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Immunopathology and Infectious Disease

Induction of Active Tolerance and Involvement of CD1d-Restricted Natural Killer T Cells in Anti-CD3 $F(ab')_2$ Treatment-Reversed New-Onset Diabetes in Nonobese Diabetic Mice

Guojiang Chen,* Gencheng Han,* Jianan Wang,* Renxi Wang,* Ruonan Xu,* Beifen Shen,* Jiahua Qian,† and Yan Li*

From the Department of Molecular Immunology, Institute of Basic Medical Sciences, Beijing, People's Republic of China; and the Vaccine Branch,† National Cancer Institute, National Institutes of Health, Bethesda, Maryland*

The application of anti-CD3 $F(ab')_2$ monoclonal antibodies has recently been expanded to treat established autoimmune diseases, including type 1 diabetes. However, the mechanism underlying their effect remains largely unclear. We report that short-phase administration of anti-CD3 $F(ab')_2$ antibodies efficiently allowed 80% of new-onset, nonobese diabetic (NOD) mice to significantly regain both normoglycemia and pancreatic β cell-specific autoantigen (ie, glutamic acid decarboxylase and insulin) tolerance, with both effects lasting more than 40 weeks. The responsible mechanism appears to involve the induction and maintenance of a population of immunoregulatory CD1d-restricted natural killer T (NKT) cells, which were marked by an enhanced Th2 response and secretion of elevated levels of interleukin-10. *In vivo* neutralization of interleukin-4 and/or interleukin-10 bioactivity abrogated this anti-CD3-mediated effect. Importantly, when the cotransfer of NKT cells from the livers of anti-CD3-treated mice and splenocytes from untreated, acutely diabetic NOD mice was performed in NOD-severe combined immunodeficient mice, the NKT cells were sufficient to either delay or prevent the onset of diabetes compared with controls where only splenocytes were introduced. These data suggest that CD1d-restricted NKT cells may play a critical role in anti-CD3 antibody-induced diabetes remission and the restoration of immune tolerance. *(Am J Pathol 2008, 172:972–979; DOI: 10.2353/ajpath.2008.070159)*

Type 1 diabetes is an autoimmune disease characterized by immunological destruction of insulin-producing pancreatic β cells and subsequent hyperglycemia.[1] The development of type 1 diabetes is influenced by multiple genetic and environmental factors, most of which remain unknown.[2,3] The nonobese diabetic (NOD) mouse strain spontaneously develops a disease similar to human type 1 diabetes as a consequence of the loss of basic tolerogenic processes designed to control self/nonself discrimination.[4]

Many studies have demonstrated that defects of several regulatory lymphocytes contribute to the pathogenesis of type 1 diabetes, including $CD4^+$ regulatory T cells (Tregs) and natural killer T (NKT) cells coexpressing T and NK cell receptors.[5–7] The number and function of these cells are impaired in NOD mice and individuals at risk for type 1 diabetes. Increasing the number by adoptive transfer,[8,9] or by augmenting function of these regulatory cells by T cell receptor engagement,[10] has potential for the prevention and treatment of diabetes. Manipulation of $CD4^+$ Tregs or NKT cells has been shown to be involved in antigen-induced tolerance to self islets.[11]

Anti-CD3 monoclonal antibody (mAb) (OKT3) is commonly considered as a category of immunomodulatory agents for silencing acute allograft rejection. Its therapeutic role in autoimmune diabetes has been recently reported that the treatment of new-established diabetes with anti-CD3 $F(ab')_2$ resulted in durable disease remission in animals or reduced usage of insulin in humans involving Th2 deviation.[12,13] A population of $CD4^+CD25^+$ Tregs has been proposed to be implicated in this effect.[14] However, whether other immunoregulatory path-

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G.C. and G.H. contributed equally to this work.

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Address reprint requests to Prof. Yan Li, Department of Molecular Immunology, Institute of Basic Medical Sciences, Taiping Road, No. 27, Beijing, 100850 People's Republic of China. E-mail: liyan62033@yahoo.com.cn.

ways required for regulation of glycemia by anti-CD3 exist need to be determined. Here, we found that CD1d-restricted NKT cells were involved in anti-CD3-mediated diabetes remission. The studies on the role of NKT cells in the induction and maintenance of self tolerance after anti-CD3 F(ab′)$_2$ treatment suggested that the number of CD1d-restricted NKT cells increased significantly in remitting NOD mice and their response deviated to Th2 phenotype. Notably, active inhibition of diabetes transfer by these cells indicated the generation of a population of protective NKT cells after anti-CD3 treatment.

Materials and Methods

Mice and Glycemia Screening

NOD.scid mice were obtained originally from the Jackson Laboratory (Bar Harbor, ME) 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 Institute of Basic Medical Sciences. The incidence of diabetes in these mice is 80 to 90% by 30 weeks of age. At 10 weeks of age, NOD mice were monitored for fasting blood glucose weekly. Diabetes was defined ≥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 laboratory. Anti-CD3 fragments were obtained by pepsin digestion. Briefly, anti-CD3 monoclonal antibody was dialyzed against citrate buffer (pH 3.5), and then 0.1 mg/ml pepsin dissolved in citrate was used at an enzyme/antibody ratio of 1:20 and incubated at 37°C for 3.5 hours, which was previously optimized. The reaction was stopped by adding 1:10 volume of 3 mol/L Tris base (pH 8.8). The pepsin-digested mAb preparation was immediately dialyzed against phosphate-buffered saline (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 kd were collected. The purity of anti-CD3 mAb F(ab′)$_2$ was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the preparation was stored in phosphate-buffered saline at 4°C until use.

Antibody Treatment

The diabetic NOD mice, within 7 days of the onset of overt diabetes, were treated intravenously with anti-CD3 F(ab′)$_2$ (40 μg/mouse) for 5 consecutive days. The untreated diabetic littermates were regarded as controls. The percentage of remission of diabetes was calculated as follows:

$$\text{Remission of diabetes (\%)} = \frac{\text{Number of mice with normoglycemia at certain time point after antibody treatment}}{\text{Total number of mice in the group}} \times 100\%$$

In some cases, NOD mice treated with anti-CD3 antibody received intraperitoneally antibody to interleukin (IL)-4 (11B11) and/or IL-10 (JES2A5) or rat IgG at a dose of 0.5 mg/mouse on days −1, 0, 2, 5, and then every 5 days. These antibodies were purchased from eBiosciences (San Diego, CA).

Lymphocyte Preparation

Single-cell suspension was prepared from spleen or pancreatic draining lymph nodes by passing them through nylon mesh. Lymphocytes from livers were isolated by 40%/80% Percoll solution.[15]

In Vitro *Proliferation Assay*

Lymphocytes from pancreatic draining lymph nodes were isolated from the anti-CD3-treated mice and from the control counterparts 3 weeks after antibody therapy. Proliferation assay was performed as described previously.[16] Briefly, lymphocytes were suspended with RPMI 1640 culture medium supplement with 10% fetal calf serum and were seeded in 96-well plate in triplicate (4×10^5 cells/well) with 10 μg/ml glutamic acid decarboxylase 65 (GAD65), 10 μg/ml $GAD_{500-585}$ (both prepared in our laboratory), 20 μg/ml porcine insulin, 15 μg/ml myelin basic protein, or 5 μg/ml concanavalin (all from Sigma Chemical Co, St. Louis, MO). On day 3 cultures were pulsed with 0.5 μCi of [^{3}H]thymidine per well for the last 16 hours, and the cells were harvested and counted by standard liquid scintillation.

Enzyme-Linked Immunosorbent Assay of Cytokine Production

Fluorescence-activated cell sorted hepatic NKT cells (5×10^4) were incubated in 96-well flat-bottomed microtiter plates in the presence of 0.5 μg/ml anti-CD3 monoclonal antibodies. In some experiments, Splenocytes (5×10^5) were incubated in the presence of 20 μg/ml recombinant $GAD_{500-585}$ protein. Supernatants were harvested after 48 hours. The levels of IL-4, interferon (IFN)-γ, and IL-10 were determined in triplicate in 0.1 ml of supernatant by sandwich enzyme-linked immunosorbent assay. The enzyme-linked immunosorbent assay kits used in this study were purchased from BD PharMingen (San Diego, CA).

Preparation for α-GalCer-Loaded CD1d:Ig Protein Staining Cocktail

α-Galactosylceramide (α-GalCer) (Toronto Research Chemicals Inc., Toronto, Canada) and CD1d:Ig (PharMingen) were mixed together in phosphate-buffered saline at appropriate

ratio according to the manufacturer's instructions and incubated at 37°C overnight. The staining cocktail was prepared by mixing α-GalCer-loaded CD1d:Ig protein with phycoerythrin-conjugated A85-1 mAb (PharMingen) at a ratio of 1:1 dimer to A85-1 mAb, and the mixture was incubated for 1 hour at room temperature in the dark.

Flow Cytometry

Antibodies used for flow cytometry analysis included fluorescein isothiocyanate-labeled anti-murine T cell receptor-β (H57-597) from eBiosciences and phycoerythrin-labeled anti-murine CD3ε (145 2C11) from PharMingen. Cells were stained in phosphate-buffered saline with 2% heat-inactivated fetal calf serum and 0.2% sodium azide and fixed using phosphate-buffered saline with 1% paraformaldehyde. The NKT cell population was identified as T cell receptor-$\beta^+\alpha$-GalCer-loading dimer$^+$ cells. For the detection of peripheral blood CD3$^+$ cells, whole blood was treated with fluorescence-activated cell sorting lysing solution (Becton Dickinson, Tokyo, Japan) to eliminate red blood cells after staining. Data collection and analysis were performed on a fluorescence-activated cell sorting Calibur flow cytometry using CellQuest software (Becton Dickinson, San Jose, CA).

Histopathology

NOD mice were sacrificed by cervical dislocation, and pancreas was immediately removed. Each pancreas was fixed with 10% buffered formalin, embedded in paraffin, sectioned at 4.5 μm, and stained with hematoxylin and eosin.[17] Insulitis grade was scored as follows: 0, normal islet; 1, mononuclear infiltration, largely in the periphery, in <25% of the islet; 2, 25 to 50% of the islets showing mononuclear infiltration; 3, >50% of the islet showing mononuclear infiltration; 4, small, retracted islet with few mononuclear cells.

Adoptive Transfer of Diabetes

Purified NKT cells (2×10^5 cells) from the liver of anti-CD3-treated or control mice were mixed with splenocytes (1×10^7 cells) from untreated, acutely diabetic NOD mice and administered intravenously into the tail veins of 4- to 8-week-old NOD.scid mice. Age-matched NOD.scid mice receiving only 1×10^7 diabetic splenocytes were used as positive control. Recipients were tested every week for diabetes and diagnosed as described above.

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.

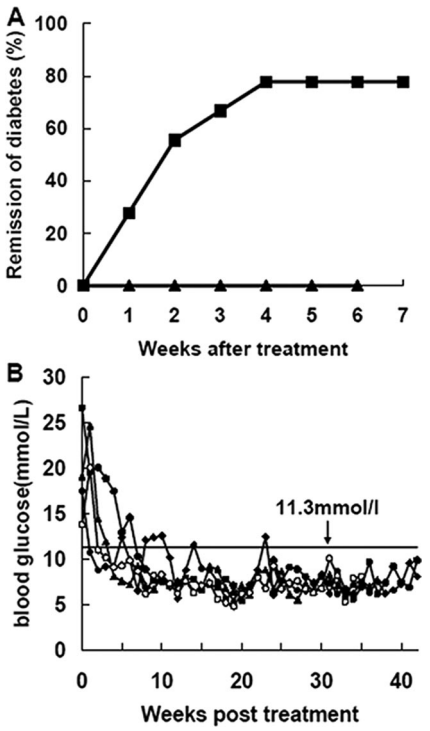


Figure 1. Anti-CD3 F(ab′)$_2$ antibody efficiently reversed new-onset diabetes in NOD mice. Antibody preparation and injection are described in Materials and Methods. **A:** New-onset diabetic NOD mice were injected with anti-CD3 F(ab′)$_2$. The diabetic untreated littermates were regarded as controls. 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 (▲, $n = 15$) and the antibody-treated group (■, $n = 20$). **B:** Representative data of glucose control from four anti-CD3 F(ab′)$_2$-treated NOD mice. Blood glucose was detected weekly after antibody treatment. A value less than 11.3 mmol/L was regarded as normal glycemic.

Results

Anti-CD3 F(ab′)$_2$ mAbs Effectively Reversed New-Onset Diabetes in NOD Mice

From 10 weeks of age, NOD mice were monitored for fasting glycemia weekly. New onset diabetic mice were randomly divided into two groups and were transfused with anti-CD3 antibody or left untreated as described in Materials and Methods. Blood glucose were screened once a week. As shown in Figure 1A, 80% (16 of 20) of mice injected with anti-CD3 F(ab′)$_2$ returned to normoglycemia by the fourth week after treatment. This effect was maintained over 40 weeks (Figure 1B), indicating that short-phase anti-CD3 F(ab′)$_2$ treatment resulted in significant and durable remission of hyperglycemia to normoglycemia in NOD mice.

Anti-CD3 F(ab′)$_2$ Treatment Diminished the Insulitis in NOD Mice

To determine whether reversal of diabetes following anti-CD3 F(ab′)$_2$ treatment correlated with reduction

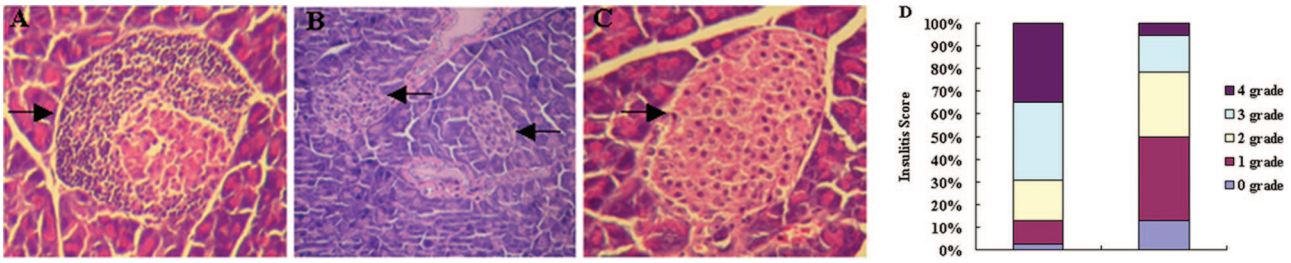


Figure 2. Anti-CD3 F(ab′)$_2$ treatment diminished insulitis in NOD mice. **A:** Representative islet from untreated diabetic mice ($n = 4$) with infiltration of numerous lymphocytes. **B:** Representative islets from mice ($n = 6$) 10 weeks after anti-CD3 F(ab′)$_2$ treatment. **C:** Representative islets from mice ($n = 5$) 20 weeks after anti-CD3 F(ab′)$_2$ treatment. **D:** Insulitis scores of pancreatic islets from anti-CD3 F(ab′)$_2$-treated mice ($n = 8$) or controls ($n = 6$). Seventy-two islets for control group and 94 islets for treated group were examined, respectively. Magnifications: **A** and **C**, ×200; **B**, ×100. **Arrows** denote islets.

and/or collapse of inflammatory infiltrates, we characterized the histology of pancreata from NOD mice 10 weeks and 20 weeks after antibody treatment. The results showed that, in contrast to the islet from the control diabetic mouse that showed much infiltration of numerous mononuclear cells (Figure 2A), anti-CD3 F(ab′)$_2$ (Figure 2, B–D) treatment diminished the insulitis in NOD mice.

Anti-CD3 F(ab′)$_2$ Treatment Had No Influence on the Number of CD3$^+$ Cells in Vivo

Anti-CD3 antibodies function mainly by depleting CD3$^+$ T cells in treating allograft rejection.[18,19] To determine whether this was available to explain the effect of anti-CD3 F(ab′)$_2$ on reversing established diabetes, the number of CD3$^+$ cells after anti-CD3 F(ab′)$_2$ treatment was examined. We found that anti-CD3 F(ab′)$_2$ treatment did not result in significant increase or decrease in CD3$^+$ cell numbers in peripheral blood (Figure 3A), spleen, or inguinal lymph nodes (Figure 3B). Therefore, reversal of diabetes by anti-CD3 F(ab′)$_2$ treatment was not due to a change in the abundance of CD3$^+$ cells.

Anti-CD3 F(ab′)$_2$ Treatment Induced Immune Tolerance to Pancreatic β-Cell Autoantigens

We next explored further the underlying mechanisms responsible for remission of diabetes by anti-CD3 F(ab′)$_2$ treatment. To determine whether anti-CD3 F(ab′)$_2$ administration diminished all T cell response or specifically inhibited the activation of autoantigen-specific T cells, we tested the T cell response to glutamic acid decarboxylase and insulin, two candidates of islet β-cell autoantigens,[20] as well as some unrelated antigens such as myelin basic protein and concanavalin as stimulators. In addition, as it is documented that pancreatic draining lymph nodes are critical sites for priming effector cells through the pathogenesis of type 1 diabetes,[21,22] we isolated mononuclear cells from pancreatic draining lymph nodes and examined the proliferation of these T cells in response to different antigens *in vitro*. When whole glutamic acid decarboxylase (GAD65) and GAD$_{500–585}$, a peptide consisting of dominant epitopes of GAD65,[23,24] as well as insulin were used as autoantigens in the *in vitro* T cell proliferation assay, we found that injection of anti-CD3 F(ab′)$_2$ antibodies resulted in significant hyporesponsiveness of lymphocytes to GAD65, GAD$_{500–585}$, and insulin compared to the control group (*$P < 0.05$ and **$P < 0.01$) (Figure 4). However, no statistical differences on the proliferative responses to concanavalin or myelin basic protein between the two groups were observed ($P > 0.05$). These results demonstrated that anti-CD3 antibody administration induced autoantigen-specific tolerance, which accounted for reversal of diabetes in NOD mice.

We have also examined in another study whether CD4$^+$ T regulatory cells are involved in anti-CD3 F(ab′)$_2$ antibody treatment-induced tolerance. Unexpectedly, both the abundance and the function of CD4$^+$ T regulatory cells are comparable to untreated controls.[25]

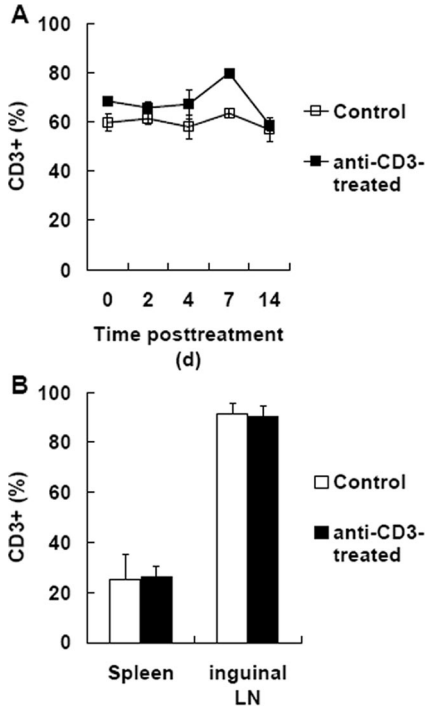
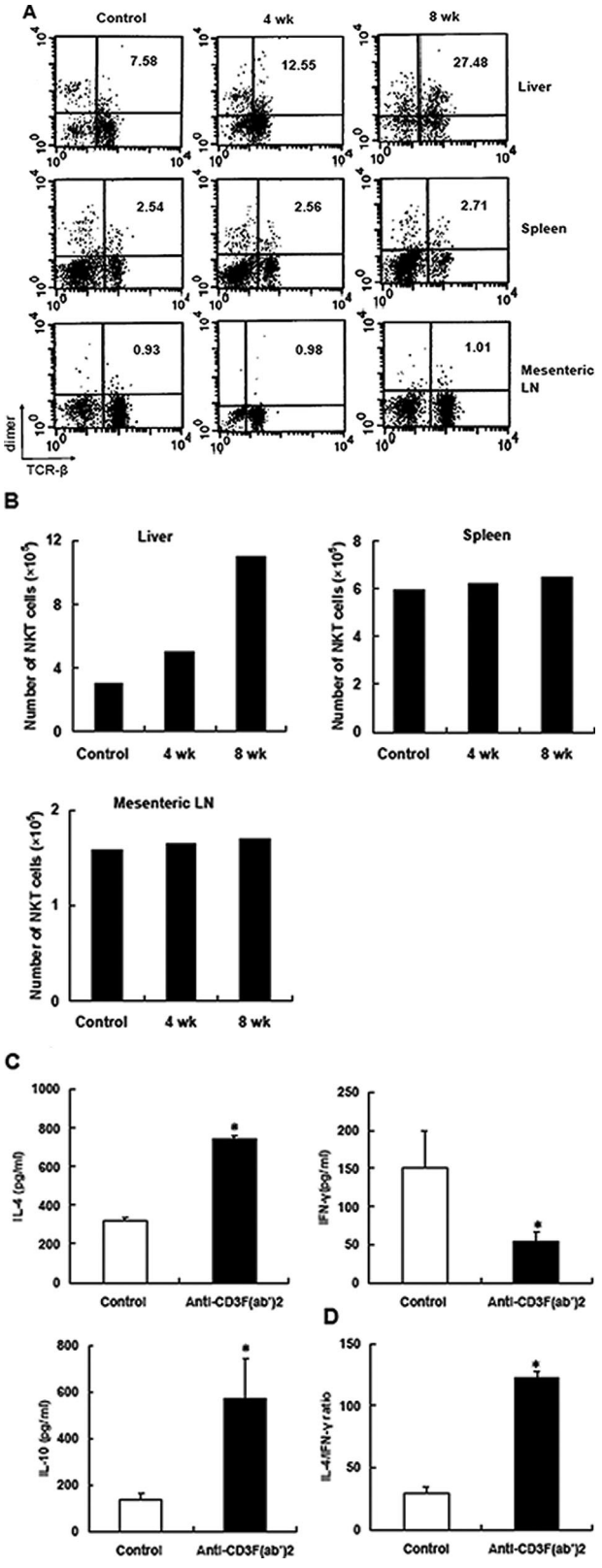


Figure 3. Anti-CD3 F(ab′)$_2$ treatment did not affect CD3$^+$ cell abundance. **A:** At different time after anti-CD3 F(ab′)$_2$ treatment, the proportions of CD3$^+$ cells were detected by PE-labeled anti-CD3ε antibody in peripheral blood. **B:** Two weeks after anti-CD3 F(ab′)$_2$ treatment, cells were collected from spleen as well as inguinal lymph nodes of NOD mice and detected by flow cytometry. Data are shown as means ± SD, $n = 4–6$.



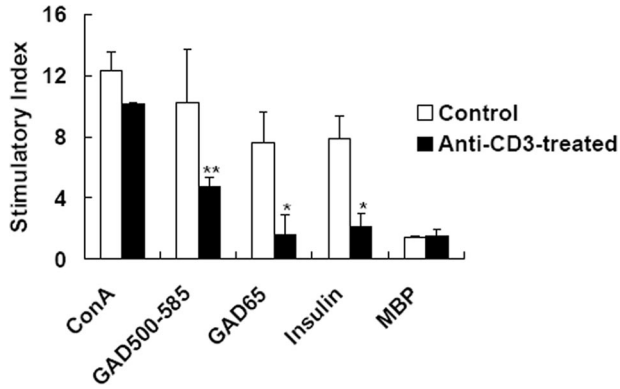


Figure 4. Anti-CD3 F(ab′)$_2$ administration diminished the T cell response in NOD mice. Lymphocytes isolated from pancreatic draining lymph nodes of anti-CD3 F(ab′)$_2$-treated NOD mice or the controls 3 weeks after antibody treatment were reacted with recombinant mouse GAD65 protein (GAD65), 500–585 part of GAD65 ($GAD_{500-585}$), porcine insulin, concanavalin, or myelin basic protein, and the cells were incubated with 0.5μCi of [^{3}H]thymidine. Proliferation was determined by [^{3}H]thymidine uptake. Data are expressed as stimulation indices (SI) ± SD of the mean from four mice with the background of 1249–3914 cpm, tested in triplicate.*$P < 0.05$, **$P < 0.01$ as compared with the control counterparts.

CD1d-Restricted NKT Cells Were Involved in Anti-CD3 F(ab′)$_2$ Antibody-Mediated Diabetes Reversion

Accumulative evidence has implicated CD1d-restricted NKT cells as a unique T lymphocyte sublineage in the regulation of immune responses associated with a broad range of diseases, including autoimmunity.[26] Amplified activity of NKT cells contributed to manipulating autoimmunity aggression via controlling pathogenic cells *in vivo* through an IL-4-dependent pathway. Thus, to determine whether CD1d-restricted NKT cells were involved in restoring immune tolerance after CD3 antibody treatment, we first tested the abundance of NKT cells (T cell receptor-β^+dimer$^+$) in anti-CD3-treated mice versus the controls. At various time points after treatment, cells were collected from different lymphoid organs of NOD mice and analyzed by flow cytometry. The percentage and absolute number of NKT cells significantly increased after treatment in liver but not in spleen and mesenteric lymph nodes (Figure 5,A and B). This is consistent with the concept that the liver compartments harbor unique microarchitecture and vascularization in favor of preferential distribution of blood-borne anti-CD3ε antibodies to the liver.[27] Furthermore, when hepatic NKT cells were isolated from remitting mice 4 weeks after antibody treat-

Figure 5. Anti-CD3 administration expanded NKT cells and enhanced anti-inflammatory response. The proportion **(A)** and absolute number **(B)** of NKT cells in various lymphoid compartments of NOD mice after anti-CD3 treatment were determined by fluorescence-activated cell sorting analysis. Representative data of three experiments are shown. **C:** Hepatic NKT cells were isolated from control or anti-CD3 F(ab′)$_2$-treated mice, respectively, and stimulated with anti-CD3 monoclonal antibodies (0.5 μg/ml) for 48 hours. The production of IL-4, IFN-γ, and IL-10 was determined in the supernatant by enzyme-linked immunosorbent assay. **D:** Splenocytes from antibody-treated or control mice 4 weeks after treatment were cultured in the presence of $GAD_{500-585}$ at the concentration of 20 μg/ml for 48 hours, and then the supernatants were collected. The levels of IL-4 and IFN-γ were determined, and the ratio of IL-4 to IFN-γ is presented. Values are shown as means ± SD of four mice. *$P < 0.05$ as compared with control mice.

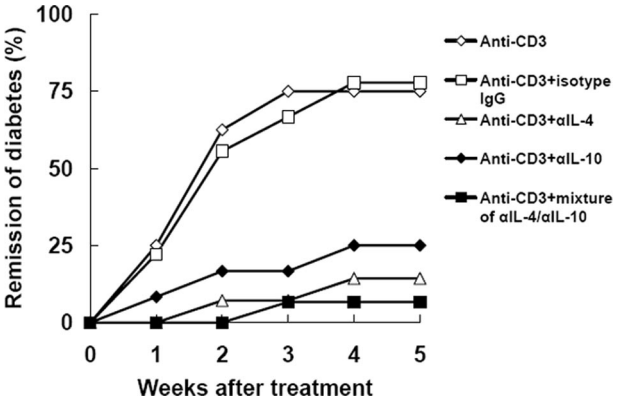


Figure 6. Neutralization of IL-4 and/or IL-10 prevented diabetes remission after anti-CD3 treatment. Diabetic NOD mice receiving CD3-specific antibody were injected intraperitoneally with neutralizing antibody to IL-4, IL-10, or a mixture of these two antibodies. As controls, anti-CD3-treated mice received isotypes or not. The remission of diabetes was evaluated for 5 weeks. N = 8–15 for each group.

ment, these NKT cells secreted increased amounts of IL-4 and IL-10 and lesser amounts of IFN-γ (Figure 5C), indicating a population of functionally immunomodulatory NKT cells occurred. In addition, we examined the level of IL-4 and IFN-γ in the supernatant of spleen cells from control or remitting mice 4 weeks after treatment. Consistent with Th2 phenotype of NKT cells, Th2 shift could also be detected in periphery (Figure 5D).

To determine a role of Th2 cytokines IL-4 and IL-10 in anti-CD3-mediated diabetes reversion, NOD mice treated with anti-CD3 were injected with antibodies specific for IL-4 and/or IL-10 to neutralize the bioactivity of these cytokines *in vivo*. In contrast to those only receiving isotype control antibodies or not, the mice receiving αIL-4, αIL-10, or combination of these two antibodies did not exhibit diabetes remission after anti-CD3 treatment (Figure 6), indicating Th2 cytokines IL-4 and IL-10 were critical for anti-CD3 effect.

To further address the protective potency of NKT cells, we fractionated NKT cells from the liver of antibody-treated mice and examined protective effect of these cells. Six- to 8-week-old NOD.scid recipients were transfused intravenously with splenocytes from diabetic NOD mice alone (diabetogenic cells) or mixed with NKT cells from antibody-treated or control NOD mice. In these experiments, 100% of mice injected with diabetogenic cells alone developed diabetes within 2 to 4 weeks of the transfer. In contrast, NKT cells from antibody-treated remitting mice significantly inhibited or delayed diabetes transfer into recipients. Whereas, no protection of NKT cells from control diabetic mice was observed (Figure 7). Taken together, these data indicated that the treatment with anti-CD3 antibodies induced a protective IL-4/IL-10-secreting subset of NKT cells.

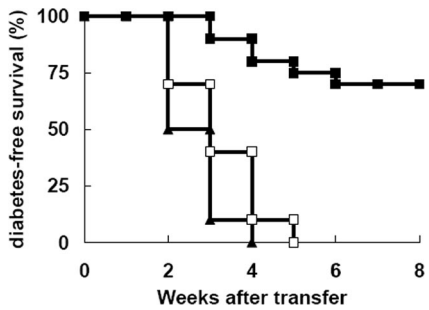


Figure 7. Expanded NKT cells suppressed the transfer of diabetes. NOD.scid females were injected with diabetic splenocytes (1×10^7 cells) alone (▲, n = 10) or mixed with isolated NKT cells (2×10^5 cells) from anti-CD3-treated (■, n = 20) or untreated mice (□, n = 20). The development of diabetes in the recipients was monitored weekly. Data were pooled from three separate experiments.

Discussion

Nonmitogenic anti-CD3 antibodies have been used to treat established immunological diseases such as type 1 diabetes, multiple sclerosis, psoriatic arthritis, and arteriosclerosis.[12,28–30] However, the underling mechanisms are largely unclear. Here our data showed that a short-term administration of CD3 antibodies was sufficient for reversing the new-onset diabetes for a long time, and the mechanisms involved the induction and maintenance of long-term immune tolerance, rather than a consequence of T cell depletion (Figures 1–4). The elements responsible for the specific tolerance were explored from different methods.

First we examined the role of $CD4^+CD25^+$ Tregs, a putative population of regulatory T cells,[31] in anti-CD3 induced glycemia reversal and tolerance restoration. Not consistent with a previous report,[14] which showed the involvement of $CD4^+CD25^+$ Tregs in CD3-antibody treated mice, neither increased number nor enhanced function of $Foxp3^+CD4^+$ T cells in NOD mice were found in our study, as depletion of $CD4^+CD25^+$ T cells using neutralizing anti-CD25 antibodies had no effect on the efficacy of anti-CD3 therapy.[25] The discrepancy could be resolved because up-regulation of $CD4^+CD25^+$ T cells is substantially due to T-cell activation rather than induction/expansion of $CD4^+$ Tregs. This prompted us to survey other pathways required for maintaining immune tolerance to self islets following anti-CD3 treatment.

In searching the T cell populations responsible for the immune tolerance in our model, we found that CD1d-restricted NKT cells, a dominant subset in mice,[32] were significantly up-regulated in anti-CD3-treated mice. These cells secreted high amounts of IL-4/IL-10 and less IFN-γ as compared to those from untreated controls (Figure 5). Further, using a cotransfer model, these cells from antibody-treated remitting mice blocked diabetes transfer, suggesting a protective function of expanded NKT cells. This is in agreement with that NKT cell-deficient NOD mice displayed exacerbated diabetes[33] and activation of NKT cells via repeated administration of CD1d ligands, for example α-GalCer, protected NOD mice from diabetes.[34,35] In addition, these functionally regulatory NKT cells perhaps derive from expansion of remnant NKT cell pool.[36]

The mechanisms by which enhanced NKT cells affect the autoimmune process need to be determined. Here we focus on the alteration of Th1/Th2 profile in NKT cells and peripheral lymphocytes. NKT cells has been described as one of the immune regulators that work through secreting different cytokines, such as IFN-γ or

IL-4, to promote different helper T cell response.[37,38] Our study indicated that the cytokine pattern of NKT cells deviated to Th2 phenotype after anti-CD3 treatment with the characteristics of decreased production of IFN-γ and increased IL-4/IL-10 production, which might contribute to suppressing peripheral pathogenic Th1 response to islets in the progression of overt autoimmunity,[39,40] although the details anti-CD3 treatment modulates NKT cell bioactivities need elucidation in the future experiments. Importantly, blockade of IL-4 and/or IL-10 activities *in vivo* led to abrogation of the diabetes-suppressive effect after anti-CD3 treatment, indicating a critical role of Th2 cytokines IL-4 and IL-10 in the NKT cell-mediated regulation of autoimmunity. These results are consistent with previous studies that underscore Th2 shift as a key mechanism of NKT-induced protection against diabetes.[34,35,41] Notably, Th2 shift could also be detected in the cytokine production patterns of spleen cells from CD3 antibody-treated mice. So in our system, CD3 antibody administration might work through regulating the activity of NKT cells that resulted in up-regulated production of IL-4/IL-10 and decreased IFN-γ production; thereafter these cells drive effector cells toward a nonpathogenic Th2 phenotype in the periphery. In some cases, NKT cells could inhibit the IL-2 and IFN-γ production and later proliferation of the effector $CD4^+$ T cells, resulting in an anergic phenotype, rather than blocking their initial activation and expansion[42] or dampening T cell differentiation into effectors directly by cell contacts.[43] Besides direct action on pathogenic T cells, the activation of NKT cells may influence other cell type, including dendritic cells, NK cells, and regulatory T cells.[44–47] Activated NKT cells induce dendritic cell maturation and accumulation in pancreatic lymph nodes, where they subsequently recruit and tolerize pathogenic T cells.[44] Once activated, NKT cells could also lead to subsequent activation of conventional NK cells.[46]

Collectively, these data have implied the involvement of CD1d-restricted NKT cells in anti-CD3 therapeutic effect on overt diabetes. Up-regulated NKT cells in anti-CD3-treated group express a Th2 shift on stimulation, which might affect the T cell balance in the peripheral system. Although the mechanisms in detail remain to be determined, our findings have important implications for understanding the mechanisms involved as well as potential modifications for the regimen of anti-CD3 therapy in the clinic.

Acknowledgment

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