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B7/CD28 Costimulation Is Essential for the Homeostasis of the CD4$^+$CD25$^+$ Immunoregulatory T Cells that Control Autoimmune Diabetes

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Summary

CD28/B7 costimulation has been implicated in the induction and progression of autoimmune diseases. Experimentally induced models of autoimmunity have been shown to be prevented or reduced in intensity in mice rendered deficient for CD28 costimulation. In sharp contrast, spontaneous diabetes is exacerbated in both B7-1/B7-2-deficient and CD28-deficient NOD mice. These mice present a profound decrease of the immunoregulatory CD4$^+$CD25$^+$ T cells, which control diabetes in prediabetic NOD mice. These cells are absent from both CD28KO and B7-1/B7-2KO mice, and the transfer of this regulatory T cell subset from control NOD animals into CD28-deficient animals can delay/prevent diabetes. The results suggest that the CD28/B7 costimulatory pathway is essential for the development and homeostasis of regulatory T cells that control spontaneous autoimmune diseases.

Introduction

The nonobese diabetic (NOD) mouse strain remains the best available model for the study of insulin-dependent type I diabetes mellitus. This mouse strain develops a complex autoimmunity controlled by multiple genes including those syntenic with genetic loci controlling susceptibility in humans (Todd, 1997). Thus, attempts to define the basic immune pathology and regulation of this disease in mice are likely to have important implications for the understanding and treatment of the human disease. Over the past few years, we and others have shown that the B7/CD28 costimulatory pathway is a major regulatory pathway for the control of immune responses (Bluestone et al., 1999). T-dependent antibody responses are severely impaired and induction of CTL to short term antigen exposure greatly reduced in the absence of CD28 signaling (Shahinian et al., 1993; Kundig et al., 1996). CD28 blockade has been shown to prevent or delay graft rejection and, in some instances, results in the development of donor-specific tolerance (Lenschow et al., 1996a). Recent studies have implicated CD28 costimulation in the induction and progression of autoimmune diseases. Experimentally, antigen-induced models of autoimmunity including arthritis, encephalomyelitis, myocarditis, thyroiditis and myasthenia gravis have all been shown to be prevented or reduced in intensity in CD28-deficient mice or when CD28 costimulation was blocked during the priming with the relevant self-antigen (Perrin et al., 1995; Bachmaier et al., 1996; Shi et al., 1998; Chang et al., 1999; Peterson et al., 1999; Tada et al., 1999). Thus, CD28 costimulation blockade is thought to be a promising approach in treatment of these diseases. However, both CD28-deficient NOD mice and transgenic NOD mice that express soluble circulating CTLA4Ig, a molecule that binds with high affinity to B7 molecules and blocks B7 costimulation, have increased severity of this autoimmune disease (Lenschow et al., 1996b).

Multiple differences may account for the discrepancy between the results in the NOD model of spontaneous autoimmunity and other experimentally induced autoimmunity models. First, contrary to experimental autoimmune diseases induced after a single injection of the relevant self-antigen (usually in a proinflammatory adjuvant such as CFA), the spontaneous diabetes observed in the NOD mice is a consequence of chronic exposures to the pathogenic self-antigens, where CD28 costimulation has been shown to be less critical. Indeed, it has been reported in CD28-deficient mice that short-term TCR stimulation induced anergy of T cells, whereas prolonged TCR stimulation induced a functional T response in vivo (Kundig et al., 1996). This may explain why CD28 is dispensable in spontaneous autoimmune diabetes. However, the relative CD28 independence of this response cannot account for the exacerbation of disease in the CD28-deficient setting. Second, as we have previously shown, the increase of diabetes in CD28-deficient NOD mice could be due to a lack of autoreactive Th2-type T cells (Lenschow et al., 1996b). Indeed, the Th1/Th2 balance of autoreactive T cells has been shown to regulate the disease, as Th2 cytokines have protective effects in this model (King and Sarvetnick, 1997). Finally, CD28 has been shown to upregulate the expression of the negative regulatory CD28 homolog CTLA-4 (Alegre et al., 1996; Finn et al., 1997). CTLA-4-deficient mice die of a massive lymphoproliferation resembling a generalized autoimmune disease (Tivol et al., 1995), and CTLA-4 has been shown to play a role in T cell peripheral tolerance (Perez et al., 1997). Blocking the B7/CTLA-4 interaction during an ongoing autoimmune process can exacerbate experimental autoimmune encephalomyelitis (Karandikar et al., 1996). Significantly, short-term therapy with anti-CTLA-4 mAbs of a diabetogenic T cell receptor transgenic mouse strain results in rapid induction of insulitis and β cell destruction (Luhder et al.,

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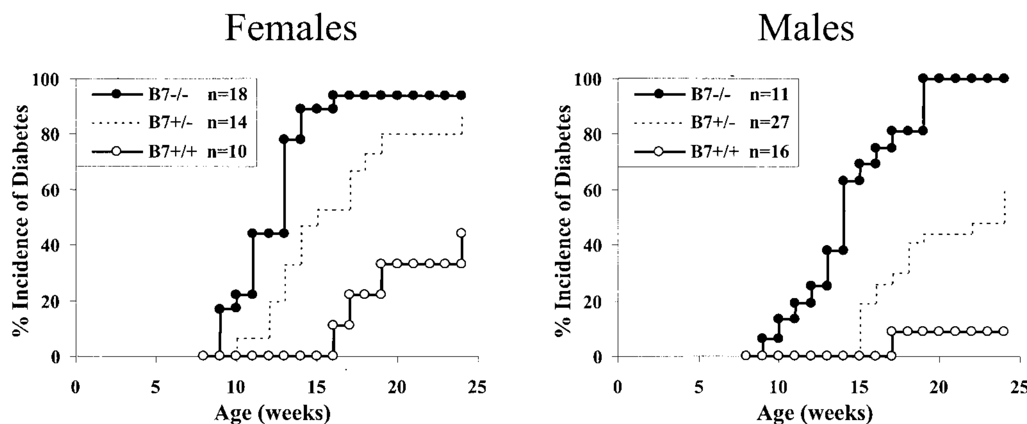

Figure 1. Increased Incidence and Early Onset of Diabetes in B7-Deficient NOD Mice

Blood glucose levels were checked weekly in B7-deficient ($B7^{-/-}$, closed circles), B7 heterozygous ($B7^{+/-}$, dashed line) and B7 wild-type ($B7^{+/+}$, open circles) females (left) and males (right). Ten to twenty-seven mice were analyzed per group.

1998). Finally, CTLA-4 polymorphisms have been implicated as a risk factor in the development of human diabetes mellitus (Donner et al., 1997; Marron et al., 1997). Thus, the blockade of CD28 might alter CTLA-4, affecting immune regulation in this model.

To further analyze the role of B7 costimulation in autoimmune diabetes, we bred B7-1/B7-2-deficient mice into the NOD background. B7-1 (CD80) and B7-2 (CD86) molecules are the only two ligands of CD28 and CTLA-4; thus, B7-deficient mice have a complete deficit in CD28 and CTLA-4 signaling. In the present study, we show that B7-1/B7-2-deficient mice develop a more severe diabetes as compared to control mice. The disease was not associated with a shift of the Th1/Th2 balance. Rather, the B7-1/B7-2-deficient NOD mice present with a profound decrease of a regulatory $CD4^{+}CD25^{+}$ T cell population. Not only are these cells absent from both CD28-deficient and B7-1/B7-2-deficient mice, but transfer of the regulatory T cell subset from control NOD animals into CD28-deficient animals can delay/prevent diabetes. The results suggest that the CD28/B7 costimulatory pathway is essential for the development and homeostasis of the regulatory T cells that control spontaneous autoimmune diseases.

Results

Increased Incidence of Diabetes in B7-1/B7-2-Deficient NOD Mice

A genetic approach was used to analyze the role of B7 costimulatory molecules in autoimmune diabetes. B7-1/B7-2 double-deficient ($B7^{-/-}$) mice were bred into the NOD background. Diabetes was assessed by weekly measurement of urinary glucose or blood glucose concentrations. After four generations of backcrosses, some but not all the diabetic susceptibility genes were fixed as only 40% of female wild type ($B7^{+/+}$) and less than 10% of male $B7^{+/+}$ mice became diabetic by 24 weeks of age as compared to 70% of females and 20% of males in our wild type NOD colony. However, despite the incomplete breeding to the NOD background, almost 100% of female and male $B7^{-/-}$ mice became diabetic by 18 weeks of age. The disease onset was earlier and more severe as compared to their littermate $B7^{+/+}$ controls (Figure 1). Interestingly, the incidence of diabetes in heterozygote $B7^{+/-}$ mice was significantly increased over that seen in the $B7^{+/+}$ animals (Figure 1). Flow cytometric analyses showed that both B7-1 and B7-2 were expressed at lower levels on resting and in vitro activated B cells, dendritic cells, and $CD4^{+}$ and $CD8^{+}$ T cells isolated from $B7^{+/-}$ mice as compared to cells from $B7^{+/+}$ littermate mice (data not shown). Thus, both alleles of *B7-1* and *B7-2* genes are functionally active and contribute to the overall level of B7 expression. These results suggest that a 50% decrease on B7 expression can dramatically alter the progression of autoimmunity in these animals.

Both Th1 and Th2 Cytokines Are Reduced in B7-Deficient NOD Mice

The role of cytokines in autoimmune diabetes has been extensively studied in NOD mice. Some cytokines, such as IFNγ, are pathogenic, whereas others, like IL-4 or TGFβ, could block the development of the disease (King and Sarvetnick, 1997; King et al., 1998). The differentiation of Th2 cells secreting IL-4, IL-5, and IL-10 requires B7/CD28 costimulation (Rulifson et al., 1997). In fact, CD28-deficient NOD mice have impaired Th2 differentiation while Th1 development is maintained (Lenschow et al., 1996b). These results suggested that an imbalance in Th1/Th2 differentiation, as evidenced in the CD28-deficient NOD mice, might be a possible mechanism for the exacerbation of diabetes observed in the B7-deficient NOD mice. Thus, T cells from B7-deficient mice were stimulated with anti-CD3 mAbs, and supernatants were harvested to determine the levels of IL-4, IL-5, and IFNγ production. First, unlike the CD28-deficient NOD mice (Lenschow et al., 1996b), the production of all cytokines was significantly reduced as compared to cytokines produced by T cells from wild-type mice (Figure 2A). In addition, isotype analysis of antibodies to glutamic acid decarboxylase (GAD), a potent autoantigen in autoimmune diabetes, showed that overall levels of antibodies were reduced in the serum of B7-deficient

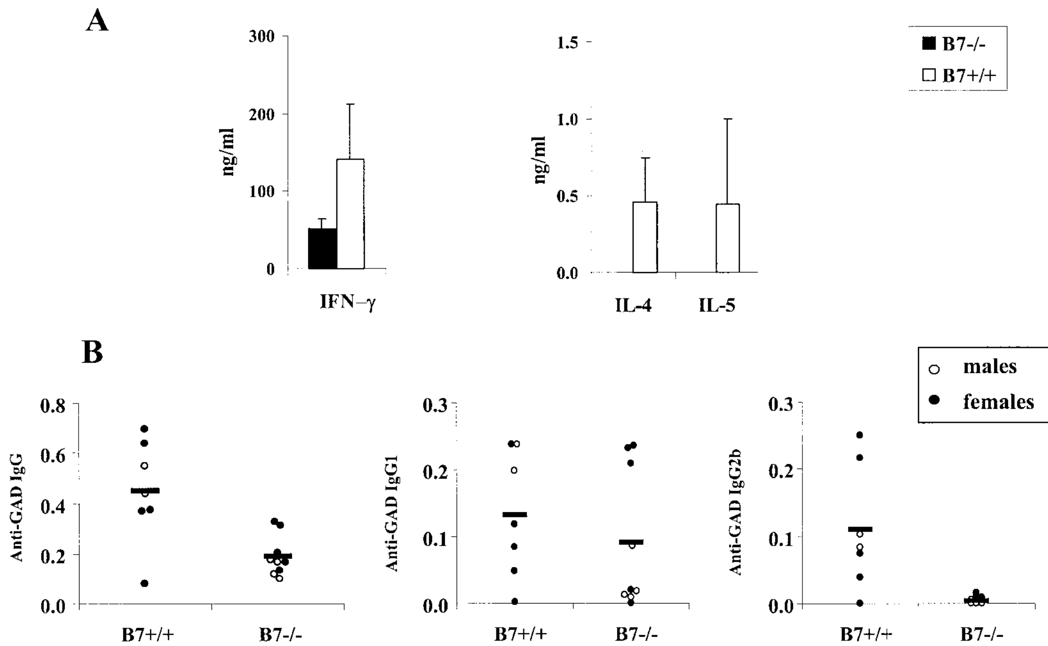

Figure 2. Both Th1 and Th2 Responses Are Reduced in B7-Deficient Mice

(A) Cytokines produced by T cells stimulated as described in the Experimental Procedures were measured by ELISA. Two $B7^{-/-}$ females and three littermate $B7^{+/+}$ females were analyzed in this experiment, which is representative of three independent experiments. Cells from males and females produced similar levels of cytokines.

(B) Reduced anti-GAD67 antibody levels in the sera of B7-deficient NOD mice. Anti-GAD67 IgG antibody, anti-GAD67 IgG1, and anti-GAD67 IgG2b isotypes in the sera of individual male and female $B7^{+/+}$ and $B7^{-/-}$ NOD mice were measured by ELISA as described in the Experimental Procedure. The results are presented as OD at 1:20 dilution of the sera.

NOD mice and that the levels of the Th1-dependent IgG2b isotype were severely decreased in these mice (Figure 2B). The Th2-dependent, IgG1 isotype was more variable. The levels of GAD-specific IgG1 antibodies were significantly reduced in five out of nine mice, but the serum levels of anti-GAD IgG1 antibodies were comparable to wild-type mice in the remaining four animals. Thus, contrary to previous observations with the CD28-deficient NOD mice, a selective decrease in Th2 cytokine production could not explain the increased disease severity in this B7-deficient NOD strain.

B7-Deficient NOD Mice Develop a Rapid Progression of Insulitis at 7–9 Weeks of Age

In order to better understand the pathogenesis of the disease, a careful study of the insulitis in B7-deficient NOD mice was undertaken. Virtually all islets isolated from 9-week-old prediabetic male and female B7-deficient mice were highly infiltrated and scored as severe insulitis. This result was in stark contrast to the wild-type NOD mice, where less than 10% of the islets from female and only 3% of the islets from male mice displayed a severe insulitis (Figure 3A). $CD4^+$ and $CD8^+$ T cells, $B220^+$ B cells, and $CD11c^+$ dendritic cells infiltrated the islets at high levels more similar to that observed in much older (15- to 20-week-old) control NOD mice (data not shown). These results suggested that the disease process was highly accelerated in the B7-deficient mice. A kinetic analysis of islet infiltration in B7-deficient mice was carried out by scoring the intensity of insulitis at different time points in young prediabetic animals. No insulitis was detected at 4 weeks of age and even at 6 weeks most of islets were not infiltrated. However, a rapid increase in the levels of islet infiltration was observed by 9 weeks of age (Figure 3B; data not shown). The increased islet infiltration was not progressive as in control NOD mice but occurred rapidly between 7 and 9 weeks of age. The accelerated kinetics of islet infiltration in B7-deficient mice suggested that a regulatory process that normally modulates the autoreactivity in the pancreas is lacking in these mice. Such a regulatory mechanism has been postulated in NOD mice based on the normally protracted time between the first appearance of insulitis (checkpoint 1) and the progression to frank diabetes (checkpoint 2) (Andre et al., 1996).

Expression of CTLA-4 on an Immunoregulatory Subset of $CD4^+$ T Cells in NOD Mice

One candidate for the control of diabetes is the regulation of T cell function via CTLA-4, a negative regulatory molecule on the surface of activated T cells (Walunas et al., 1994). The engagement of this molecule has been shown to control other autoimmune diseases, and the absence of its ligands, B7-1 and B7-2, in B7-deficient mice might account for the absence of immunoregulatory activity to control checkpoint 2. Therefore, T cells from NOD mice were examined for intracellular CTLA-4 expression where the majority of the CTLA-4 protein resides (Alegre et al., 1996). As seen in Figure 4, flow cytometric analysis of T cells from NOD mice identified a small population of CTLA-4^+ T cells in the spleen and

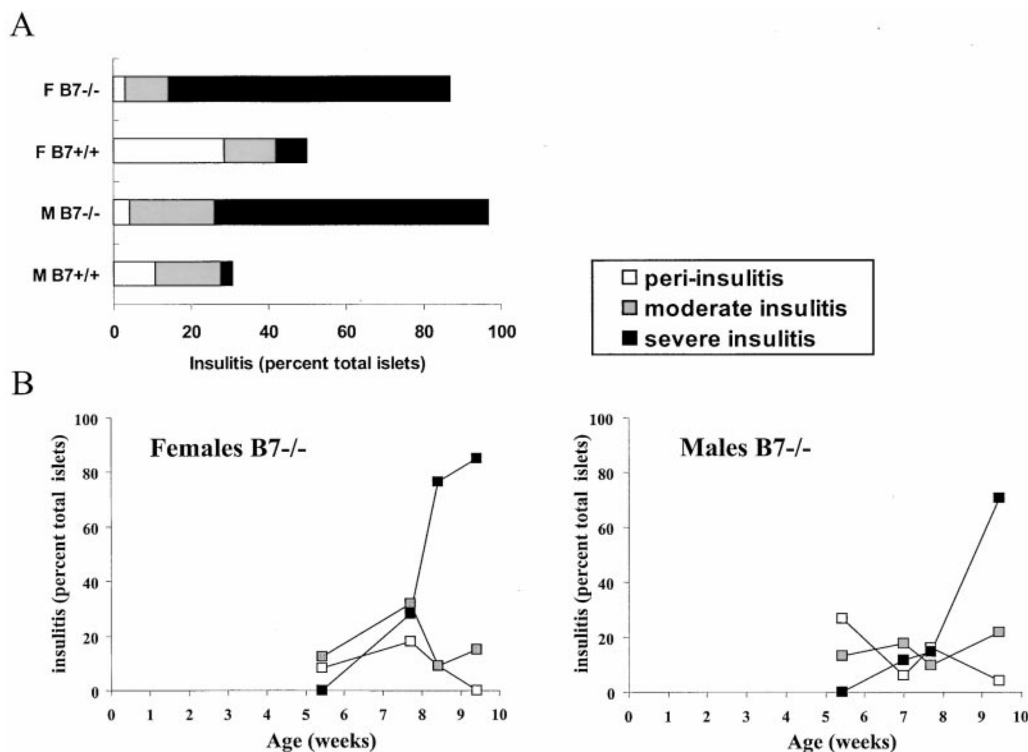

Figure 3. Increased Incidence and Rapid Progression of Insulitis in B7-Deficient NOD Mice

(A) Insulitis in 9-week-old B7$^{-/-}$ and littermate B7$^{+/+}$ control females (F) and males (M) were scored for the presence of peri-insulitis, moderate insulitis, and severe insulitis. Four mice and at least 100 islets for each group were scored, except for the male B7$^{+/+}$ where two mice and 65 islets were scored. Insulitis is reported as the percentage of infiltrated islets per total islets.

(B) Kinetics of insulitis in B7$^{-/-}$ female and male mice. Two animals (25–55 islets for females and 15–120 islets for males) from each time point were scored. Insulitis is reported as the percentage of infiltrated islets per total islets.

lymph nodes. The T cells were shown further to be small in size and express CD25 constitutively.

Previous studies have demonstrated the existence of regulatory CD4$^+$CD25$^+$ T cells that can prevent or limit autoimmunity. In the absence of this regulatory subset, normal mice develop several types of autoimmune disease spontaneously (Asano et al., 1996; Suri-Payer et al., 1998; Itoh et al., 1999). In most mouse strains, this subset represents 7%–10% of the CD4$^+$ T cell subset. In NOD mice, these cells are reduced in number, representing only 5%–6% of CD4$^+$ T cells (Figure 6A). An adoptive transfer model was used to examine the role of the CD4$^+$CD25$^+$ T cell subset in the regulation of autoimmune diabetes. Thirty million total splenocytes or CD25-depleted splenocytes from 11-week-old prediabetic NOD mice were transferred into lymphocyte-deficient NOD.SCID mice. Whereas 5 out of 14 mice transferred with total splenocytes became diabetic beginning 9 weeks after the cell transfer, virtually all of the recipients of CD25-depleted splenocytes, including the one receiving splenocytes from NOD males, developed diabetes within 5 weeks post transfer (Figure 5). In addition, in these latter mice, blood glucose levels rose from normal values (80 to 120 mg/dl) to high hyperglycemic levels of more than 300 mg/dl within 1 week, suggesting a very rapid kinetics of islet destruction. In contrast, the increase of glucose levels was more gradual in recipients of nondepleted splenocytes (Figure 5). These results suggest that a population of small CTLA-4$^+$CD25$^+$CD4$^+$ T cells are present in NOD mice that control the severity and temporal incidence of diabetes.

Absence of Immunoregulatory CD4$^+$CD25$^+$ T Cells in B7-Deficient NOD Mice

The possibility that B7 expression might affect the regulatory CD4$^+$CD25$^+$CTLA-4$^+$ T cells in NOD mice was examined by flow cytometric analyses of this subset. A profound decrease of CD4$^+$CD25$^+$ T cells was observed in the B7$^{-/-}$ NOD mice as early as 2 weeks of age and lasted for as long as analyzed (Figure 6A). Less than 1% of CD4$^+$ T cells expressed CD25 as compared to 5.4% in littermate B7$^{+/+}$ controls, corresponding to a 5- to 6-fold decrease of the absolute number of the CD4$^+$CD25$^+$ T cells in the B7$^{-/-}$ NOD, since the numbers of CD4$^+$ T cells were similar in B7$^{-/-}$ and B7$^{+/+}$ NOD mice (data not shown). There was also a partial decrease (37%) of this subset in B7$^{+/-}$ heterozygotes (Figure 6A). Thus, B7$^{-/-}$ mice are deficient in the immunoregulatory cells present in wild-type NOD mice, supporting a potential role for B7 engagement in CD4$^+$CD25$^+$ regulatory T cell development or homeostasis.

The regulation of the development or homeostasis of CD4$^+$CD25$^+$ cells by B7 costimulation could either be due to the absence of CD28 or CTLA-4 engagement. To

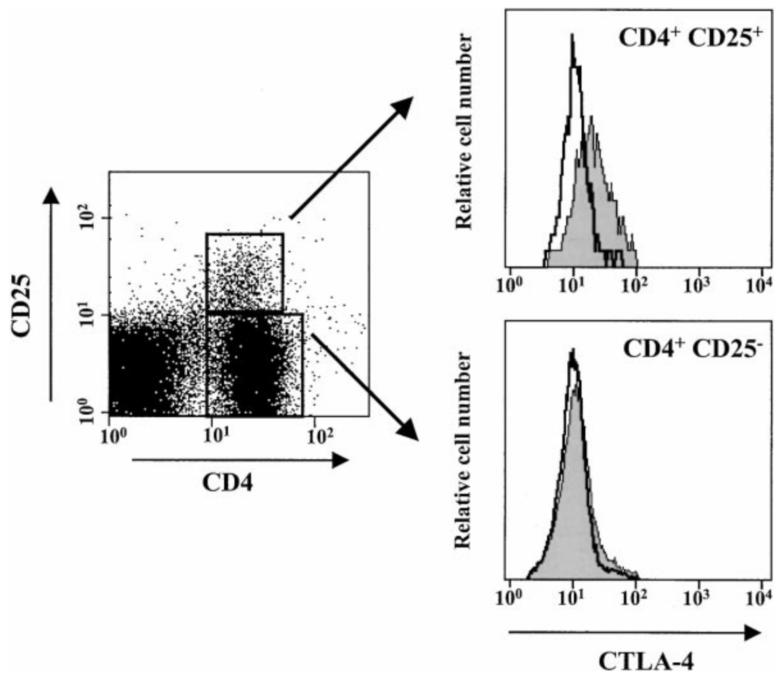

Figure 4. $CD4^+CD25^+$ Cells Expressed Intracellular CTLA-4 Constitutively

Spleen cells coexpressing CD4 and CD25 (upper panel) or expressing CD4 but not CD25 (lower panel) were gated and analyzed for intracellular CTLA-4 expression as described in Experimental Procedures. CTLA-4 expression was represented with shaded histograms and control staining was represented with thick lines. One representative experiment of three is shown.

differentiate between these two pathways, the proportion of the regulatory T cells in CD28-deficient NOD mice was determined. In these mice, only 1.5% of CD4 T cells expressed CD25 compared to 6.5% in the wild-type controls (Figure 6A; data not shown). Thus, the profound absence of these cells in CD28-deficient mice was similar to that observed in the B7-deficient mice, suggesting that the B7/CD28 interaction was critical for the homeostasis of these cells. In order to determine if B7/CD28 costimulation is required continuously for the homeostasis of this T cell subset or is required for their development, wild-type NOD mice were treated with murine CTLA4Ig, which blocks B7 costimulation. A 4- to 5-fold decrease of $CD4^+CD25^+$ cells was observed within 9 days of CTLA4Ig treatment (Figure 6B), suggesting a dynamic and permanent role for CD28/B7 ligation in the peripheral homeostasis of these cells. Moreover, the critical role for CD28 costimulation in the maintenance of this subset supports the possibility of an ongoing, active antigenic stimulus.

It is interesting to note that NOD mice treated with murine CTLA4Ig between 2 and 4 weeks of age had increased incidence and more rapid onset of autoimmune diabetes compared to PBS-treated control mice (Figure

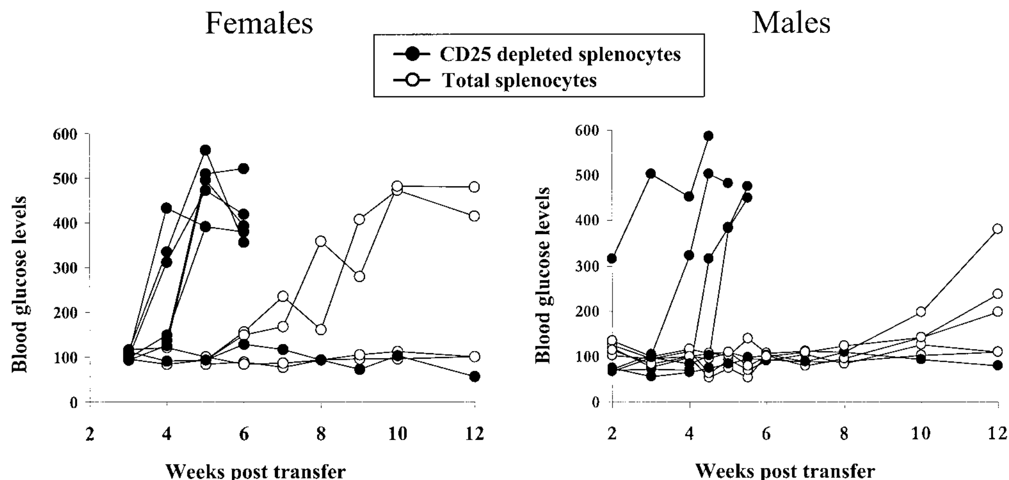

Figure 5. $CD4^+CD25^+$ Regulatory Cells Control Autoimmune Diabetes

Thirty-million total splenocytes (open circles) or splenocytes depleted of CD25-expressing cells (closed circles) from 11-week-old prediabetic female (left) or male (right) NOD mice were transferred intravenously into sex-matched NOD.SCID mice. Blood glucose levels were checked weekly starting 2–3 weeks post transfer until 12 weeks post transfer. One representative experiment of three is shown.

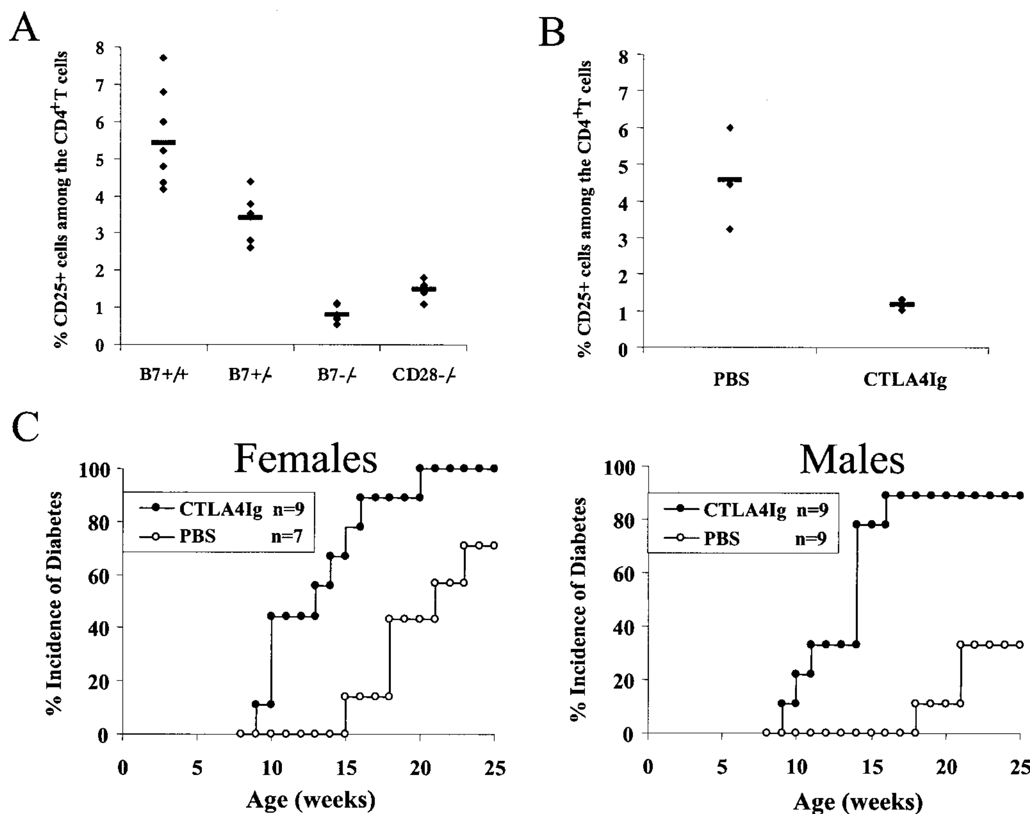

Figure 6. Absence of CD4$^+$CD25$^+$ Cells in B7/CD28-Deficient NOD Mice

After a double staining with anti-CD4 and anti-CD25 mAbs, the percentage of CD4$^+$CD25$^+$ T cells in the spleen was analyzed by flow cytometry. Each diamond represents an individual mouse and the bars represent the average for each group.

(A) The percentages of CD4$^+$CD25$^+$ cells were determined for B7$^{-/-}$ (eight mice), B7$^{+/-}$ littermates (six mice), B7$^{+/+}$ (eight mice), and CD28$^{-/-}$ (seven mice) NOD mice. The results from 2- to 13-week-old mice were pooled since similar data were observed at the different ages. Comparable percentages and differences were observed in lymph nodes.

(B) The percentages of CD4$^+$CD25$^+$ cells were determined in 6- to 8-week-old NOD mice at day 9 after five injections of 100 μg murine CTLA4Ig or PBS at days 0, 2, 4, 6, and 8. One representative experiment of two is shown. Similar changes were observed in lymph nodes.

(C) Diabetes incidence in NOD females (left) or males (right) treated every other day with 50 μg murine CTLA4Ig (closed circles) or PBS (open circles) at 2–4 weeks of age. Blood glucose levels were checked weekly.

6C). The exacerbated diabetes of murine CTLA4Ig-treated NOD mice is very similar to that seen in B7-deficient NOD mice (Figures 1 and 6C) and could be explained by the absence of CD4$^+$CD25$^+$ T cells in both groups.

CD4$^+$CD25$^+$ Regulatory T Cells Control Autoimmune Diabetes in CD28-Deficient NOD Mice

To determine if the increase of diabetes in the mice deficient in B7/CD28 costimulation was due to the absence of the CD4$^+$CD25$^+$ cells, we analyzed whether these regulatory T cells can prevent or delay diabetes induced by CD28-deficient T cells transferred in NOD.SCID mice. As expected, the majority (70%) of control recipients injected with splenocytes from diabetic CD28-deficient mice became diabetic by 10 weeks post transfer. Importantly, CD4$^+$CD25$^+$ NOD T cells but not CD25$^-$ NOD T cells prevented diabetes in this setting. Indeed, 20 out of 21 NOD.SCID mice coinjected with CD28-deficient splenocytes and regulatory T cells were nondiabetic at least 10 weeks post transfer (Figure 7A). In fact, in some cases the experiment has been carried out for more than 20 weeks with no sign of diabetes in the CD4$^+$CD25$^+$ transferred mice (data not shown). Thus, CD4$^+$CD25$^+$ NOD T cells control diabetes induced by CD28-deficient T cells. To extend these findings, we examined whether the transfer of a limited number of regulatory T cells directly into whole prediabetic CD28-deficient NOD mice could alter the progression of disease in these highly susceptible animals. All the CD28-deficient NOD mice either injected with PBS or with CD4$^+$CD25$^-$ NOD T cells became diabetic by 11 weeks of age. In contrast, in four out of the six CD28-deficient mice, the transfer of CD4$^+$CD25$^+$ NOD T cells significantly delayed the development of diabetes. In two animals, the disease did not develop through 15 weeks of age (Figure 7B). Together, these studies suggest that the deficit of CD4$^+$CD25$^+$ regulatory T cells is responsible for the exacerbation of diabetes of the B7/CD28-deficient NOD mice.

Discussion

Targeting the CD28/B7 costimulatory pathway remains among the most promising approaches to regulating

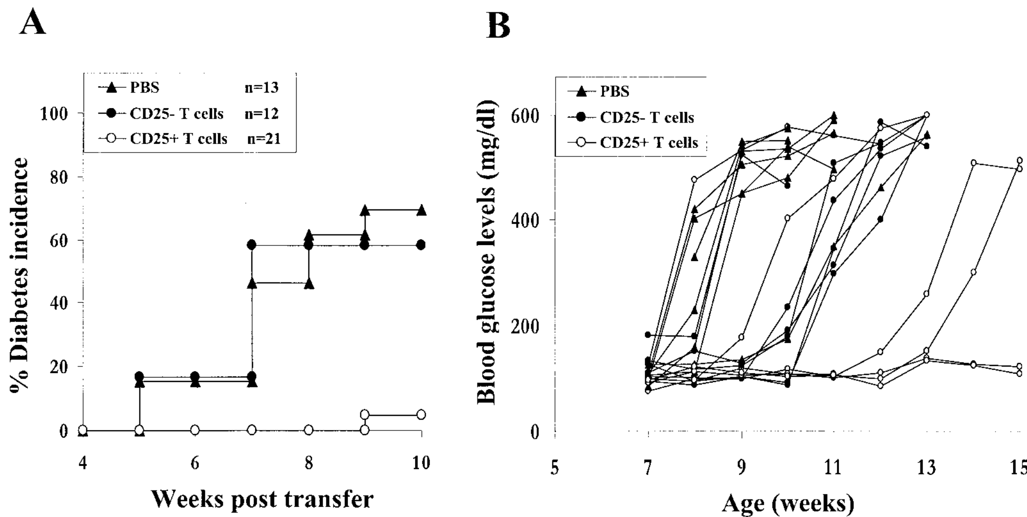

Figure 7. CD4⁺CD25⁺ Regulatory T Cells Control Diabetes Induced by CD28-Deficient T Cells

(A) Four- to ten-week-old NOD.SCID mice were coinjected with 1×10^7 splenocytes from 8- to 10-week-old diabetic CD28-deficient NOD mice and either 1.3–2 x 10^5 $CD4^+CD25^+$ NOD T cells (open circles) or 2×10^5 $CD25^-$ NOD T cells (closed circles). As a control, NOD.SCID mice were injected with 1×10^7 CD28-deficient splenocytes alone (closed triangles). Blood glucose levels were then checked weekly. The data were generated from four independent experiments with a total of 12–21 mice per group. The results with both males and females were similar and thus were pooled. (B) A total of 8×10^6 $CD4^+CD25^+$ NOD T cells (open circles), 8×10^6 $CD4^+CD25^-$ NOD T cells (closed circles), or PBS (closted triangles) were transferred in two or three injections into CD28-deficient NOD mice (5–8 weeks of age). The figure depicts blood glucose levels of individual mice generated from four independent experiments. The results with both males and females were similar and thus were pooled.

autoimmune responses. The enthusiasm derives from multiple studies demonstrating that the blockade of CD28/B7 interactions results in inhibition of induction and progression of autoimmunity in a number of experimental models (Perrin et al., 1995; Bachmaier et al., 1996; Shi et al., 1998; Chang et al., 1999; Peterson et al., 1999; Tada et al., 1999). In contrast, a previous study has shown that CD28-deficient NOD mice, defective in the CD28/B7 costimulatory pathway, have exacerbated autoimmune diabetes (Lenschow et al., 1996b). CD28-deficient mice can also mount effective immune responses in allograft rejection and some viral infections (Kundig et al., 1996; Lin et al., 1998; Szot et al., 2000). In contrast, B7-deficient mice are not able to reject allografts and exhibit a more severely compromised immune response than the CD28-deficient mice (Shahinian et al., 1993; Borriello et al., 1997; Szot et al., 2000). Therefore, B7-1/B7-2 deficient mice were bred into the NOD background to assess the role of B7 molecules in the development and progression of diabetes in this autoimmune mouse strain. The results demonstrate that not only is the engagement of B7-1 and B7-2 not necessary for the development of diabetes, but the absence of this costimulatory ligation results in exacerbation of autoimmunity.

Several explanations could have explained the phenomenon of disease exacerbation in the B7-deficient NOD setting. First, we had observed previously that the increased incidence and severity of diabetes in CD28-deficient NOD mice correlated with a profound deficit in Th2 differentiation (Lenschow et al., 1996b). Thus, the exacerbation of diabetes in B7-deficient NOD mice, which lack CD28 signaling, might have been related to ineffective Th2 development, since previous studies have shown that CD28 ligation is critical for Th2 development (Lenschow et al., 1996b; Rulifson et al., 1997) and IL-4 is a critical regulator of autoimmunity in the NOD mouse strain (King and Sarvetnick, 1997). However, Th1/Th2 skewing was not observed in B7-deficient mice. T cells isolated from B7-deficient animals were defective in both IL-4 and IFNγ production. Furthermore, total IgG levels to GAD were decreased in B7-deficient mice without an obvious decrease of the IgG1/IgG2b isotype ratio when compared to control mice.

One major difference between the CD28-deficient and B7-deficient NOD models is that B7-deficient mice lack both CD28 and CTLA-4 signaling. CTLA-4 has been implicated in the regulation of autoimmunity. One of the loci of susceptibility to autoimmune diabetes in mice and humans is closely linked to the CTLA-4 locus (Todd, 1997). Furthermore, the rapid death of CTLA-4-deficient mice suggests an important role for this molecule in immune tolerance (Tivol et al., 1995). However, several pieces of evidence suggest that the absence of CTLA-4 ligation (at least on the potentially diabetogenic cells) was not the primary cause of the increase of diabetes in B7-deficient mice. First, in our hands, anti-CTLA-4 antibody treatment (using a regimen known to cause enhanced immunity in other autoimmune settings [Karandikar et al., 1996]) does not exacerbate autoimmunity in normal NOD mice (data not shown). Furthermore, in a series of studies in which B7-deficient mice were treated with anti-CD28 to provide surrogate costimulation, some animals died of a disease similar to that observed in the CTLA-4-deficient mice, but, importantly, the others did not develop diabetes (data not shown). Finally, T cell proliferation to GAD, a unique and critical autoantigen in diabetes, was not increased in B7-deficient mice

(data not shown), suggesting that the absence of CTLA-4 ligation did not result in uncontrolled proliferation to this autoantigen. Interestingly, the absence of CTLA-4 ligation in the B7-deficient NOD mice may explain, at least in part, why Th2 differentiation was partially maintained in B7-deficient mice but not in CD28-deficient mice. Indeed, previous studies from our laboratory using blocking antibody to CTLA-4 have shown that CTLA-4 blockade promotes Th2 development (Walunas and Bluestone, 1998). Thus, CD28 and CTLA-4 signaling have opposite effects on Th2 differentiation.

The enhanced autoimmune disease in the CD28/B7 mice is best explained as a consequence of the elimination of the immunoregulatory $CD4^+CD25^+CTLA\text{-}4^+$ T cell subset essential for the protection of NOD mice from establishment of autoimmunity. The existence of a $CD4^+$ T cell population present in peripheral lymphoid tissues of prediabetic NOD mice capable of suppressing autoimmune diabetes has been known for 10 years (Boitard et al., 1989). Moreover, a population of $CD4^+$ T cells constitutively expressing CD25 controls various autoimmune diseases such as gastritis or thyroiditis in BALB/c mice (Asano et al., 1996; Suri-Payer et al., 1998; Itoh et al., 1999). In addition, the present study demonstrates that this population very efficiently controls autoimmune diabetes in NOD mice. The fact that splenocytes from prediabetic mice depleted of the $CD4^+CD25^+$ T cells induced a rapid diabetes after transfer in NOD.SCID mice suggests that they play a major role in regulating the switch from an islet nondestructive insulitis observed in prediabetic mice to the massive islet destruction seen late in the progression of the disease. Interestingly, in NOD mice a subpopulation of $CD4^+$ T cells expressing high levels of CD62L present in the thymus has been shown to play a regulatory role during the final effector phase of autoimmune diabetes (Herbelin et al., 1998). These cells could be the precursors of the immunoregulatory $CD4^+CD25^+$ T cells of peripheral lymphoid tissues, since the latter cells derived from the thymus and most of them express high levels of CD62L (Thornton and Shevach, 1998; Itoh et al., 1999). In addition, the overall number of $CD4^+CD25^+$ T cells is reduced by a factor of 1.5 in NOD mice as compared to other mouse strains (data not shown). This reduction could be a factor in the susceptibility of NOD mice to autoimmunity.

The regulatory $CD4^+CD25^+$ T cells were the only cells observed to be CTLA-4^+ in NOD mice (Figure 4). Thus, CTLA-4 may play a critical role in regulating this disease by functioning through the suppressor T cell population. Two pieces of recent data support this interpretation. First, Bachmann and colleagues have recently shown that addition of bone marrow from wild-type CTLA-4 sufficient mice protected CTLA-4-deficient mice from the lethal hyperproliferative disease (Bachmann et al., 1999). It is possible that the bone marrow allows the generation of a CTLA-4^+ regulatory cell that controls the autoimmunity. Second, a recent study showed that ligation of CTLA-4 can induce the production of TGFβ (Chen et al., 1998), a potent immune regulatory cytokine of T cell activation and autoimmune diabetes (King et al., 1998). Thus, we would speculate that a CTLA-4^+ T cell, represented in the $CD4^+CD25^+$ regulatory T cell subset, may control autoimmune diabetes in the NOD mice perhaps through the production of TGFβ or other immunosuppressive cytokine.

The profound decrease of the $CD4^+CD25^+$ regulatory T cells in B7/CD28-deficient mice was unexpected. Such a deficit in these regulatory T cells and the delay/protection of diabetes obtained after reintroducing these cells in the B7/CD28-deficient mice provide an explanation for the increase of diabetes in these mice. It remains to be determined whether B7/CD28 costimulation plays a critical role in the development of the $CD4^+CD25^+$ T cells in the thymus or in providing a survival signal in the periphery. The thymically derived $CD4^+CD25^+$ cells represent a unique lineage and have the capacity to expand in the periphery (Papiernik et al., 1998). Since $CD4^+CD25^+$ cells are missing in IL-2-deficient mice (Papiernik et al., 1998), the control of this cell subset by CD28 costimulation could be mediated by its control of IL-2 production. In this regard, short-term blockade of B7/CD28 costimulation following in vivo administration of CTLA4Ig for only 9 days induced a dramatic decrease of these cells. This result suggests that the homeostasis of these cells is under a dynamic control of CD28 costimulation and supports the possibility that these cells are encountering an ongoing signal in the periphery.

In conclusion, blocking B7 costimulation in vivo has been shown to prevent or delay autoimmune diseases and graft rejection and is a promising alternative therapy (Bluestone et al., 1999). However, the complex outcomes of such treatments must be carefully understood and monitored. The critical role of CD28/B7 engagement in regulatory T cell homeostasis adds to a growing list of "unintended" consequences of B7 blockade, including a selective defect of Th2 differentiation and increased T cell proliferation by inhibiting CTLA-4 signaling. Together, the results imply that immunomodulation of spontaneous autoimmune diseases may not be easily altered by costimulation blockade due to the critical importance of these pathways on the development and homeostasis of the regulatory T cell subset.

Experimental Procedures

Mice

B7-deficient mice (Borriello et al., 1997) were backcrossed to NOD mice for four generations and then intercrossed to generate $B7^{-/-}$, $B7^{+/-}$, and $B7^{+/+}$ NOD mice. CD28-deficient mice (Shahinian et al., 1993) were backcrossed to NOD mice for 12 generations and then intercrossed to generate $CD28^{+/+}$, $CD28^{+/-}$, and $CD28^{-/-}$ NOD mice. The genotypes were determined by PCR using B7- and CD28-specific primers. NOD mice were purchased from the Jackson Laboratory or Taconic. Typically, diabetes incidence at 24 weeks in our animal facility is 70% in females and 20% in males with NOD mice.

Assessment of Diabetes and Insulitis

Starting at 8 weeks of age, blood glucose levels were measured every week with a Lifescan glucose meter (One Touch II, Lifescan, Milpitas, CA). Mice were considered diabetic after two consecutive measurements over 300 mg/dl. The onset of diabetes was dated from the first of the sequential diabetic measurement. The scoring of islets for an inflammatory response was performed as follows. Pancreases were fixed in 3.7% paraformaldehyde and embedded in paraffin. For each animal, multiple 4 μm sections were prepared, stained with hematoxylin and eosin, and scored blindly for severity of insulitis. The insulitis was considered peri-insulitis when lymphocytes were found surrounding but not infiltrating the architecture of the islets. Insulitis was considered as moderate if less than half of the islet architecture was infiltrated with lymphocytes and severe if

more than half of the islet architecture was infiltrated with lymphocytes.

Antibodies and Recombinant Reagents

Anti-CD3 mAb (145–2C11 [Leo et al., 1987]) and anti-CTLA-4 (UC10–4F10 [Walunas et al., 1994]) were purified over a protein A–coupled Sepharose column. Biotin-labeled anti-CD25 mAbs (7D4) and FITC-labeled anti-CD4 mAb (GK1.5) were obtained from Southern Biotechnology Associates (Birmingham, AL). PE-labeled anti-CD25 mAbs (PC61) were purchased from PharMingen (San Diego, CA). The murine CTLA4Ig was kindly provided by Genetics Institute (Cambridge, MA).

Flow Cytometry Analyses

For determination of the proportion of $CD4^+CD25^+$ cells, 1×10^6 spleen cells or lymph node cells were washed in FACS buffer (0.1% BSA and 0.01% sodium-azide in 1× PBS), incubated at room temperature with 20% hybridoma supernatant of anti-FcγR mAb (2.4G2, ATCC HB-197, ATCC, Manassas, VA) for 10 min, then incubated for 30 min at 4°C with saturating amounts of FITC-coupled anti-CD4 (GK1.5, PharMingen) and biotin-coupled anti-CD25 (7D4, PharMingen), washed in FACS buffer, and revealed with PE-coupled streptavidin (Southern Biotechnology Associates) by staining 30 min at 4°C. After a final wash, cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

For analysis of intracellular CTLA-4, splenocytes were first stained in FACS buffer with PE-labeled anti-CD4 and biotin-labeled anti-CD25 revealed with PercP-coupled streptavidin, fixed with 1% PFA for 10 min at room temperature, washed with PBS containing 0.03% saponin, stained with 1 μg anti-CTLA-4 and 1% goat serum in permeabilizing buffer (PBS containing 0.3% saponin), washed, and revealed with a FITC-labeled goat anti-hamster antibody in permeabilizing buffer. For control staining, cells were similarly stained except that a control hamster antibody was used in the absence of the anti-CTLA-4 mAb.

T Cell Stimulation Assay and Cytokine Detection

Spleen cells, depleted of red blood cells, were cultivated at 1×10^6 per milliliter in a 24-well plate in culture medium (DMEM [Life Technologies, Grand Island, NY] supplemented with glutaMAX, 5% FCS [Summit Biotechnology], antibiotics, 10 mM HEPES, and 5×10^{-5} M 2-ME) in the presence of soluble anti-CD3 (145-2C11) at 1 μg/ml for 3 days. After washing, the cells were rested in culture medium without stimulation for 2 days at 37°C. Dead cells were removed after a Ficol-Hypaque gradient and replated at 0.5×10^6 cells per milliliter in culture medium in a 24-well plate previously coated with anti-CD3 at 1μg/ml in PBS. After 48 hr of restimulation, the culture supernatant was removed and analyzed for cytokines by ELISA using commercial kits from Endogen (Cambridge, MA) for IL-4 and IL-5 and using reagents kindly provided by Dr. Robert Schreiber (Washington University, St. Louis, MO) for IFNγ.

GAD Antibody ELISA

Anti-GAD67 antibody levels were determined in both male and female $B7^{+/+}$ and $B7^{-/-}$ NOD mice. Anti-GAD antibody was detected by ELISA assay as described before (Lenschow et al., 1996b). Briefly, 96-well flat-bottomed ELISA plates (Falcon) were coated overnight with 50 μl of GAD67 in coating buffer (0.5 $Na_2CO_3NaHCO_3$ [pH 9.6]) at a concentration of 10 mg/ml. Plates were washed three times with PBS-0.05% Tween 20 (PBS-T), and stored at 4°C. Antisera to be tested were diluted at appropriate concentrations with PBS-T buffer. Fifty microliters of diluted serum was added to each well. Plates were incubated at 4°C overnight. After three washings with PBS-T buffer, 50 μl 1:2000 alkaline phosphatase-conjugated goat anti-mouse IgG, anti-IgG1, or anti-IgG2b antibodies (CALTAG Laboratories, South San Francisco, CA) were added to each well, and the plates were incubated at 4°C for 4 hr. After three washings with PBS-T buffer, 50 μl substrate (5 mg p-nitrophenyl phosphate disodium in 5 ml diethylamine buffer) was added to each well. OD value was measured in ELISA reader at 405 nm 20 min later. Results are expressed as mean OD of 1:20 dilution of the sera.

Depletion of $CD4^+CD25^+$ Cells and Cell Transfer in NOD.SCID mice

Spleen cells, depleted of red blood cells, were incubated with anti-CD25 mAb (25% supernatant of 7D4 hybridoma, ATCC CRL-1698, ATCC), then cells expressing CD25 were depleted with rabbit complement during an incubation of 45 min at 37°C. Dead cells were removed after a Ficol-Hypaque gradient; viable cells were washed once with culture medium and twice with 1× PBS, and injected in the retroorbital blood sinus of NOD.SCID mice. The depletion of $CD4^+CD25^+$ cells was checked by flow cytometry using the anti-CD25 antibodies produced by the PC61 hybridoma, which does not compete for the binding of CD25 with the mAb produced by the 7D4 hybridoma that was used for complement depletion. Typically, less than 0.1% of the depleted cells expressed CD25.

Purification of $CD4^+CD25^+$ and $CD25^-$ T Cells and Cell Transfer

The $CD4^+CD25^+$ cells were purified using a technique derived from a protocol previously described (Thornton and Shevach, 1998). Cells from axillary, brachial, inguinal, cervical, mandibular, and mesenteric lymph nodes of 6- to 12-week-old NOD males were mechanically dissociated, and cells expressing CD8 ($CD8^+$ T cells) and CD24 (antigen-presenting cells) were depleted with rabbit complement at 37°C for 30 min. After a Ficol-Hypaque gradient to remove dead cells, purified $CD4^+$ T cells were cultured overnight at 3×10^6 cells per milliliter in culture medium (see above) supplemented with 20 U/ml recombinant murine IL-2 (Cetus, Emeryville, CA). Following incubation, the cells were washed twice to remove any remaining IL-2 and stained with 0.5 μg biotin-coupled anti-CD25 mAb (7D4) per 1×10^6 cells, followed by a staining with 0.1 μg FITC-coupled streptavidin (Southern Biotechnology Associates) per 1×10^6 cells. The stained cells were incubated with anti-FITC microbeads (Miltenyi Biotec, Auburn, CA) for 30 min on ice, washed, and $CD25^+$ cells were separated on a VS^+ positive selection column (Miltenyi Biotec). The purity of the $CD4^+CD25^+$ cells was ∼85%. The $CD4^+CD25^-$ T cells, which did not bind to the anti-CD25-coated beads, were harvested from the flow through during the washing steps. In some experiments, $CD25^-$ T cells were purified from lymph nodes after depletion of $CD24^+$ and $CD25^+$ cells with rabbit complement. Purified T cells were washed twice in 1× PBS and injected in the retroorbital blood sinus of CD28-deficient mice or of NOD.SCID mice as described in the figure legends.

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