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Immunoregulatory Pathways Controlling Progression of Autoimmunity in NOD Mice:

The Role of CTLA-4 and TGF- β

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Abstract

The activation, expansion, and survival of regulatory T cells (Tregs) as well as the expression of their suppressive capacities result from distinct signaling pathways involving various membrane receptors and cytokines. Multiple studies have shown that thymus-derived naturally occurring Tregs constitutively express the forkhead/winged helix transcription factor FoxP3 in addition to high levels of CD25, the negative co-stimulatory molecule CTLA-4, and the glucocorticoid-induced TNF receptor-related protein GITR. At variance, adaptive or induced Tregs acquire these phenotypic markers as they differentiate in the periphery, following adequate stimulation in the appropriate environment, together with their capacity to produce immunomodulatory cytokines (mainly, IL-4, IL-10 and TGF- β) and to display regulatory capacities. However, none of these molecules but FoxP3 are restricted to Tregs since they may also be expressed and upregulated on activated effector T cells. This explains why different hypotheses were proposed to interpret interesting reports showing that *in vivo* abrogation of CTLA-4 signaling using neutralizing CTLA-4 antibodies triggers different autoimmune or immune-mediated manifestations. Thus, an effect on pathogenic T cell effectors and/or Tregs has been proposed. Here we present and discuss recent results we obtained in the nonobese diabetic (NOD) mouse model of spontaneous autoimmune diabetes, arguing for a key role of CTLA-4 in the functional activity of Tregs. Moreover, data are presented that simultaneous blockade of CTLA4 and TGF- β further impairs immunoregulatory circuits that control disease progression.

Keywords

CTLA-4; TGF- β ; NOD; autoimmune diabetes; regulatory T cells

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Conflict of Interest

The authors declare no conflicts of interest.

Introduction

The molecular and cellular basis of T cell-mediated regulation has been the subject of numerous investigations. Identifying specific markers of regulatory T cell (Treg) subsets has become a matter of interest since several CD4⁺ T cell subsets could be exploited to prevent or treat autoimmune diseases, inflammatory disorders, allergy, infections, tumors, or to induce donor-specific transplantation tolerance.^{1,2} Among the various markers that have been characterized, the most specific one is the transcription factor FoxP3, which is constitutively expressed by naturally occurring CD4⁺CD25⁺ T cells³ and has been shown to be involved in their differentiation and the expression of their functional capacity. Thus, *foxp3* mutant *scurphy* mice that are deficient in natural CD4⁺CD25⁺ Tregs develop a severe autoimmune syndrome associated with lymphoproliferation.⁴ Similarly, in humans, mutations of the *foxp3* gene lead to the IPEX syndrome, a rare, often lethal syndrome associated with severe enteropathy and polyautoimmune manifestations, in particular polyendocrinopathy including type 1 diabetes.⁵

Among the other Treg markers identified is cytotoxic T lymphocyte antigen-4 (CTLA-4), which is highly constitutively expressed on natural CD4⁺CD25⁺ Tregs and whose expression is regulated by FoxP3.^{6,7} However, like various other Treg markers, such as CD25 or GITR, CTLA-4 is expressed on all T cell subsets, including effector T cells, upon activation.⁶ At variance with CD25 or GITR, CTLA-4 triggers negative co-stimulatory signals that inhibit activation, IL-2 production, and cell cycle progression.⁸ CTLA-4 exhibits a high affinity for CD80/CD86 and thus successfully competes with CD28 for B7 binding sites on antigen-presenting cells (APCs), thereby lowering the delivery of co-stimulation signals.⁹ Of interest, CTLA-4 within lipid rafts migrates to the immunologic synapse, where it controls TCR accumulation and/or retention of T cell receptor (TCR) complexes and interferes with TCR signaling.¹⁰ In addition, CTLA-4 reduces contact period between T cells and APCs, thus limiting proliferation and proinflammatory cytokine production.¹¹ Finally, more recent data show that CTLA-4 downregulates CD28 expression on T cells as a result of enhanced internalization and degradation of CD28.¹² It is also of interest to mention here studies showing that binding of CTLA-4 expressed on CD4⁺CD25⁺ Tregs to CD80/CD86 on dendritic cells induces downmodulation of these two B7 family members and the release of indoleamine 2,3-dioxygenase (IDO), which inhibits T cell activation.^{13,14}

Because of these negative co-stimulatory effects, blockade of CTLA-4 protects against tumor growth and viral/bacterial infections, while blockade of CD28 signaling using CTLA-4Ig appears highly effective in preventing organ transplant rejection.^{15,16} Our present data show that CTLA-4 targeting markedly enhanced progression of autoimmune diabetes, further highlighting its crucial role in self-tolerance.

Role of CTLA-4 in T Cell Homeostasis and Maintenance of Self-Tolerance

The first strong evidence in support of a key role of CTLA-4 in the control of self-reactivity stemmed from the study of mice genetically invalidated for CTLA-4 which show massive and fulminant lymphoproliferation, severe inflammation, and multiple and aggressive organ infiltration leading to early death (3–4 weeks of age).¹⁷ This lethal lymphoproliferative autoimmune syndrome is blocked upon infusion of wild-type Tregs. Another striking example is that of the autoimmune gastritis that develops after administration to very young (10-day-old) BALB/c mice of anti-CTLA-4 antibody.⁶ In this model, neutralization of CTLA-4 does not alter the number of CD4⁺CD25⁺ Tregs in adult mice.⁶ Similarly, in experimental autoimmune encephalomyelitis (EAE) induced in SJL/J mice immunized with proteolipid protein (PLP)-139-151, administration of an anti-CTLA-4 antibody dramatically

increases disease severity and inflammation in the central nervous system.¹⁸ The same effect was obtained in a transgenic model of autoimmune diabetes (BDC 2.5 mice).¹⁹

Using a T cell-mediated colitis model, Read and colleagues showed that anti-CTLA-4 antibody treatment also in this model increased disease severity via the functional inhibition of Tregs controlling intestinal inflammation and not through activation of colitogenic effector T cells.^{20,21}

These *in vivo* data suggesting a central role of CTLA-4 at the Treg level in maintaining self-tolerance were supported by *in vitro* data showing that in the conventional suppression co-culture assay, the CD4⁺CD25⁺ T cell-mediated inhibition was abolished upon addition of anti-CTLA-4 antibody.^{20,22} However, the fact that CD4⁺CD25⁺ T cells recovered from CTLA-4-deficient mice retain their inhibitory activity *in vitro* rendered the explanation more complex than it appeared.²² In spite of all these findings the contribution of CTLA-4 to the functional capacity of CD4⁺CD25⁺ Tregs and its role in the maintenance of self-tolerance remained highly debated as CD25⁺ T cells recovered from CTLA-4-deficient mice retain their inhibitory activity *in vitro*.

T Cell-Mediated Immunoregulation in Autoimmune Type 1 Diabetes

NOD mice are a good model for human type 1 diabetes. Disease spontaneously appears mostly in female mice by 12–16 weeks of age and is preceded by quite a long phase of asymptomatic “prediabetes” characterized by progressive insulinitis starting as early as 3 weeks of age. Initially, the insulinitis is not aggressive and the T cell infiltrate is composed of mononuclear cells, including CD4 and CD8 lymphocytes, confined at the periphery of the islets (peripheral insulinitis). By the time the mice are 13 weeks of age, T cells invade the islets and initiate the destruction of insulin-producing β cells (invasive insulinitis), leading to the advent of hyperglycemia when approximately 70% of the β cell mass is destroyed. Aside from showing type 1 diabetes, NOD mice exhibit a number of other autoimmune polyendocrine manifestations, notably, sialitis and thyroiditis.

There is compelling evidence from various laboratories including ours to show that development of diabetes in NOD mice is tightly controlled by Tregs. Diabetes transfer, normally observed after the infusion of T cells from diabetic mice (diabetogenic cells) into immunoincompetent syngeneic recipients (severe combined immunodeficiency SCID-NOD mice) is prevented by co-injection of CD4⁺CD25⁺ Tregs also expressing CD62L (L-selectin) from the spleen or the thymus of young prediabetic mice.^{23–25} Moreover, treatment of young NOD mice (3–4 weeks of age) with anti-CD25 antibodies, which massively deplete CD4⁺CD25^{high} Tregs, accelerates the onset of diabetes and increases its frequency in both male and female mice.²⁶ Finally, NOD mice deficient for the CD28 molecule are almost completely deprived of natural CD4⁺CD25⁺ Tregs and exhibit enhanced Th1 responses and accelerated disease.²⁷ Adoptive transfer of purified CD4⁺CD25⁺ T cells from normal prediabetic NOD mice into CD28-deficient NOD mice restores the deficit, thus delaying or preventing diabetes.²⁷ In the same vein, treatment of normal NOD mice with CTLA-4Ig, which blocks CD28/B7 interaction, leads to a major selective reduction of CD4⁺CD25⁺ T cells, also affecting both the thymus and the periphery.²⁷

It is now well accepted that the regulatory function is not confined to thymus-derived naturally occurring CD4⁺CD25⁺Foxp3⁺ Tregs. Other subsets of Tregs that fulfill the definition of “adaptive” Tregs (Th2, Th3, Tr1 cells) are generated *in vitro* and *in vivo* from CD4⁺CD25[−] precursors in the periphery under defined conditions, including the type of antigenic stimulation, the nature of the antigen-presenting cells (APCs) involved, as well as the cytokine milieu, IL-10 and TGF- β appearing as two privileged cytokines affording the

differentiation of adaptive regulatory T cells.^{28,29} Upon differentiation, these adaptive Tregs acquire the characteristic markers of Tregs such as Foxp3, GITR and CTLA-4.²

In the NOD mouse model, it has been shown that Th2 (IL-4-dependent) and Tr1 (IL-10-dependent) cells specific for defined glutamic acid decarboxylase (GAD) determinants exist in nonmanipulated animals and can efficiently protect against diabetes development.^{30,31} In addition, we have demonstrated that a subset of FoxP3⁺ cells present within a CD4⁺CD25^{low} lymphocyte subset suppresses T cell immunity in spontaneous diabetic NOD mice in a TGF- β -dependent manner, a functional property typical of “adaptive” regulatory T cells.²³ This distinct Treg subset is present in NOD but not in normal mice (BALB / c, C57BL / 6), suggesting that they arise in an attempt to regulate ongoing autoimmunity. These TGF- β -dependent adaptive CD4⁺CD25^{low} Tregs can be induced from peripheral CD4⁺CD25⁻ T lymphocytes by anti-CD3 immunotherapy and represent a major target of this therapy that has been shown to restore self-tolerance.²³ It is interesting to quote here recent reports demonstrating that engagement of CTLA-4 is required for the generation of adaptive Tregs after either TGF- β or antigen-specific stimulation.^{32,33}

Collectively, these data highlight the heterogeneity and the diversity of Tregs controlling autoimmune diabetes and suggest that the thymus-derived suppressor CD4⁺CD25^{high} FoxP3⁺ T cells function prominently to maintain self-tolerance in early steps of development and life and that the TGF- β -dependent adaptive Tregs are operational at later stages of disease progression.

CTLA-4 Is a Key Regulatory Molecule Promoting Self-Tolerance: Contribution of the TGF- β Pathway

Targeting of CTLA-4 Accelerates Diabetes Onset: The Synergistic Effect of TGF- β Blockade

On the basis of the data mentioned above and because CTLA-4's contribution to Treg function has remained controversial, we were interested in further dissecting its role in autoimmune diabetes development. In addition, because of our data described above pointing to the TGF- β dependency of the adaptive Tregs we identified in NOD mice, we explored the effect of a combined targeting of CTLA-4 and TGF- β during the early phase of life in the NOD mouse model.

Ten-day-old male and female NOD mice were injected i.p. with antibodies to CTLA-4 or to TGF- β administered alone or in combination. A total of three doses were given on d10, d17 and d24 using 0.8 mg of each antibody per injection. As shown in Figure 1, diabetes onset was accelerated after administration of anti-CTLA-4 in a significant proportion of animals: 20.8% of female and 16.6% of male mice rapidly became diabetic by one to two weeks after the last injection. At variance, treatment with anti-TGF- β alone did not accelerate diabetes onset (Fig. 1) in spite of its clear-cut insulinitis-promoting effect (Fig. 2). Of interest, fulminant diabetes was observed in NOD mice that received both anti-CTLA-4 and anti-TGF- β antibodies (named “Cocktail” in Fig. 1). Incidence of diabetes reached 35% of the female NOD mice within the 2–6 days after the last injection (i.e., 4 weeks of age). Thus, in this group, by 6 weeks of age, 50% of the females and 30% of the males had become diabetic.

Histologic analysis of pancreata recovered at different time-points after treatment disclose that anti-TGF- β antibody-treated female NOD mice exhibited a significantly more severe insulinitis pattern as compared to controls by 12–13 weeks of age (79.2% of invasive insulinitis versus 34.4% in controls) (Fig. 2). More aggressive insulinitis was also observed in animals treated with anti-CTLA-4 alone. As detailed in Figure 2, in 6-week-old females, the

proportion of intact islets was decreased as compared to controls (55.3% vs. 85.9%, respectively), while 36.8% of the islets exhibited massive and invasive infiltration compared to only 8.4% in control animals. Paralleling what is observed in terms of overt disease onset, a severe insulinitis pattern was observed in mice receiving both anti-CTLA-4 and anti-TGF- β antibodies (cocktail) (Fig. 2). By 4 weeks of age, invasive insulinitis was scored in 37.5% of the islets within the treated female group. This proportion progressively increased with age, ranging from 56%, 75.2% and 80% in 6-, 9- and 13-week-old NOD mice, respectively, versus 8.4%, 25.1%, and 34.4% in controls. were slightly but reproclucibly above threshold (Fig. 3B). Mice treated with the combination of anti-TGF- β and anti-CTLA-4 antibodies exhibited a higher incidence of gastritis, colitis, and sialitis compared to that seen when each antibody was used alone (Fig. 3A). Thus, one-third of 6-week-old male and female NOD mice showed gastritis that was associated with high serum levels of anti-H⁺ / K⁺ ATPase autoantibodies, detectable as early as 2–3 weeks after injection (Fig. 3B). Frequency of colitis and sialitis was also very high, but comparable to that observed in recipients of anti-CTLA-4 alone. It is interesting to note that animals that developed fulminant diabetes also exhibited gastritis.

As a whole, these data demonstrate that CTLA-4 plays key roles in regulatory mechanisms controlling the development of autoimmune diseases and in particular type 1 diabetes in NOD mice. Although blockade of TGF- β did not *per se* modify disease onset and Young males were also sensitive to the antibody treatment as lower numbers of normal islets were observed compared to controls, in males treated with anti-CTLA-4 alone, or, in a more clear fashion, in males receiving the cocktail treatment.

Targeting of CTLA4 Alone or in Combination with TGF- β Blockade Promotes a Polyautoimmune/Inflammatory Syndrome

Antibody-treated NOD recipients were also monitored for the occurrence of other autoimmune / inflammatory diseases, namely, gastritis, colitis, and sialitis (Fig. 3A). Control NOD males and females showed histologic signs of sialitis and colitis (which, however, was scored as discrete or moderate and was not associated with the occurrence of wasting disease). Anti-TGF- β antibody-treated mice exhibited a pattern similar to that observed in the control group, except for a more pronounced colitis in the females and some signs of discrete gastritis. Administration of anti-CTLA-4 antibody led to a higher frequency of infiltration in all the organs studied in both females and males. Anti-H⁺ / K⁺ ATPase antibody levels in the serum of 9-week-old anti-CTLA-4-treated NOD mice incidence, it exacerbated the deleterious effect of CTLA-4 neutralization. Therefore, CTLA-4 and TGF- β may act synergistically to control the autoimmune process in the NOD mouse model.

CTLA-4 Antibody Preferentially Targets Tregs

To get further insights into the mechanisms mediating the effect of anti-CTLA4 and anti-TGF- β antibodies and in particular to address the nature of the preferential targets of the antibody action (e.g., Tregs or T effectors or both), we took advantage of two different models: the CD28^{-/-} NOD mouse and the adoptive transfer model.

The CD28^{-/-} NOD Mouse Model

The use of CD28^{-/-} NOD mice that are deprived of natural CD4⁺CD25⁺ Tregs²⁷ allowed us to get further insights into the mechanisms mediating the effect of CTLA-4 and TGF- β neutralization on diabetes progression. Ten-day-old female CD28^{-/-} NOD mice received anti-CTLA-4 or both anti-CTLA-4 and anti-TGF- β antibodies (0.8 mg/injection on d10, d17, and d24 of life). As expected, 70% of control animals showed overt disease by 15 weeks of age (Fig. 4A). Selective blockade of CTLA-4 did not modify the occurrence of type 1

diabetes in CD28^{-/-} mice. These results indicate that the accelerating effect of anti-CTLA-4 on disease onset is strictly dependent on the presence of CD4⁺CD25⁺ Tregs and does not imply a direct effect on pathogenic T cells. Interestingly, CD28^{-/-} NOD mice treated with the combination of anti-CTLA-4 and anti-TGF- β antibodies showed acceleration of disease (50% diabetes at 9 weeks of age, $P < 0.007$) (Fig. 4A). This result reinforces the notion that TGF- β plays a major role in the control of diabetogenic mechanisms and at the same time it suggests, although indirectly, that the mediators of this effect are distinct from natural CD4⁺CD25⁺ Tregs. At this point one cannot exclude a role for other Tregs in this model (adaptive Tregs, NKT cells, $\gamma\delta$ T cells).²³ Moreover, it is well known that many cell types may be the source of TGF- β , including dendritic cells, and more experiments are needed to identify the precise target(s) of anti-TGF- β in this setting.

The Adoptive Transfer Model

To further confirm that Tregs rather than diabetogenic T cell effectors were the major target of anti-CTLA-4 treatment, we performed adoptive transfer experiments into NOD-SCID mice. We have previously demonstrated that diabetogenic lymphocytes mainly include T cells lacking CD25 and L-selectin (CD62L).²⁴ As shown in Figure 4B, recipients of CD25⁻CD62L⁻ T cells from diabetic donors treated with anti-CTLA-4 antibody did not exhibit any disease acceleration. A comparable insulinitis pattern was observed in anti-CTLA-4 antibody-treated versus control recipients (data not shown).

Finally, we confirmed the predominant role of CTLA-4 in the CD4⁺CD25⁺ T cell-mediated protection against autoimmune diabetes development using an adoptive co-transfer model. Diabetogenic T cells were transferred into NOD-SCID recipients in conjunction with CD4⁺CD25⁺ Tregs isolated either from the thymus or the spleen of young prediabetic NOD mice endowed, as previously demonstrated, with efficient regulatory capacities as assessed by their capacity to prevent disease transfer.^{23,24} As illustrated in Figures 4C and 4D, in NOD-SCID recipients treated with anti-CTLA-4 antibody, the protection afforded by splenic (Fig. 4C) and thymic (Fig. 4D) CD4⁺CD25⁺ Tregs was significantly abrogated. At 8 weeks from transfer 25% of NOD-SCID mice transferred with diabetogenic T cells and thymic or splenic Tregs were diabetic. After treatment with anti-CTLA4 disease incidence increased to 78% and 67% in recipients co-injected with diabetogenic cells plus CD4⁺CD25⁺ splenocytes (Fig. 4C) or CD4⁺CD25⁺ thymocytes (Fig. 4D), respectively.

Conclusions

In summary, our data show that the CTLA-4 signaling pathway clearly contributes to the regulatory capacities of thymic-derived naturally occurring Tregs in NOD mice. Blockade of this pathway abrogates Treg function, which is essential for controlling the development of autoimmune diabetes as well as other autoimmune / inflammatory diseases such as gastritis, colitis, and sialitis. Conversely, no effect on the functional capacity of diabetogenic CD25⁻CD62L⁻ effector T cells was evidenced. As such, these findings are fully in keeping with the initial data provided by Takahashi and colleagues suggesting that anti-CTLA-4 antibodies essentially target Tregs, thereby neutralizing their function and triggering autoimmune gastritis in young normal BALB / c mice.⁶

Of interest, CTLA-4 appears to synergize with TGF- β for optimal control of the autoreactive responses as combined targeting of both pathways significantly exacerbates the occurrence and severity of all autoimmune / inflammatory diseases studied. This observation can be related to the work of Zheng and colleagues, who reported the requirement of CTLA-4 for the TGF- β -mediated generation of adaptive Tregs,³² and to our data showing the importance of TGF- β -dependent adaptive Tregs in the control of autoimmune diabetes.²³ Thus, these results suggest that *in vivo* CTLA-4 signaling plays a regulatory role at two levels: first in

the mediation of the suppressive functions of naturally occurring CD4⁺CD25⁺ T cells and second in the induction and / or differentiation and / or homeostasis of adaptive Tregs. This dual effect may be of particular importance in autoimmune genetic background, as in the NOD model, where fully competent diabetogenic T cells appear at the periphery between 4 and 6 weeks of age, a long time before onset of overt disease, but where these cells are under the active control of regulatory T cell populations.²⁴

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Figure 1.

Acceleration of diabetes in NOD mice after treatment with anti-CTLA4 and / or anti-TGF- β antibodies. Ten-day-old NOD mice (male or female) were treated with antibodies to CTLA-4, TGF- β , or both to CTLA-4 and TGF- β (cocktail). Unmanipulated NOD mice or mice treated with purified mouse IgGs were used as controls (purified mouse IgGs [Jackson ImmunoResearch Laboratories]). The dose used was 0.8 mg/injection/mouse i.p. once a week on d10, d17, and d24 of life. Glycosuria measurements were performed twice a week. The TGF- β antibody used was produced by the 2G.7 hybridoma (mouse IgG2b, specific for human TGF- β 1, provided by C.J.M. Melief, Leiden University Medical Center, Leiden, the Netherlands). The anti-CTLA4 antibody used was produced by the UC10-4F10-11 hybridoma (hamster IgG, specific for mouse CTLA4). The two antibodies were produced in ascites fluid and purified by affinity chromatography. The occurrence of diabetes was plotted using the Kaplan–Meier method (i.e., a nonparametric cumulative survival plot). The statistical comparison between the curves was performed using the log-rank (Mantel–Cox) test. Incidence of diabetes was significantly accelerated after administration of CTLA-4 alone or in combination with anti-TGF- β ($P < 0.0007$ for female mice and $P < 0.04$ for male mice treated with anti-CTLA-4+anti-TGF- β antibodies versus controls, respectively).

Figure 2.

Histologic analysis of pancreas from anti-CTLA-4- and/or anti-TGF- β antibody-treated NOD mice. Female and male NOD mice treated with antibodies to CTLA-4, TGF- β , or both to CTLA-4 and TGF- β (cocktail) on d10, d17, and d24 of life were culled at various ages for histopathologic analysis of pancreata. The proportion of islets massively infiltrated with mononuclear cells increased with age and according to the treatment in the following order: IgG < TGF- β < CTLA-4 < cocktail. Invasive insulitis was observed as early as 4 or 6 weeks of age in mice injected with anti-CTLA-4+anti-TGF- β antibodies ($P < 0.0001$) or anti-CTLA-4 alone ($P < 0.008$), respectively.

Figure 3.

Polyautoimmune syndrome in NOD mice treated with anti-TGF- β , anti-CTLA-4, or both antibodies. **(A)** Histologic analysis of stomach, gut, and salivary gland of NOD mice having received antibodies against CTLA-4, TGF- β or CTLA-4 + TGF- β . Mice were killed at various ages and the severity of gastritis, colitis, and sialitis was measured and compared to control IgG-treated animals. **(B)** Anti-H⁺/K⁺ ATPase-specific autoantibody serum levels. The kinetics of anti-H⁺/K⁺ ATPase autoantibody production in mice treated with anti-CTLA-4, anti-TGF- β antibodies, or control IgGs were determined by ELISA. The x axis details the antibody treatment received by the mice and the gender of the recipients. Values above 1 are considered as a marker of gastritis. Mean antibody levels detected in recipients injected with either a mixture of antibodies against CTLA-4 and TGF- β were significantly higher than those observed in other groups.

Figure 4.

Anti-CTLA-4 antibodies preferentially target regulatory T cells *in vivo*. **(A)** Ten-day-old female NOD mice deficient for the CD28 molecule were treated with antibodies to CTLA-4 or to CTLA-4 and TGF- β (cocktail). As in conventional NOD mice, the dose used was 0.8 mg/injection/mouse i.p. once a week on d10, d17 and d24 of life. Glycosuria measurements were performed twice a week. Administration of anti-CTLA-4 antibodies alone did not accelerate diabetes incidence in CD28^{-/-} NOD mice as opposed to combination of anti-CTLA-4 and anti-TGF- β ($P < 0.007$). **(B)** NOD-SCID mice were injected with 1×10^5 pathogenic CD25⁻CD62L⁻ T splenocytes recovered from overtly diabetic mice. Cells were purified on the basis of CD62L, CD25, or CD4 expression using magnetic bead cell sorting (MACS; Miltenyi Biotech, Bergisch-Gladbach, Germany). When needed, recipients were treated with neutralizing monoclonal antibodies to CTLA-4 (1 mg/injection/mouse i.p. 3 times a week) starting 1 week after transfer and continuing for 5 consecutive weeks. **(C)** NOD-SCID recipients received diabetogenic cells alone (1×10^6 / recipient, Diab) or a mixture of diabetogenic cells with CD4⁺CD25⁺ T cells (1×10^6 / recipient) isolated from the spleen of 6 week-old NOD mice using magnetic bead cell sorting. Neutralizing monoclonal antibodies to CTLA-4 were injected (1 mg/injection/mouse i.p. 3 times a week) until all recipients injected with diabetogenic cells alone had become diabetic. Glycosuria was monitored twice a week. **(D)** NOD-SCID recipients received diabetogenic cells alone (1×10^6 /recipient, Diab) or a mixture of diabetogenic cells with CD4⁺CD25⁺ T cells (1×10^6 / recipient) isolated from the thymus of 6 week-old NOD mice. Thymocyte suspensions were depleted of CD8⁺ T cells by magnetic bead cell sorting (Miltenyi Biotech), and total CD4⁺CD25⁺ T cells were purified by FACS sorting. When needed, recipients were treated

with neutralizing monoclonal antibodies to CTLA-4 (1 mg / injection / mouse i.p. 3 times a week) as in **(B)**.