical roles in anti-CD3 antibody-reversed diabetes

To uncover the underlying mechanisms required for controlling potentially pathogenic cells and maintaining peripheral immune homeostasis, we focus on a role of soluble cytokines produced by a corrected immune system. Th2 shift has been observed in the early phase after treatment, whereas it disappeared in the late phase, implying a less importance Th2 shift

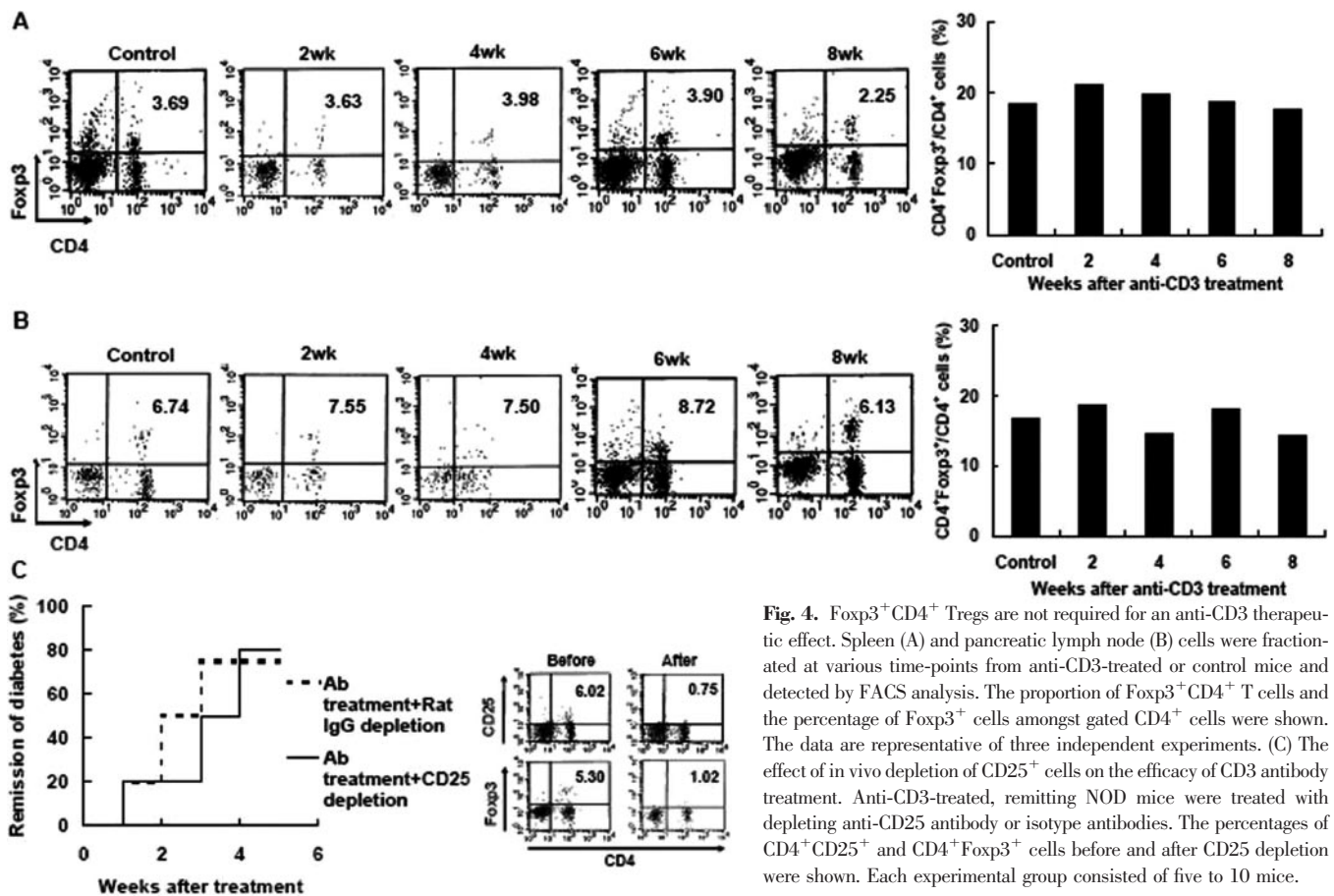


Fig. 4. Foxp3⁺CD4⁺ Tregs are not required for an anti-CD3 therapeutic effect. Spleen (A) and pancreatic lymph node (B) cells were fractionated at various time-points from anti-CD3-treated or control mice and detected by FACS analysis. The proportion of Foxp3⁺CD4⁺ T cells and the percentage of Foxp3⁺ cells amongst gated CD4⁺ cells were shown. The data are representative of three independent experiments. (C) The effect of in vivo depletion of CD25⁺ cells on the efficacy of CD3 antibody treatment. Anti-CD3-treated, remitting NOD mice were treated with depleting anti-CD25 antibody or isotype antibodies. The percentages of CD4⁺CD25⁺ and CD4⁺Foxp3⁺ cells before and after CD25 depletion were shown. Each experimental group consisted of five to 10 mice.

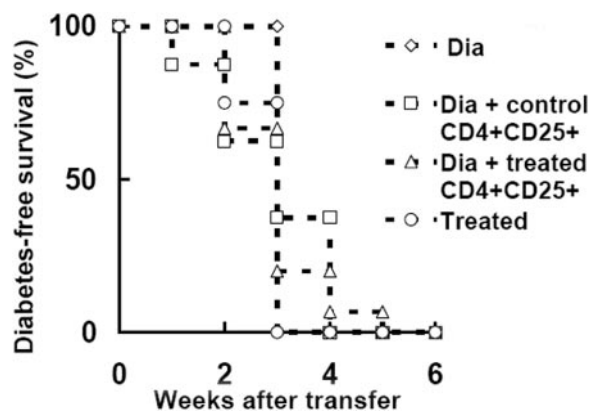


Fig. 5. Anti-CD3 administration does not induce the generation of protective Tregs in vivo. Purified CD4⁺CD25⁺ T cells from anti-CD3-treated, remitting, or control, diabetic mice were injected into NOD.scid mice combined with the splenocytes from acutely diabetic mice. As controls, some NOD.scid mice only received splenocytes of acutely diabetic or remitting mice. Each experimental group consisted of five to 15 mice. Dia, Diabetogenic splenocytes; Treated, splenocytes from anti-CD3-treated, remitting mice.

at this stage [24]. So, we concentrated on another important regulatory cytokine, TGF- β 1. As proposed in an oral tolerance model [25], augmented production of TGF- β 1 post-treatment was seen (**Fig. 6, A and B**). Although a significant increase in the plasma did not appear at 5 weeks after treatment, enhanced TGF- β 1 production by spleen cells was found (**Fig. 6B**).

To evaluate the relationship between enhanced production of TGF- β 1 and suppression of diabetogenic cells, we fractionated remitting or control mice-derived sera 10 weeks after treatment, injected them i.p. into NOD.scid mice with diabetogenic cells, and monitored diabetes development. We found that in contrast to relative inefficacy to inhibit diabetes transfer for the sera from control mice (0), as little as 0.025 ml sera (~1500 pg TGF- β 1) from remitting mice twice daily was efficient to prevent or delay the onset of diabetes, comparable with positive control using purified TGF- β 1 (**Fig. 7A**), suggesting an actively suppressive function of endogenously produced TGF- β 1. Unexpectedly, fourfold vol sera (0.1 ml) from untreated control mice also exhibited an effect of suppression on diabetes transfer (**Fig. 7B**). A plausible explanation is that TGF- β production in 0.1 ml plasma from control mice is comparable with that in 0.025 ml plasma from treated mice, and there is abundant TGF- β to prevent the onset of diabetes, indicating the defect of TGF- β in quantity instead of quality in naïve NOD mice [26]. Therefore, an increase of TGF- β effectively prevented/delayed the development of diabetes [27].

Heightened TGF- β 1 production does not derive from Foxp3⁺CD4⁺ Tregs

Almost immune cell types are able to produce TGF- β 1. To determine whether increased TGF- β 1 is secreted by Foxp3⁺CD4⁺ Tregs, we detected the level of TGF- β 1 production by spleen cells after depletion of these Tregs. The results showed that elimination of CD4⁺ Tregs did not lead to decreased production of TGF- β 1 (**Fig. 8**). These data indicated that TGF- β 1 secretion induced by anti-CD3 treatment was independent of Foxp3⁺CD4⁺ Tregs.

DISCUSSION

Anti-CD3 antibody has been demonstrated to harbor potent capacity to treat flourished autoimmune diseases [17, 28–30]. In this study, using an animal model of T1D, we showed that anti-CD3 therapy did not lead to general immunosuppression, as the anti-CD3-treated, remitting mice retained an active response to unrelated antigens, identified by skin allograft, which is in agreement with the results from Chatenoud's laboratory [17]. Furthermore, ex vivo T cell recall response assays indicated that β cell autoantigen-specific T cells from remitting mice were tolerated specially. This suggests that anti-CD3 treatment re-establishes tolerance to self-islets.

With regard to the underlying mechanisms, a previous report suggested likely involvement of CD4⁺CD25⁺ Tregs [31]; however, the results are still disputable, as CD25 can also be expressed on T cells of alternate lineages or activation states, allowing for a potential overestimation of the true Treg pool, and the expression of Foxp3, a specific lineage marker for Treg development, needs to be determined. Here, we provide evidence that Foxp3-expressing CD4 Tregs were not essential for anti-CD3 antibody-mediated diabetes reversal and tolerance by the following three ways. First, the abundance of Foxp3⁺ CD4 T cells was not up-regulated after anti-CD3 treatment in spleen and pancreatic lymph nodes (**Fig. 4, A and B**). Second,

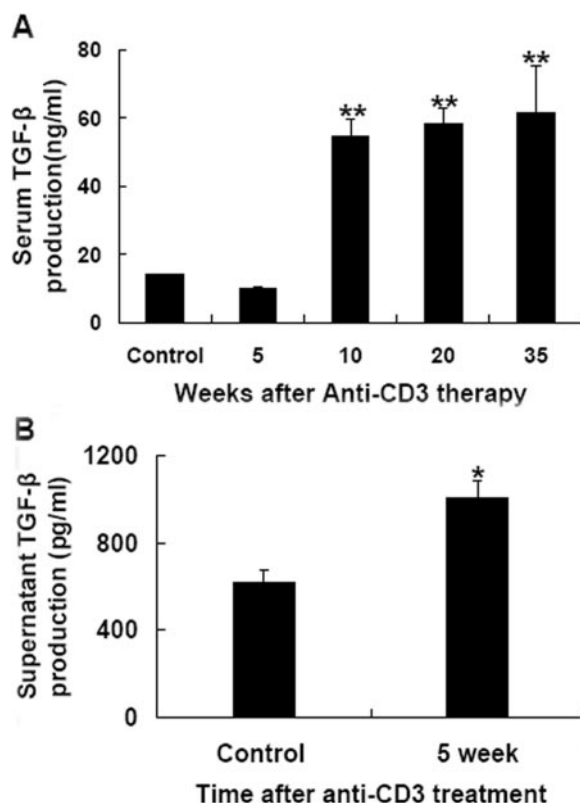


Fig. 6. Analysis of TGF- β production in the serum and supernatants from anti-CD3-treated or control mice by sandwich ELISA. (A) TGF- β production at various time-points in the serum of anti-CD3-treated or control mice were detected. (B) Fractionated splenocytes from anti-CD3-treated or control mice 5 weeks post-treatment were stimulated with rGAD65 protein (20 μ g/ml) for 48 h, and supernatants were collected for detection. Values are shown as mean \pm SD of four mice. *, $P < 0.05$; **, $P < 0.01$, compared with control mice.

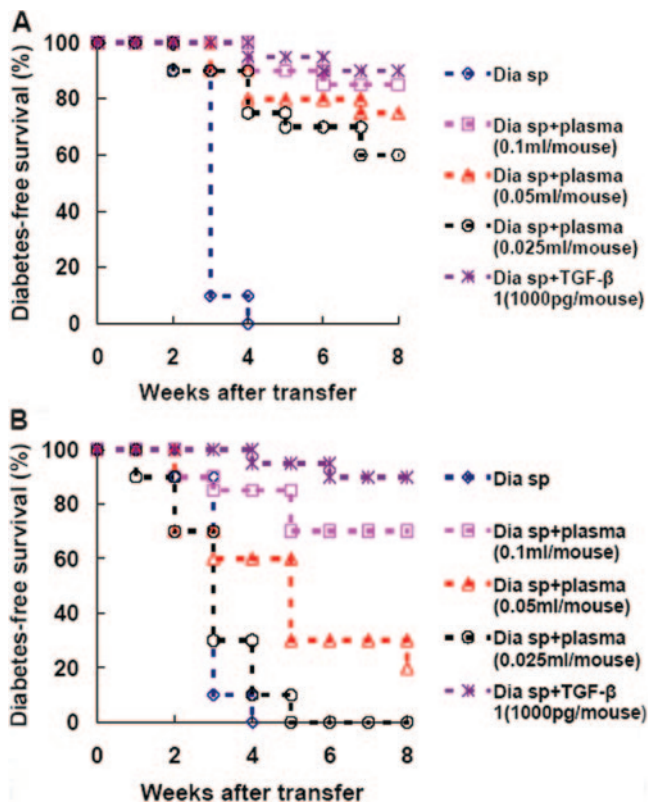


Fig. 7. TGF- β -abundant plasma from remitting mice efficiently prevent or delay the development of transferred diabetes. The plasma from anti-CD3-treated, remitting (A) or control mice (B) 10 weeks post-treatment were injected i.p. twice daily with diabetogenic cells into NOD.scid mice at an indicated titrated dose. As a positive control, TGF- β 1 cytokines and diabetogenic cells were cotransfused into NOD.scid mice at the same periodicity. The development of diabetes was screened up to 8 weeks. Each group consisted of six to 10 mice.

depletion of CD4⁺CD25⁺ T cells did not abolish therapeutic effects of anti-CD3 antibody (Fig. 4C). More importantly, fractionated CD4⁺CD25⁺ T cells from tolerated mice did not inhibit transfer of diabetes in an adoptive transfer model (Fig. 5). Our data are in keeping with recent work by Bresson et al. [32], suggesting that although anti-CD3 antibody administration increased CD4⁺CD25⁺ T cells to 20% of CD4⁺ cells, neither the percentage of CD4⁺Foxp3⁺ cells nor Foxp3 expression gated on CD4 T cells up-regulated significantly after anti-CD3 therapy. This discrepancy among the data from Von Herrath et al. [33] as well as our lab and that from the Chatenoud group [17] could be resolved by the fact that Tregs were not generated to a sufficient amount, or a majority of induced Tregs already contracted and entered into the memory phase after anti-CD3 therapy. Indeed, it has been shown that anti-CD3 mAb, just in some settings [34] but not all [33], directly promote the generation of sufficient Tregs.

In the present study, we propose a critical function of TGF- β 1 in anti-CD3-induced diabetes reversal and immune tolerance. A large amount of TGF- β 1 was produced by anti-CD3-treated, remitting mice. Notably, TGF- β 1-abundant plasma, when cotransfused with diabetogenic cells into immunodeficient mice, effectively abrogated diabetes transfer (Fig. 7), suggesting a protective role of TGF- β 1 in maintaining

peripheral tolerance by inhibiting pathogenic T cells. This is consistent with the previous reports that neutralizing endogenous TGF- β 1 led to abrogation of anti-CD3-mediated diabetes remission and recurrence of overt diabetes [31], and immunization of prediabetic NOD mice with TGF- β 1-constructed plasmid efficiently prevented the development of diabetes and effected therapeutically on newly established diabetes [27]. TGF- β 1 has been known to potently control immune response of effector T cells by acting on their differentiation, proliferation, and survival [35]. In some cases, TGF- β 1 specifically inhibits cytotoxicity of cytotoxic T cells without effecting their activation and proliferation [36]. Therefore, the details underlying that TGF- β 1 suppresses pathogenic T cells need to be clarified in further experiments.

The next important question is the source of elevated TGF- β 1 production after anti-CD3 treatment. One of TGF- β 1 sources is CD4⁺CD25⁺ Tregs, as it is demonstrated that CD4⁺CD25⁺ Tregs produce soluble and membrane-binding TGF- β 1, exerting their suppressive function [13, 37, 38]. Although the possibility of the capacity of CD4⁺CD25⁺ Tregs to secrete TGF- β 1 could not be excluded in our system; however, increased production of TGF- β 1 induced by anti-CD3 treatment clearly does not derive from CD4⁺CD25⁺ Tregs identified by depletion experiments. That is to say, even if CD4⁺CD25⁺ Tregs in remitting mice secrete TGF- β 1, the amounts of production are not sufficient to mediate an anti-CD3 therapeutic effect, as depletion of CD4⁺CD25⁺ T cells failed to down-regulate TGF- β 1 production significantly and turn over anti-CD3 efficacy. TGF- β 1 is known to be expressed by all immune cell types, including NK, dendritic cells (DC), and others. DC or NK cell-produced TGF- β 1 has the potential to actively suppress immunopathology and recover self-tolerance [16, 39, 40]. Additionally, myeloid cell-derived TGF- β 1 has been reported in a tumor-recurrence model [41]. So, the derivation of elevated TGF- β 1 needs elucidation.

Collectively, our data support an essential role of TGF- β 1 in anti-CD3-mediated diabetes reversal and tolerance restoration. TGF- β 1 inhibits pathogenic cells and resets peripheral tolerance. Importantly, CD4⁺CD25⁺ Tregs are dispensable for an

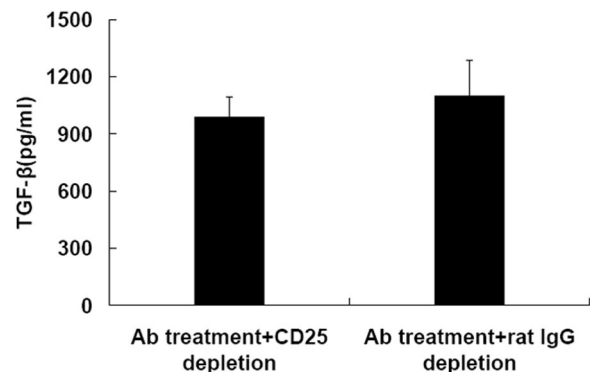


Fig. 8. The elimination of CD25⁺ T cells does not lead to reduced production of TGF- β . Splenocytes were isolated from mice 4–5 weeks after anti-CD25 or isotype antibody depletion following anti-CD3 treatment, respectively, and stimulated with rGAD_{500–585} protein (20 μ g/ml) for 48 h. The supernatants were collected for detection of TGF- β production. Values are shown as mean \pm SD. Each group consisted of four to six mice.

anti-CD3 effect and TGF- β 1 production. Although the mechanisms underlying regulation of lymphocytes by TGF- β 1 and its source need further study, our findings have important implication for the understanding of involving mechanisms responsible for an anti-CD3 therapeutic effect in diabetes and other autoimmune diseases.

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