Inhibition of autoimmune diabetes by oral administration of anti-CD3 monoclonal antibody

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Abstract
Anti-CD3 monoclonal antibody has been shown to induce tolerance and be an effective treatment for diabetes both in animal models and in human trials. We have shown that anti-CD3 monoclonal antibody given orally is biologically active in the gut and suppresses experimental autoimmune encephalitis by the induction of a regulatory T cell that expresses latency associated peptide (LAP) on its surface. In the present study we investigated the effect of oral anti-CD3 on the prevention of autoimmune diabetes in AKR mice in which low dose streptozocin (STZ) model induces autoimmunity to the beta cells of the islets. We found that oral anti-CD3 given at doses of 50 and 250 μg/feeding suppressed the incidence of diabetes in this model with best effects seen at the 50 μg/dose . Associated with suppression we observed decreased cell proliferation in the spleen and conversion of Th1 responses into Th2/Th3 responses in the periphery including in the pancreatic lymph nodes. Oral anti-CD3 increased the expression of LAP on CD4+ T cells and these cells could adoptively transfer protection. Protection by oral anti-CD3 was TGF-β dependent. Our results demonstrate that oral anti-CD3 is effective in the model of streptozocin diabetes and may be a useful form of therapy for type 1 diabetes in humans.

Introduction

Immunologic tolerance is mediated by a number of mechanisms and it is generally believed that autoimmune processes such as those that occur in type 1 diabetes are in some way related to defects in immunologic tolerance (1). One approach for the treatment of autoimmunity has been the parenteral administration of anti-CD3 monoclonal antibody which is efficacious in animal models of autoimmunity including autoimmune diabetes (2-7) EAE (8, 9) and in human trials of type 1 diabetes (10-12). Intravenous anti-CD3 is an approved therapy for transplant rejection in humans (13). We have been interested in immune therapy of autoimmune diseases by mucosal administration of autoantigens designed to induce regulatory T cells (14, 15). We have recently found that oral anti-CD3 is biologically active in the gut and induces a CD4+CD25-LAP+ regulatory T cell that suppresses EAE in a TGF-β dependent fashion (16). LAP, latency associated peptide, is the amino terminal domain of the TGF-β precursor peptide; it remains non-covalently associated with TGF-β peptide after cleavage and forms the latent TGF-β complex. We previously identified CD4+CD25-LAP+ T cells that suppress colitis by a TGF-β dependent mechanism (17).

Given this, we investigated the effect of oral anti-CD3 , in AKR mice , on the prevention of autoimmune diabetes using the low dose streptozocin (STZ) model which induces autoimmunity to β cells (18). Diabetes in STZ model can be prevented by administration of anti-T cell monoclonal antibodies and diabetes can be adoptively transferred with splenocytes from diabetic animals (19). This model is useful for the testing of novel immunotherapeutic interventions since hyperglycemia and insulitis can be easily induced in a relatively short period of time in a high percentage of animals. Furthermore, treatment can be given before irreversible tissue damage and before T cells have become sensitized to islet antigens. It has previously been shown by Herold et al that intravenous anti-CD3 is effective in the STZ model (2). Given our results in the EAE model, we investigated the effect of oral anti-CD3 in the STZ model of diabetes.

Materials and Methods

Mice

AKR/J Male mice (5-6 weeks old) were purchased from The Jackson Laboratory and housed in a pathogen-free animal facility at the Harvard Institutes of Medicine according to the animal protocol guidelines of the Committee on Animals of Harvard Medical School. For recovery of anti-CD3 after feeding, SJL mice were used (Jackson Laboratory).

Induction of diabetes by streptozotocin (STZ) and treatment protocols

Mice were injected i.p. for five consecutive days with 40mg/kg body weight of STZ (Sigma Aldrich, St. Louis, MO) in 0.01M citrate buffer (pH4.5) to induce diabetes. Day 1 was defined as the first STZ injection. An intraperitoneal glucose tolerance test (IPGTT) was performed to diagnose diabetes using a glucose meter (Hypoguard, Minneapolis, MN). Blood glucose levels were estimated from the tail vein 30 min after i.p. injection of glucose (1.33g/kg body weight). Mice over 250 mg/dl of glucose level for two or more consecutive weeks were considered as diabetic.

Anti-CD3 monoclonal antibody, whole antibody and (145-2C11) $F(ab')_2$ fragments were purchased from Bio Express Inc (West Lebanon, NH). Hamster IgG whole antibody and $F(ab')_2$ fragments were purchased from Jackson Immuno Research Laboratories Inc (Charlestown, MA) and were used as control immunoglobulin (Ig). Mice were fed with anti-CD3 Ab, control IgG or PBS followed by STZ injection (40mg/kg) daily for five consecutive days (day1-5). In combination with anti-CD3 Ab feeding in some experiments, mice were injected with 500µg of neutralizing anti-TGF-β Ab (Bio Express Inc, West Lebanon, NH) or mouse IgG (Jackson Immuno Research Laboratories Inc. Charlestown, MA) as control immunoglobulin on days 0, 2, 4, 6 and 14.

Histological examination of pancreatic islets

For histological examination of pancreatic islets, the pancreas was removed on day 28 and fixed in 10% buffered formalin. Specimens embedded in paraffin wax were cut by microtome and sections were stained with hematoxylin and eosin. The slides were analyzed for presence of insulitis by a person without knowledge of the identity of samples.

Proliferation and cytokine assays

Single cell suspensions were prepared from spleen and mesenteric lymph node (MLN). To measure proliferation, cells were plated in 96 well round bottom plates (2.5×10^5 cells/well) in cRPMI containing: RPMI 1640 medium with 10% FBS (BioWhittaker, Walkersville, MD), 100 U/ml penicillin (BioWhittaker), 100 µg/ml Streptomycin (BioWhittaker), 10 mM Hepes (BioWhittaker) and stimulated with soluble anti-CD3 Ab (BD Pharmingen, San Diego, CA). After 48h in culture at 37°C with 7% CO_2, 1µCi [^{3}H]-thymidine was added to each well and cells were harvested 12 hrs later. Proliferation was detected using a 1450 Microbeta liquid scintillation counter (Perkin Elmer, Boston, MA). For cytokine production cells were cultured at 2.5×10^5 cells/well in X-VIVO 20 medium (BioWhittaker) and stimulated with 1µg/ml anti-CD3 Ab. The culture supernatants were collected at 48hr for IL-2, IL-4, IL-10 and IFN-γ and at 72hr for TGF- β. ELISA assays for IL-2, IL-4, IL-10 and IFN-γ were performed as previously reported (20). TGF-β was assayed using a mouse/rat/porcine TGF-β1 immunoassay kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

Flow cytometry analysis

Flow cytometry analysis was performed on single cell suspensions isolated from MLN, spleen and pancreatic lymph node (PLN). Cells were stained in PBS with 2% BSA and fixed with 1% paraformaldehyde. Flow cytometry analysis was performed by FACScan using Cellquest software. The antibodies used were anti-CD16/CD32 Ab (2.4G2) as Fc-blocker, FITC-conjugated anti-CD4 Ab (L3T4), PE-conjugated anti-CD3 Ab (145 2C11), PE-conjugated anti-CD25 Ab (7D4), APC-labeled streptavidin, PE-conjugated anti-CD62L Ab (MEL14), PE-conjugated anti-CD45RB Ab (16A) (BD Pharmingen) and biotinylated anti-human LAP Ab (BAF246; XO02) (R&D Systems).

Cell purification and culture

CD4+ T cells from spleen cells were purified using a mouse T cell CD4 enrichment column kit (R&D Systems) according to the manufacture's

instructions. LAP+CD4+ cells were purified by immunomagnetic cell sorting (MACS) (Miltenyi BIOTEC, Auburn, CA). All procedures were performed in accordance with the manufacture's instructions. Purified CD4+ T cells were stained with biotinylated anti-human LAP Ab and streptavidin-magnetic microbeads in the presence of Fc-block. Then LAP+CD4+ cells were positively separated and $LAP^{-}CD4+$ cells were negatively separated by MACS column. In some experiments, CD25+ cells were excluded from LAP-CD4+ cells or CD4+ T cells followed by staining with biotinylated anti-human LAP Ab and streptavidin-magnetic microbeads. Cells stained in a combination of PE-conjugated anti-CD25 Ab and anti-PE-magnetic microbeads were separated into CD25+ cells and CD25- cells by MACS column. To prepare APCs depleted of T cells, spleen cells from naïve mice were stained with a combination of PE-conjugated anti-CD3 Ab and anti-PE magnetic microbeads and stained cells were negatively isolated by MACS column. Purified cells were used for cytokine assays or suppressive function assays. For cytokine assays, 2.5 x 10^5 LAP+ CD4+ cells, LAP-CD25+CD4+ cells or LAP-CD25-CD4+ cells were cultured with 10 μg/ml plate-bound anti-CD3 Ab in X-VIVO 20 (BioWhittaker) in 96 well round bottom plates. The culture supernatants were collected at 48hr for IL-2, IL-4, IL-10 and IFN-γ and at 72hr for TGF-β. For measuring suppressive function in a co-culture system, titrated purified cells were cultured with an equal ratio of responder cells (LAP-CD25-CD4+ cells) and APCs and stimulated with 1μg/ml soluble anti-CD3 Ab in cRPMI. Co-cultured cells were maintained for 60hr and 1 μCi ^{3}H-thymidine was added at 12hr before harvest. In some experiments, 50 μg/ml neutralizing antibody, anti-IL-10 Ab (JES052A5) or anti-TGF-β Ab (1D11) purchased from R&D systems, was added to the culture system.

Adoptive Transfer

One day after the last feeding, CD4+ T cell or LAP-CD4+ cells were purified from spleen and MLN of mice fed with 50μg anti-CD3 Ab or control IgG for five consecutive days. The purified cells (2.5×10^6) were injected i.v. to naïve AKR/J mice on day 0. STZ (40mg/kg) was administrated to the recipient AKR/J mice from day 1 to 5 and IPGTT was performed on days 7, 14, 21 and 28 using a glucose meter.

Recovery of anti-CD3 from the intestines of mice fed anti-CD3

Six hours after feeding, the upper part of the small intestine from three mice fed 50 ug anti-CD3 or control isotype antibody was digested using 1ml PBS containing 10 mg collagenase D (Roche) for 30 min at 37°C. The tissue was homogenized and centrifuged for 30 min at 70K rpm. After centrifugation, the soluble fraction was added to a protein-G column and the bound IgG was eluted 105 μg in the presence of proteinase inhibitors (Roche). The amount of total protein added to each well was 0.05μg, 0.26μg and 0.52μg.

The biologic activity and the concentration of the eluted IgG was ascertained by its ability to induce T-cell proliferation of naïve splenocytes as compared to known concentrations of anti-CD3 antibody. The culture conditions for proliferation with anti CD3 were as follows; single cell suspensions were prepared from spleens of naïve SJL mice. Cells were plated in 96 well round bottom plates (2.5x105 cells/well) in EX-VIVO20 and stimulated with either soluble known concentrations of anti-CD3 antibody (0.0025, 0.005, 0.01

ug/ml) or with 0.5-5 ul eluates.After 72 h in culture 1uCi [3H] thymidine was added to each well and cells were harvested 12 hours later. Each experiment was repeated twice. The statistics used was an unpaired Student's t-test.

Statistics analysis

Statistical significance was calculated using ANOVA with bonferroni post-hoc analysis, the unpaired Student's t test assuming unequal variance or a $\chi 2$ test for in vitro assays and using the Mantel-Cox log-rank test or $\chi 2$ test for in vivo assays.

Results

Oral anti-CD3 Ab treatment prevents the development of diabetes induced by STZ

Mice were orally administered with 5, 50, or 250 μg anti-CD3 Ab and injected with STZ to induce diabetes on days 1 to 5. Thereafter, blood glucoses levels were monitored once a week and mice with over 250 mg/dl of glucose level by IPGTT for two or more consecutive weeks were classified as diabetic. As shown in Fig.1, diabetes progression was not suppressed in mice fed with 5 μg anti-CD3 Ab compared to the PBS group or control IgG groups. On the other hand, mice treated with 50 μg or 250 μg anti-CD3 Ab had significantly reduced development of diabetes compared to mice treated with either PBS or control IgG. On day 14, 67% (8/12) of mice treated with PBS and 83% (10/12) of mice treated with control IgG were diagnosed as diabetic respectively. However, development of diabetes in mice treated with anti-CD3 Ab was only 33% (4/12). Mice treated with 50ug were followed for up to 42 days. At this time point, there was no statistically significant difference between the treated and control group although there were less diabetic animals in the treated group (8/12) than in the control group (10/12). Significant suppression of diabetes was also observed in animals fed 250 μg anti-CD3. Correspondingly, as shown in Fig.1 D, the blood glucose levels on day 14 were also significantly reduced in mice treated with 50 μg anti-CD3 Ab compared to mice treated with either PBS or control IgG. Moreover, as shown in Table 1, the mice treated with 50 μg anti-CD3 Ab had a significantly lower incidence of insulitis in the pancreas than that of mice treated with either control IgG or PBS on day 28.

Oral anti-CD3 Ab treatment suppresses IL-2 IFN-γ cell proliferation and enhances secretion of TGF-β. It has been reported that IV anti-CD3 mAb can treat diabetes in NOD mice and the effect is related to the immunoregulatory function of TGF-β (7). Therefore, we investigated whether the regulatory function in the periphery was enhanced in the STZ model after 50 μg oral anti-CD3 Ab treatment and STZ injection. On day 6, spleens were removed from each group and cells were cultured with soluble anti-CD3 Ab to measure cell proliferation and cytokine production. As shown in Fig. 2, spleen cells from mice treated with either PBS or control IgG proliferated to anti-CD3 Ab in a dose dependent manner. On the other hand, proliferation of spleen cells from mice treated with oral anti-CD3 Ab was significantly suppressed compared to either PBS group or control IgG group ($p < 0.009$). For cytokines, in comparison to both the PBS group and the control IgG group, IL-2 ($p < 0.04$) and IFN-γ ($p < 0.008$) secretion was significantly decreased and TGF-β secretion ($p < 0.01$) significantly increased in the spleens of mice fed with anti-CD3 Ab.

Recovery of biologically active anti-CD3 from the intestines of mice fed anti-CD3.
We previously found that oral anti-CD3 accumulates in the villi of fed mice after feeding as measured by immunohistochemistry (16). In order to determine whether we could recover biologically active anti-CD3 after feeding, mice were fed 50 ug anti-CD3 or isotype control antibody and 6 hours after feeding, anti-CD3 or isotype control antibody was extracted from the upper intestine using a protein-G column. The biologic activity of the eluted anti-CD3 was tested by measuring its ability to stimulate T-cell proliferation and compared to known concentrations of anti-CD3. As shown in Figure 3, biologically active anti-CD3 could be eluted from oral anti-CD3 fed animals as measured by proliferation and was equivalent to approximately 0.01ug/ml of anti-CD3.

Increase of LAP^+ on $CD4^+$ cells after oral administration of anti-CD3 Ab. To address if any cell populations with regulatory function were induced after oral anti-CD3 mesenteric lymph node (MLN), spleen and pancreatic lymph node (PLN) cells from each group were prepared on day 6. Cells were stained with a combination of anti-CD4 and antibody to CD25, latency associated peptide (LAP), CD45RB and CD62L. The frequency of CD25+CD4+ cells did not change in mesenteric lymph node (MLN), spleen or pancreatic lymph node in any groups (Table 2), whereas the frequency of LAP+CD4+ cells in spleen and pancreatic lymph node significantly increased in mice treated with oral anti-CD3 Ab compared to either PBS group or control IgG group. The frequency returned to control levels by day 21. No significant changes in frequency of either $CD45RB^{low}CD4+$ or CD62L+CD4+ were observed in MLN spleen and pancreatic lymph node after oral anti-CD3 (not shown) and as in our studies of oral anti-CD3 in the EAE model, we did not find that CD3 expression was decreased and/or down regulated after oral anti-CD3 treatment (16).

LAP+CD4+ cells from mice treated with oral anti-CD3 Ab have enhanced regulatory function in vitro. To investigate whether the in vitro suppressive function of LAP+CD4+ cell was enhanced after oral anti-CD3, purified LAP+CD4+ cells and LAP-CD25+CD4+ cells from spleen on day 6 were cultured with LAP-CD25-CD4+ cells as responder cells, APCs and soluble 1 µg/ml anti-CD3 Ab. As shown in Fig. 4A,B LAP+CD4+ cells, LAP-CD25+CD4+ cells and LAP+CD25-CD4+ cells were anergic to anti-CD3 Ab stimulation in vitro and they suppressed responder cell proliferation in a dose dependent fashion. The suppressive function of LAP+CD4+ cells was augmented more than 2 to 3 folds after oral anti-CD3 treatment compared to that of control IgG treated mice. However, suppressive function of LAP-CD25+CD4+ cells was not affected by anti-CD3 treatment. To rule out a possible effect of CD25+ cells on LAP+CD4+ cell suppressive function, CD25+ cells were excluded from LAP+CD4+ cells. As shown in Fig. 4B LAP+CD25-CD4+ cells also suppressed responder cells with increased suppressive function after oral anti-CD3 treatment. We next examined in vitro kinetics of suppressive function of LAP+CD4+ cells after anti-CD3 feeding. LAP+CD4+ cells from mice fed with anti-CD3 Ab had enhanced suppressive function compared to LAP+CD4+ cells from mice fed with

IgG on day 6 and 14, which was no longer observed on day 21 (Fig. 4C).

To determine whether the in vitro suppressive function of LAP+CD25-CD4+ cells after oral anti-CD3 is mediated through IL-10 or TGF-β neutralizing anti-IL-10 Ab or anti-TGF-β Ab was added in vitro. As shown in Fig. 4D, neutralizing anti-IL-10 Ab had no effect whereas neutralizing anti-TGF-β Ab partially reversed the suppressive function by LAP+CD25-CD4+ cells.

Neutralization of TGF-β in vivo abrogates the effect of oral anti-CD3 on diabetes. To determine whether TGF-β is involved in preventing diabetes progression following oral anti-CD3 in vivo, 500 μg neutralizing anti-TGF-β Ab was given on days 0, 2, 4, 6 and 14 and blood glucose levels were measured on day 21. As shown in Table 3, when mice treated with oral anti-CD3 Ab were injected with neutralizing anti-TGF-β Ab, the preventive effect on diabetes progression was abrogated.

Adoptive transfer of diabetes prevention following oral anti-CD3 is dependent on LAP+ T cells. We investigated whether the suppressive function of oral anti-CD3 could be adoptively transferred to naïve recipients. To investigate this, CD4+ T cells or CD4+ cells depleted of LAP+ cells from spleen and MLN of mice fed with anti-CD3 Ab or control IgG were transferred to naïve AKR/J mice on day 0. STZ was injected to the recipient AKR/J mice on day 1-5 and blood glucose was monitored once weekly. As shown in Fig. 5, development of diabetes was significantly suppressed in the mice transferred with CD4+ T cells from mice fed anti-CD3 compared to the mice transferred with CD4+ T cells from mice fed control IgG. No suppression was observed when CD4+LAP- cells were transferred. Of note, depletion of LAP+ cells from control Ig fed animals also enhanced the incidence of diabetes. These results confirm that LAP+CD4+ cells play a crucial role in prevention of autoimmune diseases followed by restoration of peripheral immune tolerance and that the regulatory function of LAP^+CD4^+ cells was reinforced by anti-CD3 feeding.

Discussion

In the present study we demonstrate the effectiveness of oral anti-CD3 in the STZ model of diabetes. Oral anti-CD3 induces a LAP+ regulatory T cell that can adoptively transfer protection and functions in a TGF-β dependent fashion. We do not know at this time whether LAP+ cells are a subset of Th3 cells though our working hypothesis is that they are precursors of Th3 cells (21) and either differentiate into Th3 cells upon secretion of TGF-β or induce Treg cells by the secretion of TGF-β. Once Th3 cells are stimulated they may lose LAP on their surface.

Our results are analogous to what we observed with administration of oral anti-CD3 in the mouse model of EAE (16). One of the features of the induction of regulatory T cells by oral anti-CD3 is a dose response in which lower doses are more effective. This was seen in the EAE model in which 5μg was the most effective dose, no effect was observed at 50μg and worsening of EAE occurred with 500μg. This dose response correlated with the induction of regulatory cells in the mesenteric lymph node. Thus, it appears that the dose of anti-CD3 is very important for the induction of regulatory T cells and that LAP+ cells will not be induced if the signal delivered to the TCR by anti-CD-3 is either too weak or too

strong. We observed a similar dose response pattern in the STZ model although, the most effective dose was 50μg and we also observed positive effects at 250μg. We did not test doses higher than 250μg. The shifted dose response may be related to different strains being used and the use of CFA for the induction of EAE versus the use of STZ to induce diabetes. Of note, at 42 days after treatment of animals with 50ug oral anti-CD3, there was no statistically significant difference between the treated and control group although there were less diabetic animals in the treated group (8/12) than in the control group (10/12). For a more prolonged effect, repeat or chronic dosing may be required.

Unlike the EAE model in which oral anti-CD3 enhanced recovery in animals treated at the peak of disease, we did not observe a protective effect of oral anti-CD3 once animals were diabetic. The lack of effect may be related to the fact that the STZ diabetes model is an induced model with rapid onset. The NOD model of diabetes serves as one of the primary models for human disease and we are currently testing the effect of oral anti-CD3 in the NOD model given both as prevention and therapeutically at the onset of diabetes. In preliminary experiments we have found that oral anti-CD3 is effective in preventing diabetes when given to neonatal NOD mice (unpublished). Although the cause of type 1 diabetes is not known, there is the suggestion that the gut may play a role as the site at which newborns are sensitized to islet antigens related to cross-reactivity with cows milk and/or defective mechanisms of mucosal tolerance. (22-24). Oral anti-CD3 may thus serve to boost the natural induction of regulatory T cells at the gut mucosa.

There is a difference in the effect of anti-CD3 depending on the route it is given (IV vs. oral) and when it is administered. In the EAE model, IV anti-CD3 is effective given at the height of the disease but not prior to disease induction (9). This phenomenon is felt to be related to the mechanism of action of IV anti-CD3. It is believed that IV anti-CD3 kills Th1 cells that have been generated and are present at the height of disease. Oral anti-CD3 on the other hand, is effective prior to disease onset in the EAE model as it directly induces regulatory T cells which prevent the induction of effector cells. In the EAE model, it is also effective at the height of disease presumably by boosting a naturally occurring regulatory T cell response that occurs prior to the recovery phase (20). The effect of oral anti-CD3 appears to be different in the STZ diabetes model. We postulate that unlike EAE, regulatory T cells have not been generated in the STZ diabetes model and that the induction of regulatory cells would not be effective in later stages of STZ induced diabetes unless Th1 responses are also downregulated. Consistent with this, in the NOD model of diabetes, IV anti-CD3 appears to eliminate Th1 milieu allowing the emergence of regulatory T cells at a late time point. Unlike IV anti-CD3, oral anti-CD3 directly induces regulatory T cells, thus in diabetes it is effective prophylactically and may also be effective following IV anti-CD3 to boost a naturally occurring T cell response that arises after IV anti-CD3 (25).

Following oral anti-CD3, LAP+ regulatory T cells are first induced in the mesenteric lymph node after which they migrate to sites of inflammation(16). Thus, we observed increased numbers of LAP+ T cells in the popliteal lymph nodes in EAE animals following immunization with PLP/CFA. In the present study we

observed an increase of LAP+ T cells in the pancreatic lymph nodes of mice induced for diabetes with STZ. It thus appears that one of the properties of LAP+ regulatory T cells is their ability to migrate to site of inflammation where they exert their regulatory effects. In our studies of anti-CD3 in the EAE model (16), we found that anti-CD3 appears in the villi of fed mice within one hour after feeding. We now present data that biologically active anti-CD3 can be recovered from the intestine as measured by the ability of recovered antibody to stimulate naïve T cells in vitro.

The immunologic effects of oral anti-CD3 are antigen non-specific since anti-CD3 binds to CD3 on the surface of T cells independent of the antigen specificity of the T cell receptor. Thus it would be expected to be effective in other models of autoimmunity besides EAE. The present study is the first demonstration that oral anti-CD3 is effective in an autoimmune model different from EAE and suggests that oral anti-CD3 may be applicable for the treatment of human type 1 diabetes. For clinical application, it may be more effective given to subjects at risk rather than those with new onset diabetes, or following treatment with IV anti-CD3 in new onset subjects.

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Table 1. Incidence of insulitis in pancreas after 50μg anti-CD3 Ab

Feeding	i.p.	Islets No.	Normal Islets (%)	Peri-Insulitis (%)	Invasive Insulitis (%)
PBS	STZ	72	50 (69%)	15 (21%)	7 (10%)
Control IgG	STZ	88	62 (71%)	15 (17%)	11 (13%)
Anti-CD3 ab	STZ	135	125 (93%)**	8 (6%)**	2 (2%)**
None	None	66	66 (100%)	0 (0%)	0 (0%)

Sections of pancreas from 6-8 mice/group (non-treated group; n=4) on day 28 were scored for the presence of insulitis. Pooled insulitis score data were statistically analyzed by χ^2 test.

**; $p<0.01$ compared with PBS or control IgG group.

Table 2. Percentages of $CD4^+$ cell populations in MLN, Spleen and Pancreatic LN

Feeding	i.p.	MLN		Spleen		Pancreatic LN	
		$CD25^+CD4^+$	LAP^+CD4^+	$CD25^+CD4^+$	LAP^+CD4^+	$CD25^+CD4^+$	LAP^+CD4^+
PBS	STZ	7.9±1.1	2.2±0.7	8.4±0.7	3.0±0.4	13.8±1.9	3.7±0.7
Ctl IgG	STZ	7.4±1.1	2.4±0.7	8.0±0.6	3.2±0.6	12.6±2.3	3.9±0.6
αCD3 Ab	STZ	7.6±1.4	2.5±0.6	8.5±0.9	4.3±0.5**	13.3±2.2	4.5±0.7*

Mean and S.D. from three experiments (n=10-14) are shown.

*; $p<0.05$ **; $p<0.01$ compared with either PBS group or control IgG group

Table 3. Neutralization of TGF-β reverses the protective effect of oral anti-CD3 In STZ-induced diabetes

Treatment		Incidence of diabetes	Blood glucose (mg/dl)
Feeding	i.p.		
PBS		11/12	381±87
Control IgG	Control IgG	10/12	394±113
Control IgG	anti-TGF-β	10/12	355±73
anti-CD3 Ab	Control IgG	6/12*	275±73*
anti-CD3 Ab	anti-TGF-β	11/12	358±81

Mean and SD from three experiments are shown. Mice were fed with 50 μg anti-CD3 Ab and injected with STZ on day 1-5. Neutralizing anti-TGF-β Ab was injected via i.p. at day 0, 2, 4, 6 and 14. Blood glucose was measured at day 21. *; $p<0.05$ compared with other groups.

Figure Legends

Figure 1. Oral administration of anti-CD3 Ab suppress incidence of STZ induced diabetes in AKR mice. AKR mice were fed with 5 μg (A), 50 μg (B) or 250 μg (C) of anti-CD3 Ab and injected with STZ from day 1 to 5 and monitored once a week. Mice with blood glucose level readings over 250 mg/dl by IPTTG were diagnosed as diabetic. △; PBS + STZ (n=12), ○; Control IgG + STZ (n=12), ●; Anti-CD3 Ab + STZ (n=12), ◇; Non-treated (n=4). (D) Blood glucose levels measured at day 14 by IPGTT in mice treated with 50 μg anti-CD3 Ab and injected with STZ. Both incidence of diabetes and the blood glucose levels on day 14 were significantly suppressed in mice fed with 50 μg or 250 μg anti-CD3 Ab compared to mice fed with either control IgG or PBS ($p< 0.05$).

Figure 2. Cell proliferation and cytokine production after oral anti-CD3. AKR mice were fed with 50 μg anti-CD3 Ab, injected with STZ from day 1 to 5 and sacrificed on day 6. Spleen cells ($2.5x10^5$ cells/well) were cultured with 1 μg/ml anti-CD3 Ab in cRPMI medium for 60hr. ^{3}H-thymidine was added in the last 12hr of culture. To measure cytokine production, cells were cultured with 1 μg/ml anti-CD3 Ab in X-VIVO 20 medium. Supernatants were collected at 48hr for IL-2, IL-4, IL-10 and IFN-γ, at 72hr for TGF-β. Mean and S.E. are shown. For proliferation *; $p< 0.009$ compared to either control IgG or PBS group; for IL-2 $p < 0.04$, for INF-γ $p < 0.008$, for TGF-β $p < 0.01$. Similar results were obtained in three independent experiments.

Figure 3. Activity of eluted anti-CD3 IgG from the intestine of mice fed with anti-CD3 as measured by T cell proliferation. Three Mice were fed with 50 ug anti-CD3 or isotype control antibody and 6 hours after feeding the upper intestine was processed to elute anti-CD3. The eluted material contained 105μg / ml of protein. Splenocytes from naïve mice were cultured with anti-CD3 or intestinal eluates and proliferation induced by eluates was measured and compared to cells stimulated with known concentrations of anti-CD3 as described in Materials and Methods. Eluates from antiCD3 fed mice had a higher proliferation as compared to control fed mice, p=.001.

Figure 4. Enhancement of LAP^+CD4^+ in vitro suppression after oral anti-CD3. Mice were fed with 50 μg anti-CD3 Ab and injected with STZ from day 1 to 5 and sacrificed on day 6. LAP^+CD4^+, $LAP^-CD25^+CD4^+$ or $LAP^+CD25^-CD4^+$ cells as modulator were purified from spleens as described in Materials and Methods. $LAP^-CD25^-CD4^+$ cells used as responder cells were purified from spleens of mice fed with control IgG. (A) Titrated modulator cells from control IgG or anti-CD3 Ab group were cocultured with responder cells ($2.0x10^5$ cell/well) in presence of APCs and 1 μg/ml anti-CD3 Ab. (B) Titrated modulator $LAP^+CD25^-CD4^+$ cells from control IgG or anti-CD3 Ab group were cocultured with responder ($1.0x10^5$ cell/well) in presence of APCs and 1 μg/ml anti-CD3 Ab. (C) On day 6, 14 or 21, modulator LAP^+CD4^+ cells ($1.0x10^5$ cell/well) from control IgG or anti-CD3 Ab group were cocultured with responder cells ($2.0x10^5$ cell/well) in presence of APCs and 1 μg/ml anti-CD3 Ab. Modulator LAP^+CD4^+ cells from mice fed anti-CD3 were more suppressive ($p=0.0011$ for day 6 and $p= 0.0016$ for day 14) than those from control IgG fed mice. (D) Modulator $LAP^+CD25^-CD4^+$ cells ($0.5x10^5$ cell/well) from spleens of mice fed with anti-CD3 Ab were co-cultured with responder

cells ($1.0x10^5$ cell/well) in presence of APCs and 1 μg/ml anti-CD3 Ab. To neutralize IL-10 or TGF-β, either 50 μg/ml anti-IL-10 Ab or 50 μg/ml anti-TGF-β Ab were added in culture. Anti-TGF-β significantly increased proliferation compared to medium (p=0.026) or to anti IL-10 (p=0.025). These results are representative of two or three independent experiments.

Figure 5. Adoptive transfer of $CD4^+$ cells, but not LAP^-CD4^+ cells, from mice fed with anti-CD3 Ab suppresses development of diabetes in recipient mice. $CD4^+$ T cell or LAP^-CD4^+ cells were purified one day after the last feeding for 5 consecutive days from spleens and MLN of mice fed with 50μg control IgG or anti-CD3 Ab. Purified cells (2.5×10^6) were injected i.v. to naïve AKR/J mice on day 0. Recipient mice were injected with 40mg STZ from day 1 to 5 and blood glucose level was monitored by IPGTT once weekly. ●; $CD4^+$ T cells from anti-CD3 Ab fed mice (n=12), ▲; $CD4^+$ T cells from control IgG Ab fed mice (n=12), ○; $CD4^+LAP^-$ T cells from anti-CD3 Ab fed mice (n=12), △; $CD4^+LAP^-$ T cells from control IgG fed mice (n=12). Mice transferred with CD4+ cells from anti CD3 fed mice developed significantly less diabetes (p=0.05) than those that were given CD4+ cells from control IgG fed mice or LAP- cells from either CD3 or control IgG fed mice.

Fig. 1

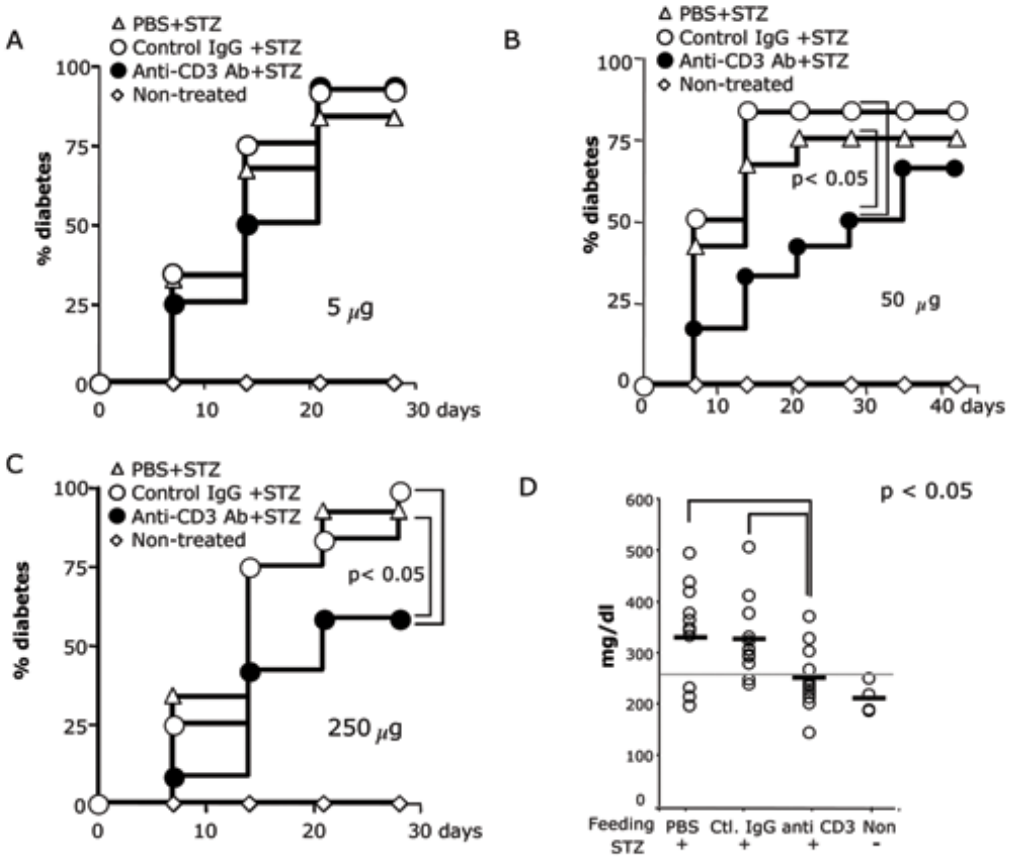

A
PBS+STZ
Control IgG +STZ
Anti-CD3 Ab+STZ
Non-treated
% diabetes
5 μg
30 days
B
p< 0.05
50 μg
40 days
C
250 μg
D
p < 0.05
mg/dl
Feeding
STZ
PBS
Ctl. IgG
anti CD3
Non

Fig. 2

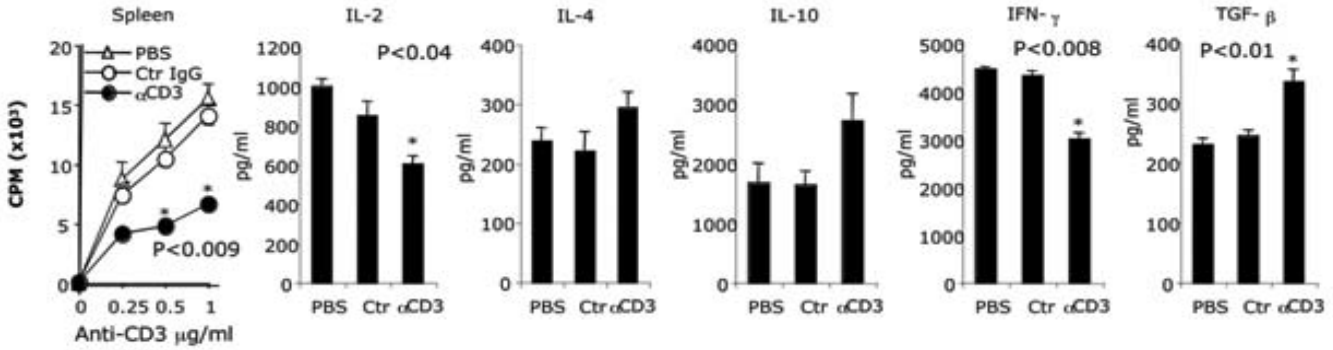
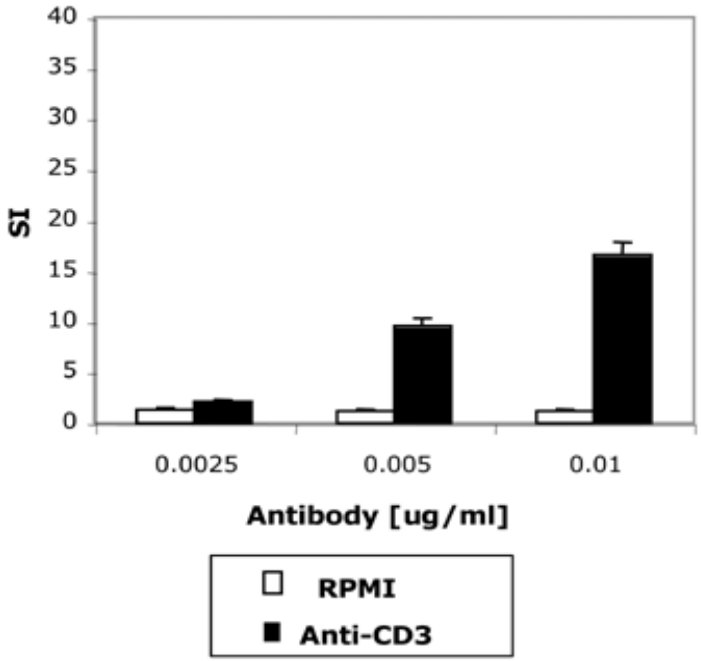

Spleen
PBS
Ctr IgG
αCD3
CPM (x10³)
P<0.009
Anti-CD3 μg/ml
IL-2
P<0.04
IL-4
IL-10
IFN-γ
P<0.008
TGF-β
P<0.01
pg/ml
PBS
Ctr
αCD3

Fig 3


40
35
30
25
20
15
10
5
0
SI
0.0025
0.005
0.01
Antibody [ug/ml]
RPMI
Anti-CD3

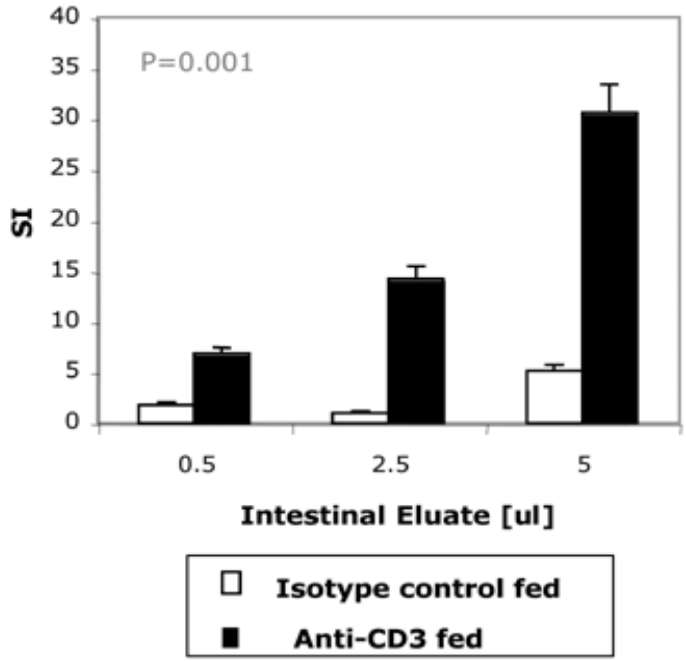

P=0.001
40
35
30
25
20
15
10
5
0
SI
0.5
2.5
5
Intestinal Eluate [ul]
Isotype control fed
Anti-CD3 fed

Fig. 4

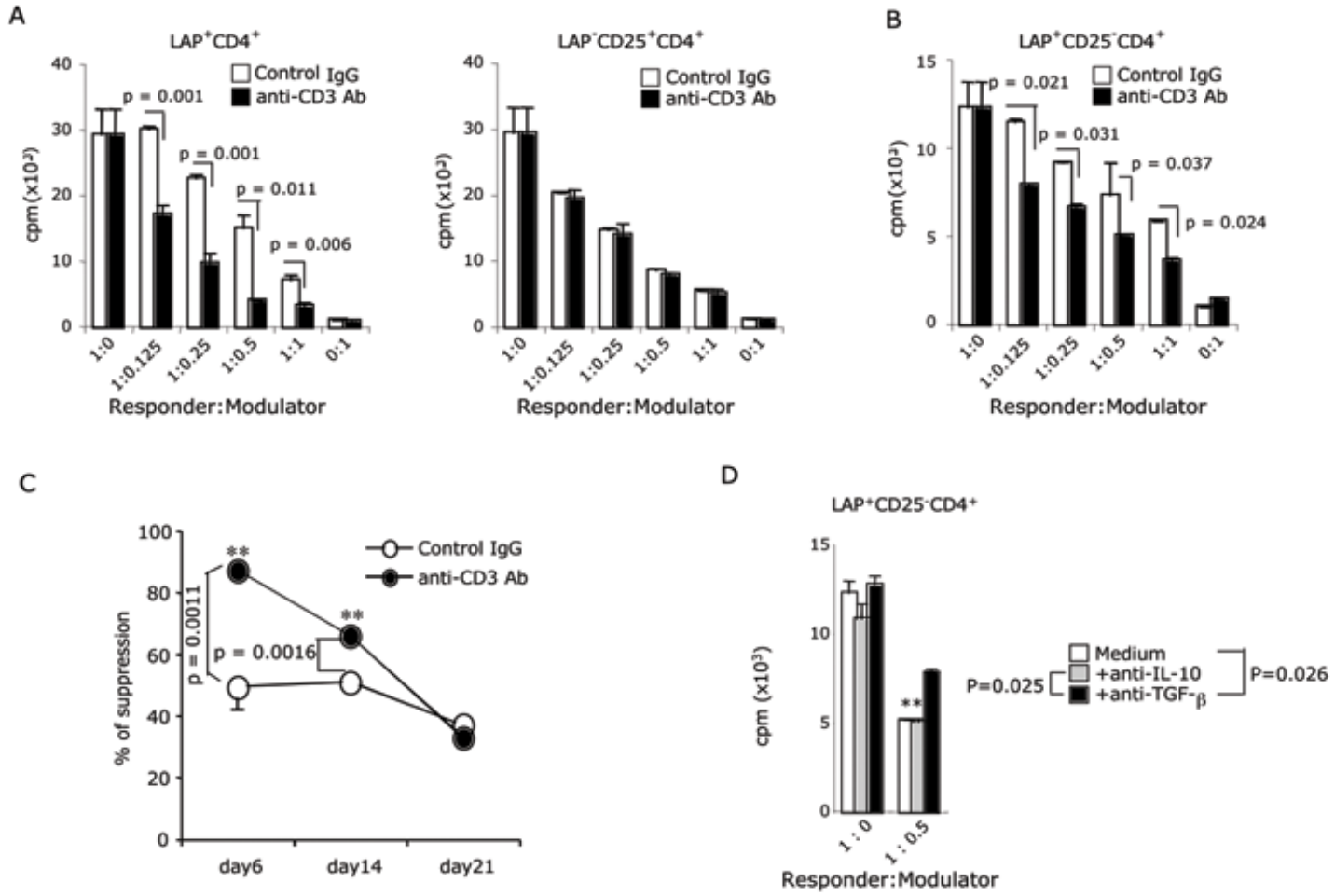
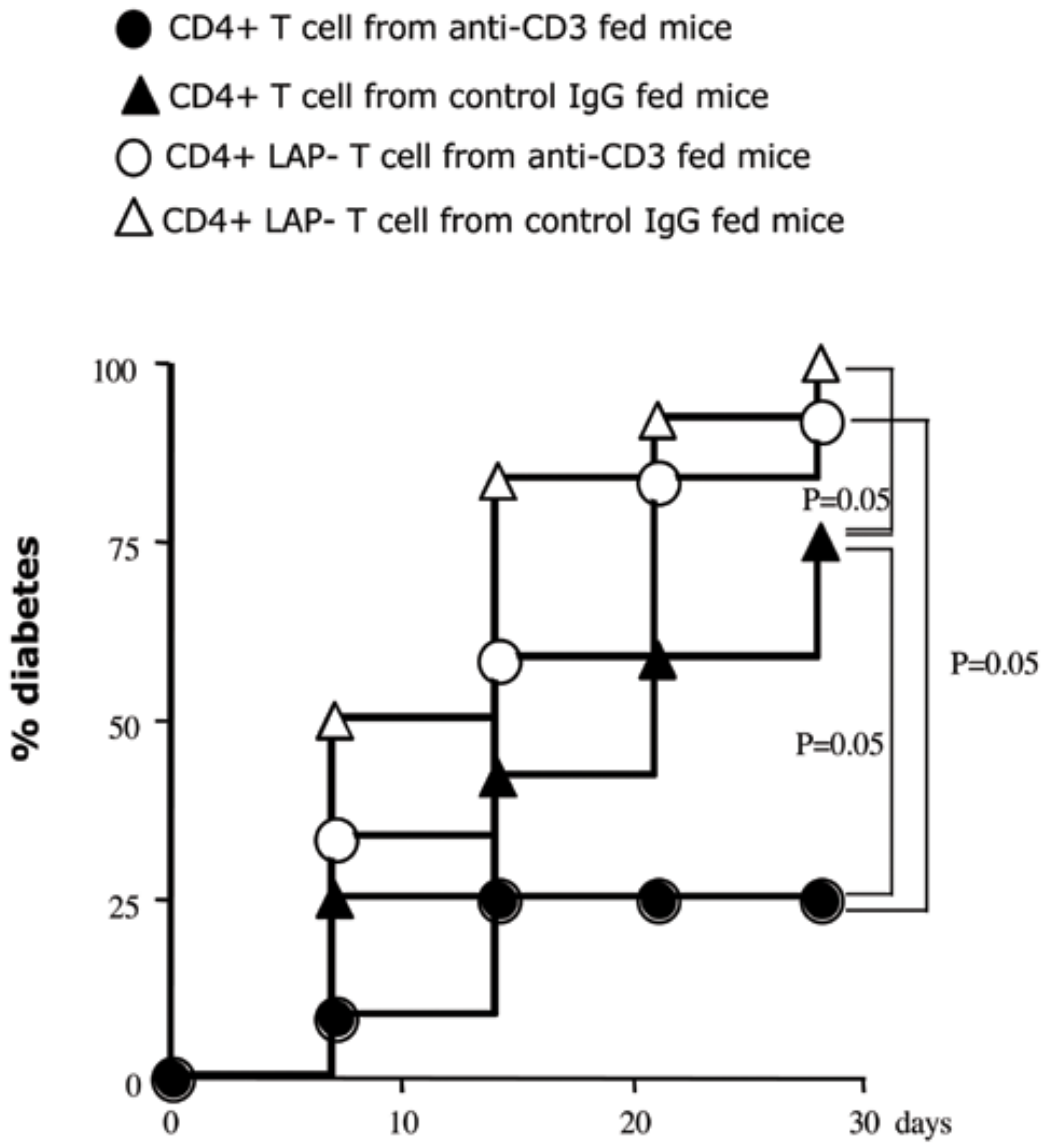

A
LAP+CD4+
Control IgG
anti-CD3 Ab
p = 0.001
p = 0.001
p = 0.011
p = 0.006
cpm (x10³)
Responder:Modulator
LAP-CD25+CD4+
B
LAP+CD25-CD4+
p = 0.021
p = 0.031
p = 0.037
p = 0.024
C
% of suppression
p = 0.0011
p = 0.0016
day6
day14
day21
D
Medium
+anti-IL-10
+anti-TGF-β
P=0.025
P=0.026
1 : 0
1 : 0.5

Fig. 5


CD4+ T cell from anti-CD3 fed mice
CD4+ T cell from control IgG fed mice
CD4+ LAP- T cell from anti-CD3 fed mice
CD4+ LAP- T cell from control IgG fed mice
% diabetes
100
75
50
25
0
0
10
20
30 days
P=0.05
P=0.05
P=0.05