

# Suppression and Acceleration of Autoimmune Diabetes by Neutralization of Endogenous Interleukin-12 in NOD Mice

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**A corpus of evidence suggests that T-helper type 1 (Th1)-dependent cellular immunity plays a pivotal role in the pathogenesis of autoimmune diabetes. This study was intended to find ways to prevent the development of NOD diabetes using a neutralizing anti-interleukin (IL)-12 antibody (C17.8) that inhibits Th1 cell differentiation. When C17.8 was administered from 5 to 30 weeks of age, NOD mice exhibited suppression of both insulinitis and diabetes. However, when C17.8 administration ceased at 15 weeks of age, 8 of 13 recipients showed diabetes at 30 weeks of age. These results suggest that IL-12 plays an important role not only in the development of effector cells but also in their activation. In contrast, when C17.8 was injected into 2-week-old female NOD mice for 6 consecutive days, all 16 recipients showed diabetes at 30 weeks of age, whereas 12 of 20 control mice became diabetic. This result suggests that depletion of endogenous IL-12 at a young age results in the enhancement of diabetes. Flow cytometric analysis indicated that activated memory T-cells were present in higher numbers after C17.8 treatment. Transfer of spleen cells from 15-week-old C17.8-treated NOD mice to NOD-*scid* mice resulted in an earlier onset and a higher incidence of diabetes. Furthermore, administration of C17.8 to 2-week-old NOD mice also resulted in a much earlier onset of diabetes. These results suggest that short-term treatment with anti-IL-12 antibody prohibits IL-2 production at a young age, which may influence the expansion and apoptosis of pathogenic T-cells, resulting in the acceleration of autoimmune diabetes. *Diabetes* 49:1998–2006, 2000**

**A** corpus of evidence suggests that type 1 diabetes is caused by chronic autoimmune inflammation in pancreatic islets. Several autoantigens have been identified by autoantibodies in the sera of acutely diabetic and prediabetic subjects, including GAD, IA-2, and insulin (1–5). Recent studies have revealed that the cellular immune response is the major effector mechanism in the destruction of pancreatic  $\beta$ -cells in human subjects with type 1 diabetes (6–8). Among T-cells, T-helper type 1 (Th1) cells that secrete interleukin (IL)-2 and interferon (IFN)- $\gamma$  are suspected to be the key effector cells responsible for the destruction of pancreatic  $\beta$ -cells (9). Nonobese diabetic (NOD) mice, which spontaneously develop autoimmune insulinitis and diabetes, have a striking resemblance to the human type 1 diabetes phenotype and have been used as an experimental model of the human disease. In this model, Th1 cells are considered to be involved in the induction of autoimmune diabetes. For example, diabetogenic T-cell clones derived from NOD mice exhibit a Th1 phenotype (10). Systemic administration of IL-4, IL-10, or the anti-IFN- $\gamma$  monoclonal antibody prevented the onset of diabetes (11–15). Expression of IFN- $\gamma$  in pancreatic islet-infiltrating cells correlated with  $\beta$ -cell destruction and the development of diabetes in NOD mice (16).

IL-12, which is released from macrophages and dendritic cells, is a major inducer of Th1 cell differentiation (17–19). Antagonists of IL-12 have been shown to inhibit autoimmune diabetes in NOD mice (20,21). Furthermore, administration of IL-12 to young female NOD mice was reported to accelerate the onset of diabetes in association with a decrease in IL-4 production by islet-infiltrating lymphocytes (22). These results suggest that IL-12 may play a critical role in the development of diabetogenic effector Th1 cells. Recently, we demonstrated that syngeneic islet grafts transduced with an IL-12 antagonist gene can prevent autoimmune rejection in diabetic NOD mice, suggesting that IL-12 plays an important role not only in the development of Th1 cells but also in the activation of the effector cells responsible for the destruction of pancreatic  $\beta$ -cells (23). Anti-IL-12 antibody was administered to NOD mice in the present study to further explore the role of IL-12. Long-term administration of anti-IL-12 antibody prevented insulinitis and diabetes. On the other hand, short-term administration of anti-IL-12 antibody at 2 weeks of age resulted in an increased incidence of diabetes. This

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ATCC, American *Type Culture* Collection; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibody; NK, natural killer; Th1, T-helper type 1.

enhancement effect was observed only at younger ages. Furthermore, administration of anti-IL-2 antibody at these young ages also resulted in an acceleration of autoimmune diabetes, but anti-IFN- $\gamma$  did not. The various apparent roles of IL-12 in the developmental processes of type 1 diabetes are discussed.

## RESEARCH DESIGN AND METHODS

**Mice.** NOD/Shi/Kbe mice were maintained in the Institute for Experimental Animals, Kobe University School of Medicine. In our NOD colony, incidence of diabetes at 30 weeks of age is 60% in female mice and 5% in male mice. All animals were handled under the Guidelines for Animal Experiments in Kobe University School of Medicine. NOD-*scid* mice were purchased from the Central Institute of Experimental Animals (Kawasaki, Japan).

**Monoclonal antibodies.** Hybridoma cell lines S4B6 (anti-IL-2, rat IgG2a), 11B11 (anti-IL-4, rat IgG2a), JES5-2A5 (anti-IL-10, rat IgG2a), and R4-6A2 (anti-IFN- $\gamma$ , rat IgG2a) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The C17.8 (anti-IL-12, rat IgG2a) hybridoma was provided by Dr. Giorgio Trinchieri (Wistar Institute, Philadelphia). Isotype-matched M18/2 (anti-CD18, rat IgG2a; ATCC) was used as a control antibody because it does not block cell-mediated target lysis *in vitro* (24). Hybridoma cells were injected intraperitoneally into CD1 *nu/nu* mice primed with tetramethylpentadecane (Aldrich Chemicals, Milwaukee, WI). Monoclonal antibodies were harvested as ascites and were affinity-purified using a protein G column.

**Long-term treatment of NOD mice with anti-IL-12 monoclonal antibody.** Female NOD mice were injected intraperitoneally with 500  $\mu$ g C17.8 twice a week at 5–30 or 5–15 weeks of age. As a control, NOD mice were treated with 500  $\mu$ g M18/2 (nonfunctional anti-CD18 monoclonal antibody [mAb]) during the same periods. The incidence of diabetes was monitored until 30 weeks of age. Urinary glucose was monitored three times a week, and blood glucose was measured if urinary glucose became positive, or at 30 weeks of age. The mice were diagnosed as diabetic when nonfasting blood glucose was >16.7 mmol/l (300 mg/dl) on 2 consecutive days. At 30 weeks of age, mice were killed for histological examination for pancreatic insulinitis.

**Short-term treatment of young NOD mice with anti-IL-12 mAb.** Fourteen-day-old female NOD mice were injected intraperitoneally with 200  $\mu$ g C17.8 or M18/2 for 6 consecutive days. As a control, 10-week-old female mice were injected intraperitoneally with 500  $\mu$ g C17.8 for 6 consecutive days. The incidence of diabetes was monitored until 30 weeks of age.

**Histology.** Normoglycemic NOD mice that were treated with mAb were killed for histological examination at 8, 36, and 64 days after injection (28, 56, and 84 days of age). Each pancreas removed from the NOD mice was fixed in 10% formalin solution. Paraffin-embedded sections were stained with hematoxylin and eosin, and the severity of insulinitis was assessed by the following criteria: 0, normal islet; 1, mononuclear cell infiltration in <25% of the islet; 2, 25–50% of the islet infiltrated; 3, 50–75% infiltrated; and 4, >75% infiltrated or small retracted islets.

**Flow cytometric analysis.** Spleens were removed from 28- and 56-day-old female NOD mice that had received C17.8 or M18/2 at 2 weeks of age. Spleen cells were prepared by depleting red blood cells with 0.015 mol/l Tris-0.83% NH<sub>4</sub>Cl.

Spleen cells were analyzed by two-color fluorescent staining. Cells were first incubated with antibodies against CD25 (7D4, rat IgM; ATCC), CD44 (IM7, rat-IgG2b; PharMingen, San Diego, CA), CD45RB (23G2, rat IgG2a; ATCC), CD54 (KAT-1, rat IgG2a; PharMingen), or CD62L (Mel-14, rat IgG2a; PharMingen) at 4°C for 30 min. After washing, cells were further incubated with fluorescein-conjugated anti-rat IgM or fluorescein-conjugated anti-rat IgG antibodies (Cappel, Westchester, PA) at 4°C for 30 min. After washing, cells were further incubated with normal rat IgG and then with phycoerythrin-conjugated anti-CD4 (RM4-5, rat IgG2a; PharMingen) or anti-CD8 (53-6.7, rat IgG2a; PharMingen) mAbs at 4°C for 30 min. Fluorescence intensity of the stained cells was analyzed on a FACS 440 flow cytometer (Becton Dickinson, San Jose, CA).

**Adoptive transfer of diabetes to NOD-*scid* mice.** At 10 or 15 weeks of age, spleen cells were isolated from normoglycemic NOD mice that had been treated with C17.8 or M18/2 at 2 weeks of age. NOD-*scid* mice 8–10 weeks old were intravenously injected with  $3 \times 10^7$  spleen cells/mouse. All recipients were monitored for the development of diabetes until 15 weeks after transfer.

**Evaluation of regulatory T-cell activity by adoptive transfer.** To evaluate suppressor activity, splenic T-cells from 8-week-old NOD mice that had received C17.8 treatment at 2 weeks of age were purified on a nylon-wool column. These splenic T-cells ( $1.5 \times 10^7$  cells/recipient) were injected intravenously into NOD-*scid* recipient mice together with spleen cells ( $3 \times 10^7$  cells/recipient) from acutely diabetic NOD mice. As a control, diabetic spleen cells were transferred with splenic T-cells from M18/2-treated female NOD mice into NOD-*scid* recipients. The occurrence of diabetes was monitored three

times a week by measuring urine and blood glucose.

**Cytokine production stimulated by lipopolysaccharide in C17.8-treated NOD mice.** Mice that had been treated with C17.8 or M18 at 2 weeks of age were injected intraperitoneally with 1 mg lipopolysaccharide (LPS) at 28 or 56 days of age. At 10 h after LPS injection, serum was sampled from the retro-orbital vein for the measurement of cytokines. Spleen cells isolated from these mice were cultured in 10% fetal calf serum (FCS)-RPMI 1640 medium without stimulation. After 48 h of culture, supernatants were harvested. Concentrations of IL-2, IL-4, IFN- $\gamma$ , and IL-10 in the sera and culture supernatants were evaluated by sandwich enzyme-linked immunosorbent assay (ELISA), as recommended by the manufacturer (PharMingen). Briefly, anti-cytokine mAb-coated wells were incubated with 100  $\mu$ l culture supernatant for 16 h, followed by incubation with the relevant biotinylated second mAb. After adding avidin-biotinylated horseradish peroxidase solution (Vector Laboratories, Burlingame, CA) for 30 min, o-phenylenediamine solution was added to the wells for color development. Recombinant cytokines were purchased from PharMingen and used as standards.

**Cytokine production by splenic T-cells from C17.8-treated NOD mice.** Spleen cells isolated from 4- and 8-week-old mice that had been treated with C17.8 at 2 weeks of age were cultured in 200  $\mu$ l 10% FCS-RPMI 1640 medium in flat-bottomed 96-well microculture plates, precoated with 5  $\mu$ g/ml anti-CD3- $\epsilon$  (145-2C11, hamster IgG; PharMingen). The culture supernatants were harvested at 24 h for measurement of IL-2 and at 48 h for the measurement of other cytokines (IL-4, IL-10, and IFN- $\gamma$ ) after stimulation.

**Administration of anti-cytokine antibodies to young NOD mice.** NOD mice were injected intraperitoneally with 200  $\mu$ g M18, R4-6A2 (anti-IFN- $\gamma$ ) or S4B6 (anti-IL-2) for 6 consecutive days at 14 days of age. The cumulative incidence of diabetes was monitored until 30 weeks of age. At 30 weeks of age, the mice were killed for histological examination for pancreatic insulinitis.

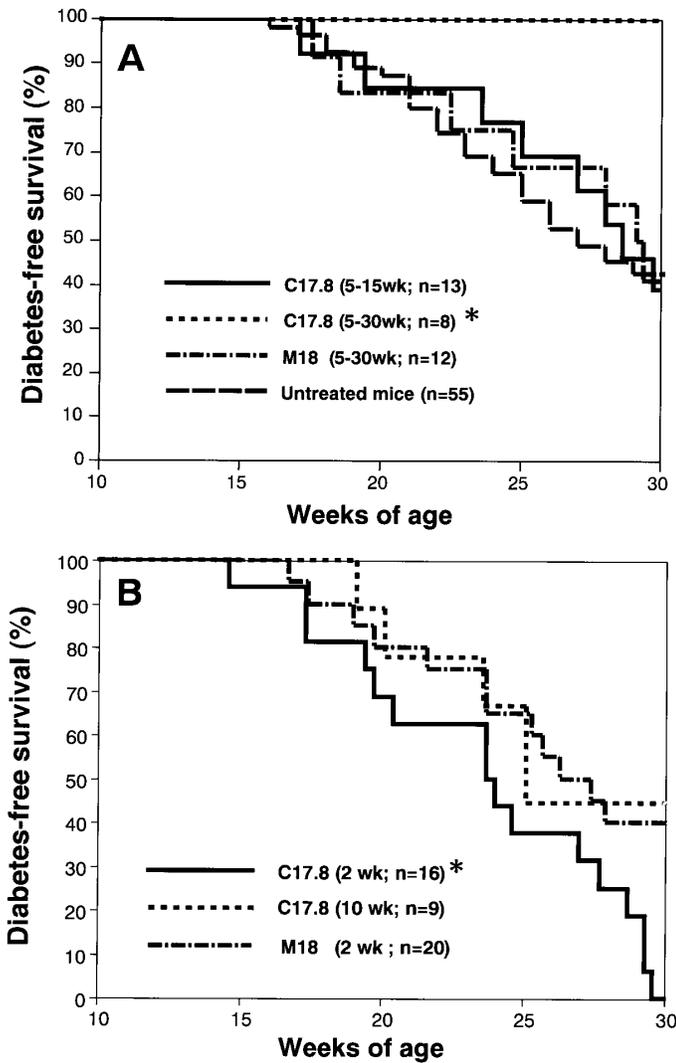
**Statistical analysis.** Statistical analysis of the incidence of diabetes was performed using the Kaplan-Meier method. Statistical analysis of the insulinitis scores and flow cytometry results were performed using the Mann-Whitney *U* test. *P* values <0.05 were considered significant.

## RESULTS

**Long-term administration of anti-IL-12 mAb to NOD mice.** A neutralizing anti-IL-12 mAb (C17.8), which can bind IL-12 p40 and p70, was administered to NOD mice to evaluate whether abrogation of endogenous IL-12 can prevent autoimmune diabetes. When C17.8 was injected from 5 to 30 weeks of age, overt diabetes was completely prevented (Fig. 1A). In contrast, when administration of C17.8 was discontinued at 15 weeks of age, NOD mice developed diabetes with a similar pattern of onset and incidence as M18-treated mice. This similarity suggests that IL-12 plays an important role in the effector phase of the autoimmune destruction of pancreatic  $\beta$ -cells.

Histological examination indicated that the administration of C17.8 significantly decreased the degree of insulinitis when the antibody was administered up until 30 weeks of age (Table 1). On the other hand, pancreatic insulinitis in 30-week-old NOD mice that had received C17.8 during 5–15 weeks of age showed progressive insulinitis similar to that seen in M18-treated NOD mice. These results suggest that abrogation of IL-12 suppressed autoimmune destruction of pancreatic islets but did not remove the autoreactive T-cells causing insulinitis. Thus, endogenous IL-12 appears to play an important role in the activation of effector T-cells.

**Short-term administration of anti-IL-12 mAbs to young NOD mice.** For 6 consecutive days, C17.8 was administered to 2-week-old NOD mice to evaluate the role of IL-12 in the early phase of autoimmune diabetes. Unexpectedly, all 16 C17.8-treated NOD mice exhibited overt diabetes at 30 weeks of age, whereas M18-treated NOD mice showed only 60% incidence of diabetes (Fig. 1B). On the other hand, only 56% of NOD mice injected with C17.8 at 10 weeks of age showed overt diabetes. This finding suggests that abroga-



**FIG. 1.** The effect of long- and short-term administration of anti-IL-12 antibody on diabetes in NOD mice. **A:** Female NOD mice were injected intraperitoneally with 500 µg C17.8 twice a week during 5–30 weeks ( $n = 8$ ) or 5–15 weeks ( $n = 12$ ) of age. Control mice were treated with 500 µg M18 during 5–30 weeks ( $n = 13$ ). **B:** Two-week-old female NOD mice were injected intraperitoneally with 200 µg C17.8 ( $n = 16$ ) or M18 ( $n = 20$ ) for 6 consecutive days. As a control, 10-week-old female NOD mice were injected intraperitoneally with 500 µg C17.8 for 6 consecutive days ( $n = 9$ ). Diabetes was monitored until 30 weeks of age. \* $P < 0.05$  compared with M18-treated mice (Kaplan-Meier).

tion of endogenous IL-12 at the time at which insulinitis is initiated may influence the later autoimmune process directed against pancreatic islets and may enhance the development of overt diabetes. To evaluate the later autoimmune response directed against islets, the degree of insulinitis was evaluated at 8, 36, or 64 days after the administration of C17.8 to 2-week-old NOD mice (Fig. 2). Although insulinitis had not progressed at 8 days after injection of C17.8, it was significantly enhanced in the C17.8-treated mice at 36 and 64 days compared with the insulinitis in the M18-treated mice.

**Effect of short-term anti-IL-12 treatment on cytokine production.** To evaluate the in vivo effect of anti-IL-12 mAbs, NOD mice that received C17.8 at 2 weeks of age were injected intraperitoneally with LPS at 4 or 8 weeks of age, and serum cytokine levels were measured by ELISA. As shown in Fig. 3A, the elevation of serum IL-2 and IFN- $\gamma$  (Th1 cytokines) was abolished in the C17.8-treated mice at 4 weeks of age, whereas the levels of IL-4 and IL-10 (Th2 cytokines) were either significantly enhanced or unchanged. In contrast, serum IL-2 and IFN- $\gamma$  had increased, whereas IL-4 and IL-10 had decreased, in the C17.8-treated mice at 8 weeks of age. Similar results were obtained when cytokine production was assessed in vitro after spleen cell culture (Fig. 3B). These results indicate that the administration of C17.8 at 2 weeks of age effectively suppressed Th1 cytokine production at 4 weeks of age but enhanced the Th1 cytokine production at 8 weeks of age in association with the suppression of Th2 cytokine production. Spleen cells obtained from C17.8- or M18-treated mice were stimulated by immobilized anti-CD3 mAbs in vitro (Fig. 3C) to assess whether administration of anti-IL-12 suppresses the development of Th1 cells or their production of cytokines. Comparable production of IL-2 and IFN- $\gamma$  was observed in spleen cells from the C17.8-treated mice and the M18-treated mice at 4 weeks of age, suggesting that treatment with C17.8 did not suppress the development of Th1 cells but suppressed Th1 cytokine production over this period. It was noted again that production of IL-2 and IFN- $\gamma$  was enhanced, whereas production of IL-4 and IL-10 was suppressed at 8 weeks of age, suggesting that more Th1 cells had developed in the C17.8-treated mice over this period.

**Evaluation of lymphocyte changes in short-term C17.8-treated NOD mice.** To evaluate quantitative and qualitative changes in lymphocyte subpopulations after short-term administration of anti-IL-12 mAbs, cell surface markers of spleen cells were examined at 4, 8, and 20 weeks of age (8, 36, and 120 days after injection) (Table 2). Spleen cell numbers and CD4/CD8 ratios had not been altered, even after treatment with C17.8. However, some lymphocyte phenotypes appeared

**TABLE 1**  
Degree of insulinitis after long-term administration of anti-IL-12

mAb	Duration of treatment (weeks of age)	Diabetic incidence (diabetes/total)	Number of histologically examined mice	Degree of insulinitis (%)					Insulinitis score (mean $\pm$ SD)
				0	1	2	3	4	
M18	5–15	58% (7/12)	5	1.5	6.1	21.3	29.4	41.6	3.04 $\pm$ 0.11
C17.8	5–15	62% (8/13)	5	1.1	6.6	15.4	33.5	43.4	3.03 $\pm$ 0.12
M18	5–30	58% (7/12)	5	0.9	8.0	14.1	31.9	46.0	3.12 $\pm$ 0.16
C17.8	5–30	0% (0/8)	5	11.6	20.9	25.6	28.8	13.0	2.10 $\pm$ 0.06*

M18 or C17.8 mAb (500 µg) were injected intraperitoneally twice a week during 5–15 or 5–30 weeks of age. Five nondiabetic mice of each group were killed for histological examination at 30 weeks of age. At least 20 islets per mice were examined for degree of insulinitis as described in RESEARCH DESIGN AND METHODS. \* $P < 0.05$  compared with M18 (control) group (Mann-Whitney  $U$  test).

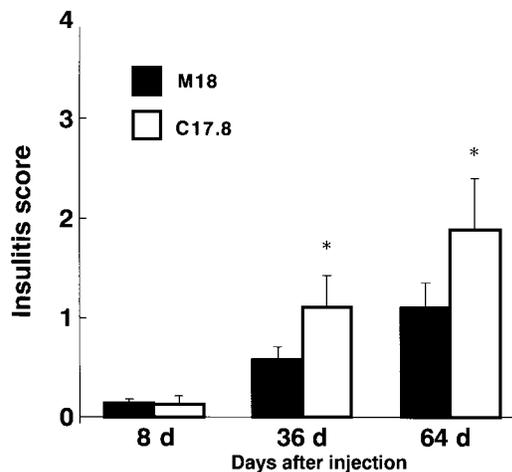


FIG. 2. The effect of short-term administration of anti-IL-12 antibody to young NOD mice on the development of insulinitis. Two-week-old female NOD mice were injected intraperitoneally with 200  $\mu$ g C17.8 or M18 for 6 consecutive days. Five NOD mice in each group were killed for histological examination at 8, 36, or 64 days after the injection of antibodies. Each pancreas removed from NOD mice was fixed in 10% formalin solution. Paraffin-embedded sections were stained with hematoxylin and eosin, and the severity of insulinitis was assessed using the criteria described RESEARCH DESIGN AND METHODS. Data are presented as the mean  $\pm$  SD of five mice in each group. \* $P < 0.05$  compared with M18-treated mice (Mann-Whitney  $U$  test).

to have changed. At 8 days after the final injection of C17.8, CD25<sup>+</sup> and CD44<sup>high</sup> cells in both CD4<sup>+</sup> and CD8<sup>+</sup> subsets were increased in the C17.8-treated mice. This result suggests that activated memory T-cells were increased by the administration of C17.8. At 5 weeks after the final injection of C17.8, the accumulation of activated memory T-cells was more evident. These results suggest that abrogation of IL-12 at 2–4 weeks of age resulted in progressive accumulation of activated memory T-cells, which may include autoreactive diabetogenic T-cells.

**Diabetogenic activity of spleen cells from C17.8-treated NOD mice.** Adoptive transfer experiments were performed to evaluate the diabetogenic effector activity in the NOD mice that received C17.8 at 2 weeks of age. When spleen cells from M18-treated mice were transferred to NOD-*scid* mice, 50% (3/6) of the recipients exhibited late-onset diabetes (>11 weeks after transfer). Similarly, transfer of spleen cells from the C17.8-treated mice at 10 weeks of age resulted in 62.5% diabetes (5/8) at 10 weeks after transfer (Fig. 4A). These results suggest that diabetogenic effector cells did not develop sufficiently at 10 weeks of age, regardless of C17.8 treatment. However, when spleen cells were transferred from 15-week-old M18-treated normoglycemic mice, 100% of recipient mice became overtly diabetic within 7 weeks after transfer, indicating that 15-week-old NOD mice have increased diabetogenic effector cells relative to 10-week-old NOD mice (Fig. 4B). Notably, spleen cells removed from the C17.8-treated normoglycemic NOD mice at 15 weeks of age caused diabetes within 4 weeks after transfer. These results suggest that short-term anti-IL-12 treatment at 2 weeks of age resulted in the accumulation of diabetogenic T-cells at 15 weeks of age.

**Suppressor activity of splenic T-cells from C17.8-treated NOD mice.** Transfer experiments were performed (Fig. 5) to evaluate the effect of C17.8 treatment at 2 weeks of

age on the development of regulatory T-cells that suppress the onset of diabetes. Splenic T-cells from 8-week-old NOD mice that had received C17.8 or M18 at 2 weeks of age were transferred to NOD-*scid* mice along with spleen cells from diabetic NOD mice. When the spleen cells from diabetic NOD mice alone were transferred, the recipients rapidly became diabetic within 6 weeks. Cotransfer of splenic T-cells from the M18-treated mice resulted in a significant retardation in the onset of overt diabetes, suggesting that regulatory T-cells exist in 8-week-old NOD mice. In contrast, cotransfer of splenic T-cells from the C17.8-treated mice resulted in a rapid onset of overt diabetes comparable to that induced by the transfer of diabetic spleen cells alone. These results suggest that development of regulatory T-cells was suppressed in the C17.8-treated NOD mice by IL-12 depletion at 2 weeks of age.

**Effect of anti-Th1 cytokine antibodies on the development of diabetes.** To see whether the decrease of Th1 cytokines by IL-12 depletion or abrogation of the direct effect of IL-12 itself resulted in a higher incidence of diabetes, neutralizing mAbs against IL-2 and IFN- $\gamma$  were administered to 2-week-old NOD mice on 6 consecutive days in a treatment similar to the administration of anti-IL-12 mAbs (Fig. 6). The treatment with anti-IL-2 (S4B6) mAbs resulted in an incidence of 100% overt diabetes, as was the case with C17.8 treatment (Fig. 1B), whereas only 60% of the mice treated with M18 were diabetic at 30 weeks of age. The median age of onset of overt diabetes was 23.7, 23.9, and 17.1 weeks in the C17.8-, M18-, and S4B6-treated NOD mice, respectively, suggesting that abrogation of IL-2 accelerated the development of diabetes compared with M18 or C17.8 treatment. On the other hand, treatment with anti-IFN- $\gamma$  (R4-6A2) mAbs resulted in a decreased incidence of overt diabetes (2/14). These results suggest that loss of IL-2, secondary to the depletion of IL-12, was responsible for the enhancement of an autoimmune response against pancreatic  $\beta$ -cells, resulting in the exacerbation of overt diabetes.

## DISCUSSION

This study shows that long-term treatment with anti-IL-12 antibody from 5–30 weeks of age can prevent the development of insulinitis and diabetes in NOD mice (Fig. 1A). This preventive effect of IL-12 depletion on spontaneous NOD diabetes is consistent with previous observations that administration of IL-12 p40 homodimers, which antagonize the active form of IL-12 p40/p35 heterodimers, prevents insulinitis and overt diabetes both in cyclophosphamide-induced and spontaneous NOD diabetes (20,21). Furthermore, it has also been reported that administration of IL-12 in a mouse experimental autoimmune encephalomyelitis model enhanced the relapse and neutralization of IL-12 by anti-IL-12 antibody-prevented spontaneous relapse (25). These studies suggest that reduced Th1 cell activity and enhanced Th2 cell activity are correlated with amelioration of organ-specific autoimmune diseases. In addition, administration of IL-12 to young NOD mice is reported to accelerate spontaneous diabetes (22). When these data are taken together, it appears that IL-12 plays an important role in the development of effector Th1 cells, and, thus, the neutralization of IL-12 may lead to a deviation toward the Th2-dominant state, which can persistently prevent Th1 cell activities.

Interestingly, when anti-IL-12 antibody was administered to 2-week-old NOD mice for 6 days, the incidence of diabetes was

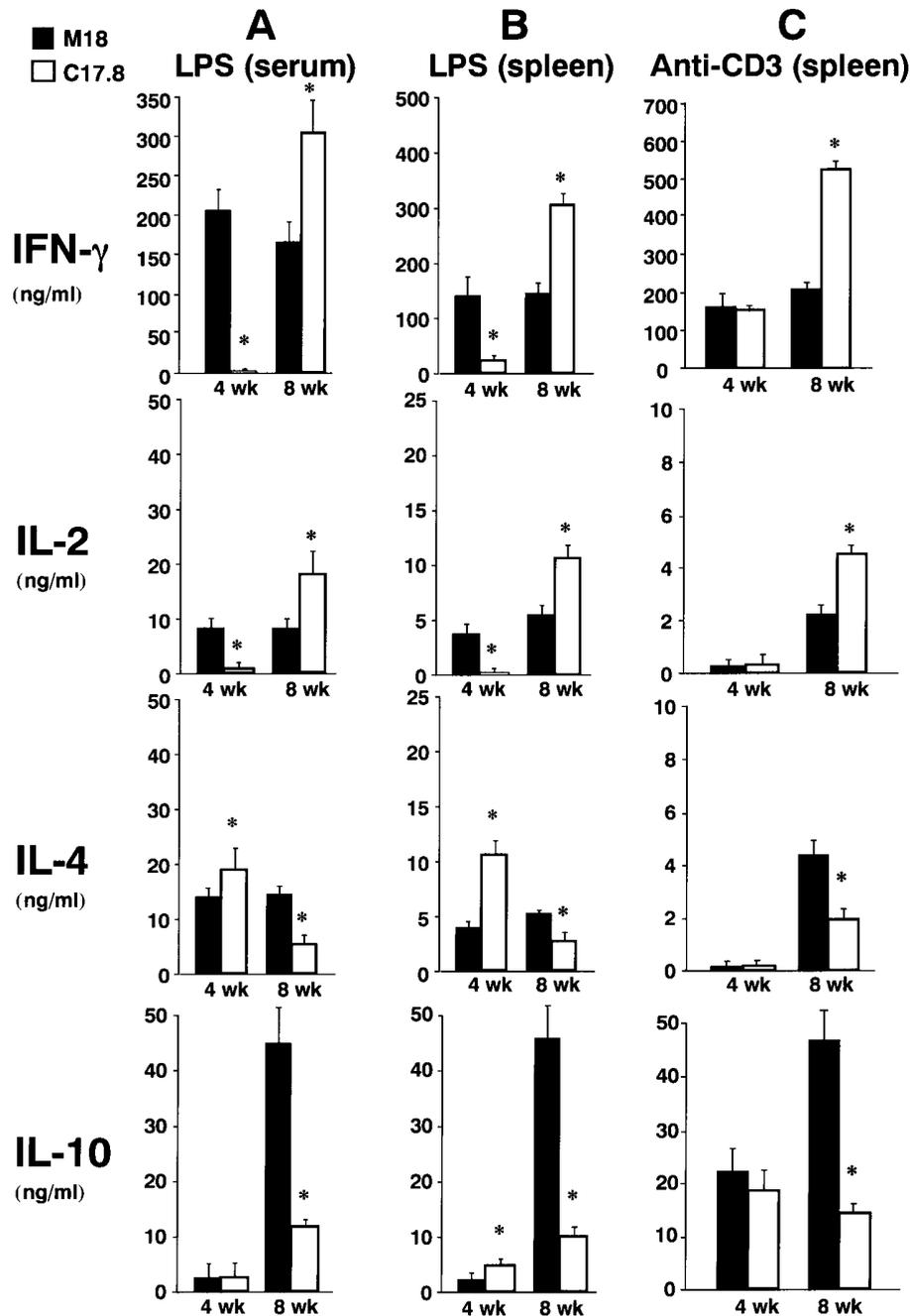


FIG. 3. Cytokine production in anti-IL-12-treated NOD mice after LPS or anti-CD3 antibody stimulation. Mice that received C17.8 or M18 at 2 weeks of age were injected intraperitoneally with 1 mg LPS at 4 or 8 weeks of age. At 10 h after LPS injection, serum was sampled for measurement of cytokine content (A) and spleen cells were cultured in 10% FCS-RPMI 1640 medium without stimulation. After incubation for 48 h, culture supernatants were harvested for the measurement of IL-2, IFN- $\gamma$ , IL-4, and IL-10 contents (B). Data are presented as mean  $\pm$  SD of five mice in each group. \* $P < 0.05$  compared with the M18 group. C: Spleen cells from 4- and 8-week-old mice that had been treated with C17.8 mAb at 2 weeks of age were stimulated with immobilized anti-CD3 mAb (5  $\mu$ g/ml). Culture supernatants were harvested at 48 h after stimulation. IL-2, IL-4, IL-10, and IFN- $\gamma$  contents were evaluated by ELISA. Data are presented as mean  $\pm$  SD of five mice in each group. \* $P < 0.05$  compared with the M18 group.

significantly increased to 100%, in contrast with an incidence of 60% in control IgG (M18)-treated NOD mice examined at 30 weeks of age (Fig. 1B). At 8 days after the injection of anti-IL-12 antibody, LPS stimulation in vivo resulted in the apparent suppression of Th1 cytokine production and the enhancement of Th2 cytokine production (Fig. 3), suggesting that the administration of anti-IL-12 antibody effectively neutralized endogenous IL-12 and could disturb Th1 cell activation in vivo. At 36 days

after the injection of anti-IL-12 antibody, however, a predominance of Th1 cells was observed after in vitro and in vivo stimulation. Furthermore, an enhancement of diabetes transfer activity was observed at 15 weeks of age. This phenomenon cannot be explained simply by a rebound enhancement of the Th1 cell population because administration of anti-IL-12 antibody at 10 weeks of age did not influence the incidence of diabetes. Furthermore, activated memory T-cells had already begun to

TABLE 2  
Flow cytometric analysis of spleen cells from C17.8-treated NOD mice

Age and mAb	n	Cell number		CD4 <sup>+</sup>				CD8 <sup>+</sup>				
		( $\times 10^7$ )	CD4 <sup>+</sup> (%)*	CD8 <sup>+</sup> (%)*	CD25 <sup>+</sup> (%)†	CD54 <sup>+</sup> (%)†	CD44 <sup>high</sup> (%)†	CD62L <sup>high</sup> (%)†	CD25 <sup>+</sup> (%)†	CD54 <sup>+</sup> (%)†	CD44 <sup>high</sup> (%)†	CD62L <sup>high</sup> (%)†
4 weeks of age												
M18	5	4.88 ± 0.45	17.8 ± 1.5	5.54 ± 1.35	6.56 ± 1.3	31.0 ± 3.9	20.2 ± 0.63	72.2 ± 1.10	9.2 ± 1.7	18.6 ± 0.9	16.7 ± 1.27	74.0 ± 1.36
C17.8	5	4.23 ± 0.89	17.8 ± 5.7	6.27 ± 0.19	15.3 ± 8.4‡	32.9 ± 6.1	29.9 ± 0.58‡	69.9 ± 0.86	17.7 ± 8.7‡	24.2 ± 9.0‡	27.9 ± 0.62‡	76.0 ± 0.00
8 weeks of age												
M18	5	5.51 ± 0.45	26.4 ± 5.9	12.5 ± 1.3	14.1 ± 1.6	14.9 ± 1.6	36.5 ± 0.21	62.8 ± 2.73	9.2 ± 1.5	55.2 ± 4.1	29.4 ± 1.14	66.3 ± 1.53
C17.8	5	5.11 ± 1.02	22.5 ± 8.8	12.7 ± 2.1	17.9 ± 4.5	20.3 ± 5.3‡	41.3 ± 0.33‡	59.4 ± 0.49	15.1 ± 3.2‡	71.0 ± 4.1‡	33.2 ± 0.78‡	66.2 ± 0.12
20 weeks of age												
M18	5	6.53 ± 0.30	30.4 ± 0.74	12.0 ± 0.55	6.64 ± 2.48	23.4 ± 4.5	46.1 ± 0.49	40.7 ± 0.49	10.4 ± 13.1	49.5 ± 5.5	38.0 ± 6.48	23.2 ± 1.5
C17.8	5	6.55 ± 0.23	28.7 ± 1.58	12.2 ± 0.29	5.67 ± 1.14	37.8 ± 3.9‡	52.5 ± 5.34‡	33.8 ± 0.42	9.7 ± 1.3	55.8 ± 6.3	35.9 ± 2.08	19.3 ± 0.92‡

Data are means ± SD of five to six mice in each group. \*Percentage in total spleen cells; †percentage in CD4<sup>+</sup> or CD8<sup>+</sup> T-cells; ‡*P* < 0.05 compared with M18 (control) group (Mann-Whitney *U* test).

accumulate, even when neutralization of endogenous IL-12 was effective at 8 days after injection. These results suggest that reduced Th1 cell activity or relatively enhanced Th2 cell activity in NOD mice at a young age results in the development of many more effector cells in the later stages of autoimmune diabetes. However, it has been generally accepted that CD4<sup>+</sup> Th1 cells secreting IL-2 and IFN- $\gamma$  play a key role in the destruction of pancreatic  $\beta$ -cells. The administration of anti-IFN- $\gamma$  antibodies abolished cyclophosphamide-induced and transferred diabetes in NOD mice (15). The transfer of islet-specific CD4<sup>+</sup> T-cell clones, secreting IL-2 and IFN- $\gamma$ , has been shown to induce autoimmune diabetes in young NOD and NOD-*scid* mice (26). In addition, the administration of IL-10, which is known to suppress Th1 cells, was reported to prevent spontaneous diabetes in NOD mice (13). These studies imply that Th2 cells might inhibit the pathogenic Th1 cell response to pancreatic  $\beta$ -cells. However, recent studies revealed that administration of IL-4 and transgenic NOD mice producing IL-10 in pancreatic islets generated nonsuppressive Th2 cell populations (12,27). Islet-specific Th2-like cells failed to protect against transfer of diabetes but instead enhanced autoimmune diabetes (28). Therefore, enhancement of Th2 cell activity may not always be related to suppression of autoimmune diabetes. Moreover, transgenic mice producing IL-10 in pancreatic islets have been reported to develop accelerated diabetes, whereas administration of IL-10 at an adult age prevented diabetes (13,14,29,30). However, there is no evidence that enhanced Th2 activity at a young age accelerates autoimmune diabetes in NOD mice. It is difficult to explain the enhancement of autoimmune diabetes in IL-12-depleted NOD mice by the cytokine balance theory alone.

The enhancement of autoimmune diabetes by IL-12 neutralization was limited only at a young age. A suite of previous studies has suggested a peculiar feature of this young age in the development of autoimmune diabetes in NOD mice. First, *in vivo* administration of anti-CD86 antibody prevented autoimmune diabetes only at an early age in NOD mice (31). Second, administration of tumor necrosis factor- $\alpha$  accelerated autoimmune diabetes at an early age, whereas at a later age, diabetes was prevented (32–34). Third, inhibition of intercellular adhesion molecule-1/leukocyte function-associated antigen-1 or CD40/CD40L interactions abolished autoimmune diabetes only at an early age (35,36). Fourth, neonatal thymectomy prevented diabetes, whereas thymectomy at

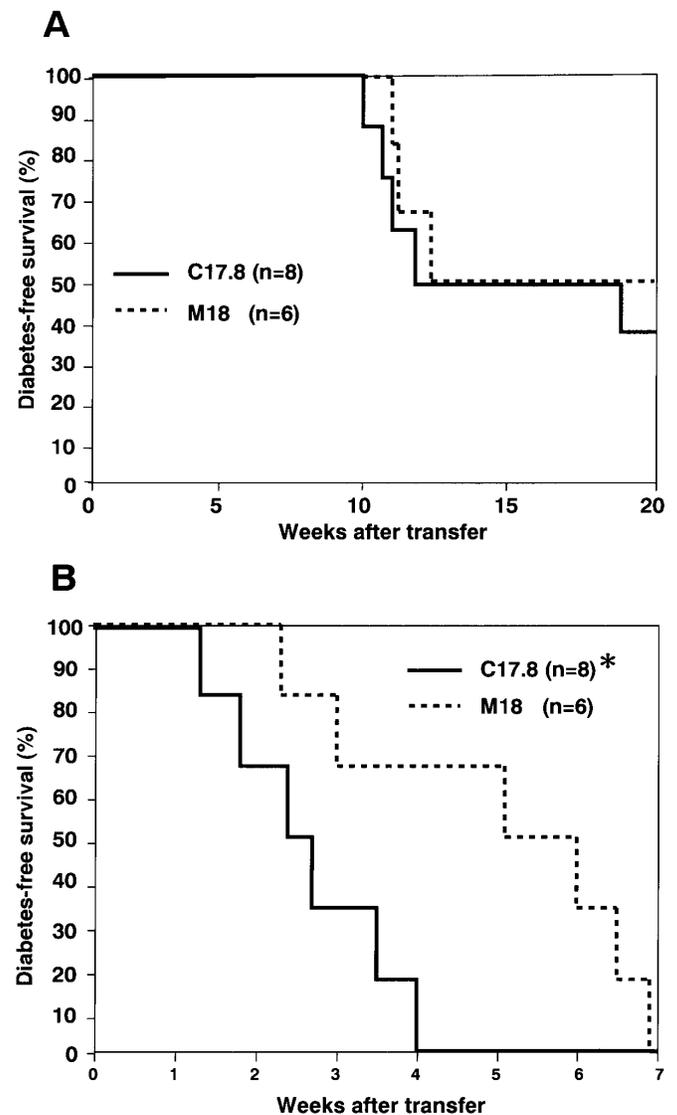
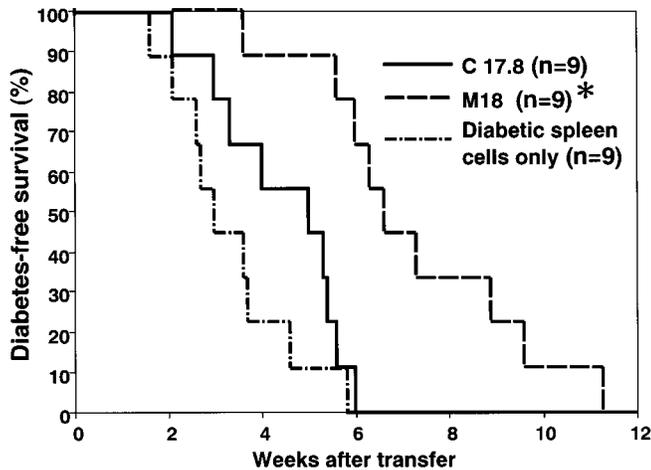


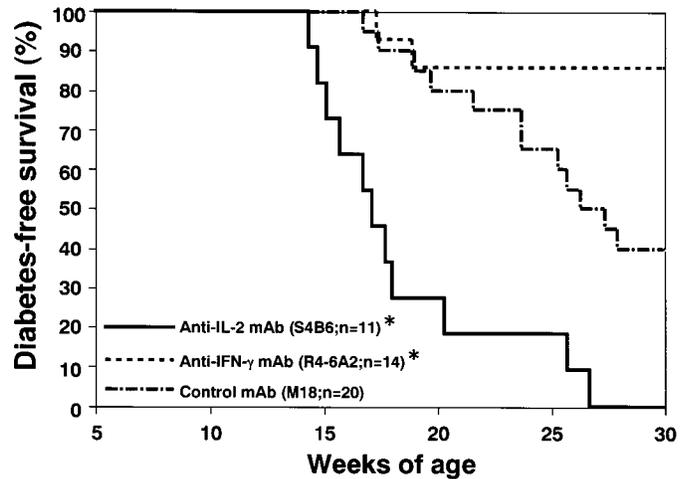
FIG. 4. Adoptive transfer of diabetes from anti-IL-12-treated NOD mice. Spleen cells ( $3 \times 10^7$ ) from 10-week-old (A) or 15-week-old (B) NOD mice that had been treated with C17.8 or M18 at 2 weeks of age were intravenously injected into NOD-*scid* mice. Development of diabetes was monitored for the indicated periods. \**P* < 0.05 compared with M18-treated mice (Kaplan-Meier).



**FIG. 5.** Regulatory activity of splenic T-cells from anti-IL-12-treated NOD mice. Splenic T-cells were isolated and purified from 8-week-old NOD mice that had received C17.8 treatment at 2 weeks of age. These splenic T-cells ( $1.5 \times 10^7$  cells/recipient) were injected intravenously into NOD-*scid* mice together with spleen cells ( $3 \times 10^7$  cells/recipient) from acutely diabetic NOD mice. Development of diabetes was monitored for 12 weeks. \* $P < 0.05$  compared with M18-treated mice (Kaplan-Meier).

3 weeks of age accelerated autoimmune diabetes (37). The last observation suggests that regulatory T-cells, which can suppress autoimmune diabetes, develop after 3 weeks of age in NOD mice. This suppressive activity was detected at 7–8 weeks of age by cotransfer with diabetogenic splenic T-cells, as shown in the present study (Fig. 5) and by others (38). Thus, regulatory T-cells seem to develop from 2 to 3 weeks of age until 7–10 weeks of age and thereafter decrease in activity. The critical timing for the acceleration or inhibition of autoimmune diabetes by treatment with anti-IL-12 appears to be at least partly determined by its effect on the development of regulatory T-cells. Our present study shows that treatment with anti-IL-12 at a young age inhibits the development of regulatory T-cells (Fig. 5). It has been consistently reported that IL-2 treatment starting at an early age can inhibit diabetes development in NOD mice by inducing immunoregulatory T-cells (39). Possible candidates for the regulatory T-cells are the natural killer (NK) T-cells that secrete IL-4 and IFN- $\gamma$ . It has been reported recently that NOD mice have a reduced number of NKT cells, and adoptive transfer of NKT cells could inhibit autoimmune diabetes (40,41). Further studies are required for the characterization of regulatory T-cells and for evaluation of the effects of IL-12 on these cells.

Interestingly, at 2 weeks after antibody treatment, when the production of Th1 cytokines was inhibited by anti-IL-12 antibody, CD25<sup>+</sup> CD44<sup>high</sup> T-cells of both CD4<sup>+</sup> and CD8<sup>+</sup> subsets were increased at the periphery. This result suggests that a certain T-cell population, which may contain progenitors of diabetogenic T-cells, was activated in the IL-12- and/or Th1 cytokine-depleted condition. A much greater acceleration of diabetes was observed after depletion of IL-2 at a young age than after anti-IL-12 antibody treatment. On the other hand, depletion of IFN- $\gamma$  resulted in a lower incidence of diabetes in the present study. These observations are consistent with observations in various knockout NOD mice. In IFN- $\gamma$ -deficient NOD mice, insulinitis has been shown, but with a lower incidence of overt diabetes, suggesting that IFN- $\gamma$  con-



**FIG. 6.** The effect of anti-IL-2 or anti-IFN- $\gamma$  antibody on the development of diabetes. NOD mice were injected intraperitoneally with 200  $\mu$ g M18 (control mAb), R4-6A2 (anti-IFN- $\gamma$  mAb), or S4B6 (anti-IL-2 mAb) for 6 consecutive days at 2 weeks of age. The cumulative incidence of diabetes was monitored until 30 weeks of age. \* $P < 0.05$  compared with M18-treated mice (Kaplan-Meier).

tributes to the progression of diabetes rather than to the initiation of insulinitis (42). On the other hand, IL-2 or IL-2R knockout mice have shown autoimmune lymphoproliferative diseases (43–45). It has also been shown that IL-2 plays a critical role in the induction of peripheral tolerance by rendering activated T-cells susceptible to apoptosis by activation-induced cell death (46–48). Because the anti-IL-12 treatment inhibited IL-2 production, as shown in Fig. 3, it might result in the accumulation of autoreactive T-cells, a large part of which should be depleted by apoptosis in the presence of IL-2.

The long-term abrogation of endogenous IL-12 from 5 to 30 weeks of age resulted in a significant reduction in insulinitis and complete prevention of overt diabetes. However, when anti-IL-12 mAb treatment was stopped at 15 weeks of age, no preventive effect was observed compared with the control mAb treatment (Table 1). This result suggests that the effector cells that destroy pancreatic  $\beta$ -cells remained intact, even after a rather long-term depletion of IL-12 at 5–15 weeks of age. Therefore, IL-12 might act to stimulate effector cells rather than to support the development of effector cells at an adult age. It has been consistently reported that the effects of IL-12 antagonists vary depending on the age when neutralization of endogenous IL-12 is initiated. Administration of IL-12 p40 homodimers during 3–12 weeks of age ameliorated spontaneous and cyclophosphamide-accelerated diabetes, whereas administration from 9 weeks of age did not (21). Furthermore, administration of anti-IL-12 antibody (the same mAbs used in this study) to NOD mice from 4 to 13 weeks of age delayed the onset of diabetes by  $\sim 10$  weeks (49). These studies started administration of IL-12 antagonists before the initiation of insulinitis. Our present study showed that neutralization of endogenous IL-12 during 5–15 weeks of age did not prevent diabetes, whereas neutralization during 5–30 weeks of age did. These results suggest that the progenitors of effector Th1 cells that developed before 5 weeks of age, when peri-insulinitis was already observed in our NOD colony, are sufficient to cause diabetes at later stages and that complete neutralization of endogenous IL-12 during 15–30 weeks

of age might prohibit the clonal expansion or differentiation of effector Th1 cells from Th0 cells. Our previous study, in which IL-12 p40-transduced syngeneic islets were transplanted into diabetic NOD mice, revealed that the IL-12 antagonist at the grafted site can prohibit autoimmune destruction of islet grafts (23). In this case, protracted survival of the grafts was well correlated with downregulation of Th1 cytokines and enhancement of Th2 cytokines. In contrast, a previous study showed that IL-4- or IL-10-transduced islet grafts were not protected from autoimmune rejection (50). IL-12 acts not only to develop Th1 cells but also to activate Th1 cells, CTL, and NK cells (18). Therefore, neutralization of IL-12 at a later stage of NOD diabetes might prohibit the activation of effector Th1 cells, resulting in suppression of autoimmune diabetes. Contrary to this interpretation, however, it has been reported that the administration of the anti-IL-12 antagonist from 9 weeks of age only slightly inhibited spontaneous diabetes (20,21). This discrepancy may be explained by the lower dose and different timing of the administration of the IL-12 antagonists. This report showed a Th1-dominant state at the onset of diabetes even in the IL-12 p40-treated mice, suggesting that the dose of IL-12 antagonist was insufficient. Another study reported that administration of anti-IL-12 antibody from 18 weeks of age did not prevent diabetes in NOD mice (49). Administration of antibody was so late that the dose might not have been adequate to neutralize endogenous IL-12 in the pancreas. A recent study revealed that IL-12-deficient NOD mice still exhibited insulinitis and diabetes (51). Complete deletion of IL-12 from the ontogeny of NOD mice resulted in the development of autoreactive Th1 cells, possibly engendered by IL-18 and unknown factors, and CD4<sup>+</sup> and CD8<sup>+</sup> cells were recruited into pancreatic islets by the qualitative change of these T-cells. The timing and degree of IL-12 suppression may affect the development and activity of autoreactive T-cells, giving rise to these different results. Thus, IL-12 appears to play an important role not only in the development of Th1 effector T-cells but also in the activation of effector cells at later stages of NOD diabetes.

In summary, IL-12 plays an important role not only in the development of Th1 effector T-cells but also in the activation of effector T-cells at the effector phase in autoimmune diabetes. In the initial phase, abrogation of endogenous IL-12 and IL-2 can cause accumulation of the progenitors of effector T-cells, either by attenuating the regulatory T-cells or breaking the peripheral tolerance by inhibiting activation-induced cell death. From data derived from fine mapping using linkage analysis and congenic mice, the IL-2 gene has been proposed as one of the Idd genes in NOD mice (52). It has recently been reported that resistance to T-cell apoptosis by deprivation of IL-2 is increased early in the lives of NOD mice (53). Lower production and/or activity of IL-2 in NOD mice may provide a suitable precondition for the expansion of autoreactive T-cells in autoimmune diabetes (52,54,55). Further study on the initial phase of autoimmune diabetes and the role of regulatory T-cells in autoimmune models will reveal the etiology of autoimmune disease and the underlying peripheral tolerance mechanism.

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