Interleukin 4 Reverses T Cell Proliferative Unresponsiveness and Prevents the Onset of Diabetes in Nonobese Diabetic Mice

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Summary

Beginning at the time of insulitis (7 wk of age), $CD4^+$ and $CD8^+$ mature thymocytes from nonobese diabetic (NOD) mice exhibit a proliferative unresponsiveness in vitro after T cell receptor (TCR) crosslinking. This unresponsiveness does not result from either insulitis or thymic involution and is long lasting, i.e., persists until diabetes onset (24 wk of age). We previously proposed that it represents a form of thymic T cell anergy that predisposes to diabetes onset. This hypothesis was tested in the present study by further investigating the mechanism responsible for NOD thymic T cell proliferative unresponsiveness and determining whether reversal of this unresponsiveness protects NOD mice from diabetes. Interleukin 4 (IL-4) secretion by thymocytes from >7-wk-old NOD mice was virtually undetectable after treatment with either anti-TCR α/β, anti-CD3, or Concanavalin A (Con A) compared with those by thymocytes from age- and sex-matched control BALB/c mice stimulated under identical conditions. NOD thymocytes stimulated by anti-TCR α/β or anti-CD3 secreted less IL-2 than did similarly activated BALB/c thymocytes. However, since equivalent levels of IL-2 were secreted by Con A–activated NOD and BALB/c thymocytes, the unresponsiveness of NOD thymic T cells does not appear to be dependent on reduced IL-2 secretion. The surface density and dissociation constant of the high affinity IL-2 receptor of Con A–activated thymocytes from both strains are also similar. The patterns of unresponsiveness and lymphokine secretion seen in anti-TCR/CD3-activated NOD thymic T cells were also observed in activated NOD peripheral spleen T cells. Exogenous recombinant (r)IL-2 only partially reverses NOD thymocyte proliferative unresponsiveness to anti-CD3, and this is mediated by the inability of IL-2 to stimulate a complete IL-4 secretion response. In contrast, exogenous rIL-4 reverses the unresponsiveness of both NOD thymic and peripheral T cells completely, and this is associated with the complete restoration of an IL-2 secretion response. Furthermore, the in vivo administration of rIL-4 to prediabetic NOD mice protects them from diabetes. Thus, the ability of rIL-4 to reverse completely the NOD thymic and peripheral T cell proliferative defect in vitro and protect against diabetes in vivo provides further support for a causal relationship between this T cell proliferative unresponsiveness and susceptibility to diabetes in NOD mice.

During T cell development, the thymus has a central role in the induction and maintenance of immunological self-tolerance, which is a prerequisite for the prevention of autoimmune diseases (1, 2). The mechanisms responsible for this self-tolerance depend on the ability of developing immature thymocytes to proliferative in response to antigenic and/or mitogenic stimuli presented by thymic APCs (1–4). Therefore, the identity and functional status of T cells that are exported from the thymus to the periphery depend on their intrathymic proliferative capacity. Accordingly, if a thymic T cell after it first encounters a self-thymic Ag becomes functionally inactivated and enters a long-lasting state of prolifer-

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ative unresponsiveness, i.e., a state of anergy, this may lead to changes in the function and/or repertoire of peripheral T cells that could ultimately lead to a breakdown in self-tolerance and autoimmune disease (1–6). Hence, if regulatory T cells that normally confer protection from diabetes become anergic either in the thymus or periphery during the early prediabetic stages of the disease, this event could result in diabetes.

Our previous findings, that thymic and peripheral T cell unresponsiveness after TCR crosslinking correlates with the time of insulitis and persists until the onset of overt disease in nonobese diabetic (NOD)[2] mice (7, 8), raised the possibility that this age-dependent event predisposes to diabetes onset. In this report, we tested this possibility by investigating the mechanisms that elicit thymic and peripheral T cell proliferative unresponsiveness in prediabetic NOD mice and determining whether treatment protocols that reverse this unresponsiveness can protect these mice from diabetes. We analyzed whether CD4$^+$ and CD8$^+$ thymic and splenic T cells from >7-wk-old NOD mice are unresponsive after TCR crosslinking due to a defect(s) in their production of or response to IL-2 and/or IL-4. Interaction between the IL-2- and IL-4-stimulated signaling pathways is important for the regulation of T cell proliferation and maturation, and both IL-2 and IL-4-dependent proliferation of Ag-specific peripheral CD4$^+$ Th0 (produce IL-2 and IL-4) cell clones are sensitive to anergy induction (reviewed in references 8–11). We report that after crosslinking by anti-TCR α/β or anti-CD3 mAbs, the secretion of both IL-2 and IL-4 by NOD thymocytes is reduced considerably. Similar results were obtained with anti-TCR/CD3-activated peripheral T cells. However, while the activation of NOD thymic T cells by Con A elicits levels of IL-2 secretion and high affinity IL-2R (HIL-2R) expression/binding capacity comparable to that of control BALB/c mice, their level of IL-4 secretion remains low. By comparison, Con A–activated NOD peripheral T cells secrete significantly less IL-2 and IL-4 than similarly activated BALB/c peripheral T cells. Exogenously added rIL-4 not only restores the in vitro NOD thymic and peripheral T cell proliferative responses to the higher level of control BALB/c thymic and peripheral T cells but also protects NOD mice from developing diabetes in vivo. These observations demonstrate that NOD T cell proliferative unresponsiveness to TCR crosslinking may result from a TCR-mediated defect that leads to decreases in IL-4 and IL-2 secretion. They also raise the intriguing possibility that this thymic and peripheral T cell proliferative unresponsiveness is a causative factor that predisposes to susceptibility to type I diabetes in NOD mice.

Materials and Methods

Mice. In vitro assays of thymic and splenic T cell function were performed using prediabetic male and female inbred NOD/Del mice between 4 and 12 wk of age. These mice were screened for the absence of glycosuria (Diastix; Miles Laboratories, Rexdale, Ontario), and were maintained in the Department of Comparative Medicine's specific pathogen-free mouse facility at the University of Toronto. This colony derived from a breeding nucleus of NOD/LtAlt mice provided by Dr. B. Singh from the University of Alberta (Edmonton, Alberta) breeding colony. The onset of insulitis in NOD/Del mice occurs at 7 wk of age, and diabetes incidence currently is 50–60% in females and 10% in males by 24 wk of age. Age- and sex-matched BALB/cJ mice obtained from the Department of Comparative Medicine mouse facility were used as controls. The specific pathogen-free NOD/Lt and NOD/Jd colonies at The Jackson Laboratory (Bar Harbor, ME) and the Hospital for Sick Children, respectively, were used to study the in vivo effects of IL-4. Diabetes incidence in the NOD/Lt colony is currently 60–80% in females and 40% in males by 24 wk of age, and the incidence in the NOD/Jd colony is currently 92% in females and 10% in males by 24 wk of age.

Reagents and mAbs. A cell supernatant containing an mAb (PC61, rat IgG1; reference 12) that detects the HIL-2R was kindly provided by Dr. H. R. MacDonald (Ludwig Institute for Cancer Research, Lausanne, Switzerland). FITC-labeled goat anti-mouse IgG and goat anti–rat IgG (H+L) were purchased from Jackson ImmunoResearch Inc. (West Grove, PA). Ascites containing the H57-597 anti-TCR α/β mAb (13) were generously provided by Dr. R. Kubo (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). The 145-2C11 anti-CD3ϵ mAb (14) was kindly supplied by Dr. J. Bluestone (University of Chicago, Chicago, IL). The 11B11 anti-IL-4 mAb (15) was kindly provided by Dr. W. E. Paul (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The S4B6 anti-IL-2 mAb was kindly provided by Dr. T. R. Mosmann (University of Alberta, Edmonton, Alberta) (16). Murine rIL-2 was kindly provided by Dr. G. Mills (Oncology Research, Toronto General Hospital, Toronto, Ontario). Murine rIL-4 was obtained from either the supernatant of murine IL-4 cDNA–transfected X63Ag8-653 myeloma cells (17) (kindly supplied by Dr. F. Melchers, Basel Institute, Basel, Switzerland), a baculovirus system that expresses a vector containing the murine rIL-4 gene (18) (kindly provided by Dr. W. E. Paul), or from Dr. T. Higgins (Sterling Drug, Inc., Malvern, PA). The IL-4-dependent T cell line CT.4S (19) was also generously supplied by Dr. W. E. Paul. The IL-2-dependent CTLL line (20) was a kind gift from Dr. M. Pierres (Centre d'Immunologie de Marseille-Luminy, Marseille, France). [^{3}H]Thymidine was obtained from Amersham (Oakville, Ontario). [^{125}I]rIL-2 (sp act 20–50 μCi/μg) was obtained from NEN (-DuPont Canada Inc., Mississauga, Ontario).

T Cell Isolation and Activation. NOD/Del and BALB/c mice were killed by cervical dislocation, thymi and spleens were removed, and single cell suspensions were prepared. Erythrocytes were removed from spleen cell suspensions by treatment for 3 min with 0.1 mM EDTA, 155 mM NH_4Cl, and 10 mM $KHCO_3$. Thymocytes and spleen cells were washed and suspended in high-glucose DMEM (HGDMEM) containing 10% heat-inactivated FCS, 10 mM Hepes buffer, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 μM 2-ME (all purchased from Gibco Laboratories, Grand Island, NY). Purified splenic T cells were obtained by passage of the cell suspensions through a nylon wool column. Cells isolated in this way consisted of ⩾95% CD3$^+$ T cells, as estimated by fluorescence microscopy using the 145-2C11 anti-CD3 mAb (14). T cells (final concentration, 10^6/ml) were cultured for 72 h at 37°C in round-bottomed 96-well plates in the presence of 2.5 μg/ml Con A with or without rIL-2 or rIL-4, and were then used for either flow cytometric anal-

[2] *Abbreviations used in this paper:* HIL-2R, high affinity IL-2 receptor; NOD, nonobese diabetic; SMLR, syngeneic mixed lymphocyte reaction.

yses or assayed for cell proliferation. Alternatively, anti-TCR α/β– and anti-CD3-induced proliferation were performed by culturing thymocytes for 72 h at 37°C in round-bottomed 96-well plates precoated with a 1:1,000 dilution of ascites containing the H57-597 anti-TCR α/β mAb or a 1:200 dilution of hybridoma supernatant containing the anti-CD3 mAb. Irradiated (3,000 rad, γ irradiation) and mitomycin C (50 mg/ml; 30 min at 37°C)-treated syngeneic splenocytes (final concentration, 2×10^6/ml) were added when thymocytes were activated by anti-CD3, as described (7). [^{3}H]Thymidine (1 μCi/well) was added 18 h before termination of culture. Cultures were harvested using a 96-well cell harvester (Skatron Inc., Sterling, VA), and the extent of cell proliferation was determined by assay of the amount of [^{3}H]thymidine incorporation using a rack β counter (LKB Instruments, Inc., Gaithersburg, MD). IL-2 or IL-4 production by activated thymocytes or splenic T cells (10^6 cells/ml) was quantified by culturing cells for 48 h at 37°C in round-bottomed 96-well plates in the presence of either plate-bound anti-TCR α/β and anti-CD3 mAbs or Con A (2.5 μg/ml), and by assay of the culture supernatant for their IL-2 and IL-4 content.

Flow Cytometric Analysis. Thymocytes activated by Con A in the presence or absence of IL-2 (800 U/ml) were harvested after 72 h in culture and washed three times in PBS containing 0.01 M α-methylmannoside to remove residual Con A. Cells were layered above 30% Percoll and centrifuged at 800 g for 20 min to recover viable cells, further washed, and resuspended in PBS containing 0.5% FCS. Viable cells (2×10^5) were incubated for 30 min at 4°C in 200 μl of undiluted anti-HIL-2R mAb–containing supernatant, washed once with PBS plus 0.5% FCS, and further incubated for 30 min at 4°C with 4 mg of FITC-labeled goat anti–rat IgG (H+L) that was previously absorbed on mouse Ig. The stimulated T cell blasts were distinguished from the unactivated cells based on their forward light scatter vs. 90° light scatter characteristics. Stained cells (10^4 cells/sample) were enumerated using an Epics V flow cytometer (Coulter Electronics, Hialeah, FL) equipped with a three-decade logarithmic amplifier. Specific staining was obtained after subtracting the background value of second antibody alone from values obtained in the presence of both antibodies.

IL-2 Binding Assay. An IL-2 binding assay was performed as described by Robb et al. (21) with minor modifications. Cells were harvested, washed three times, and counted in binding medium (RPMI 1640 supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 μM 2-ME). They were then resuspended in 3 ml of binding medium, pH 3, for 30 s on ice, washed with ice-cold medium, and resuspended in binding medium. Cells (0.5–1.0 $\times 10^6$) in a final reaction volume of 100 μl were added in triplicate to flat-bottomed 96-well plates containing twofold serial dilutions of ^{125}I-labeled human rIL-2 (starting concentration, 4–6 nM). To determine the specificity of binding, unlabeled rIL-2 was added as competitor in 500-fold excess of [^{125}I]rIL-2. After incubation for 2 h at 4°C, cell suspensions were transferred to Eppendorf tubes and the bound and free [^{125}I]rIL-2 were separated by centrifugation through 100 μl of an oil mixture (80% dibutyl phthalate and 20% olive oil). The radioactivity in the cell pellets and supernatants containing the bound and the free [^{125}I]rIL-2, respectively, was determined in a gamma counter. The number of binding sites per cell and the dissociation constants (K_d) of the HIL-2R were calculated by Scatchard analysis.

IL-2 and IL-4 Secretion Assays. Secreted IL-2 or IL-4 activity was measured using the IL-2-dependent CTLL line or IL-4-dependent CT.4S cell line, respectively, as described (18, 20). Twofold serial dilutions of test supernatant were added to cultures containing either 1.5×10^4 CTLL cells or 5×10^3 CT.4S cells in flat-bottomed 96-well plates in a final volume of 100 μl/well for 24 h or 48 h, respectively. Cell proliferation was assessed by addition of 1 μCi/well of [^{3}H]thymidine 6 h (IL-2) or 18 h (IL-4) before termination of culture, and [^{3}H]thymidine incorporation was determined by liquid scintillation counting.

IL-4 Treatment In Vivo. In the first experiment, NOD/Lt females from four separate litters were randomized at 6 wk of age, and a group of 12 females received twice weekly intraperitoneal injections of 500 U (50 ng) murine rIL-4 (sp act, 10^7 U/mg; Sterling Drug). The control group of 12 females received injections of the vehicle (PBS + 1% serum from 6-wk-old prediabetic NOD/Lt females) to provide carrier protein. The mice were maintained under specific pathogen-free conditions at The Jackson Laboratory and allowed free access to food (Old Guilford 96W pellets; Emory Morse Co., Guilford, CT) and chlorinated drinking water. Mice were tested weekly for glycosuria using Tes-Tape™ (kindly provided by Eli Lilly Co., Indianapolis, IN). Diabetes was diagnosed when mice were glycosuric for at least a consecutive 2 wk. At the end of a 14-wk treatment period, two normoglycemic females from the control and treatment groups, respectively, were killed for analysis, and the remainder of the nondiabetic mice were aged to 52 wk without further treatment. At death, pancreas, submandibular salivary glands, and kidneys from each mouse were fixed in Bouin's solution, embedded in paraffin, sectioned, and stained. Aldehyde fuchsin staining of pancreas sections sampled at three different nonoverlapping levels was used to compare the extent to which insulitic infiltrates had reduced the mass of granulated β cells. Splenic leukocyte populations were enumerated by FACScan® (Becton Dickinson & Co., Mountain View, CA) analyses using the anti-Thy-1.2 (clone HO-13-4.9), anti-CD4 (clone GK 1.5), and anti-CD8 (clone 53-6.72) mAbs in ascites form at 1:100, 1:200, and 1:200 dilutions, respectively, as described (7). Analyses of *Escherichia coli* LPS–stimulated IL-1 secretion from peritoneal macrophages and of T cell immunoregulation after activation in a syngeneic mixed lymphocyte reaction (SMLR) were performed as described (22).

In a second experiment, a group of eight 3-wk-old female NOD/Jd mice from two separate litters received twice weekly intraperitoneal injections of 500 U (50 ng in 250 μl) of a murine rIL-4 (sp act, ~10^7 U/mg)-containing supernatant derived from Sf9 Drosophila cells infected with a baculovirus that expresses a vector containing the murine rIL-4 gene (18). The control group of six age-matched NOD females received injections of supernatant (250 μl) derived from Sf9 cells infected with the wild-type baculovirus vector that does not contain the murine IL-4 gene (18). Treatment of both groups of mice was continued for 12 wk until the mice were 15 wk of age, after which treatment ceased. The mice were maintained at the Hospital for Sick Children, tested weekly for glycosuria, and diagnosed for diabetes, as described in the first experiment.

Results

Expression of HIL-2R on NOD Con A–activated Thymic T Cell Blasts Is Normal. Engagement of the TCR/CD3 complex by a mitogenic lectin or Ag/MHC generally leads to a rapid increase in HIL-2R expression followed by IL-2 production and stimulation of T cell proliferation (23). Alternatively, if such TCR stimulation results in a decrease in or lack of expression of HIL-2R, then T cell proliferative unresponsiveness ensues (24). In Con A-activated CD4$^+$ and CD8$^+$

spleen T cells from 10-wk-old NOD/ShiKbe mice, HIL-2R expression is reduced about threefold (25). The latter result raised the possibility that the proliferative unresponsiveness of NOD thymocytes from >7-wk-old mice to Con A stimulation (7) is mediated by a diminished expression of HIL-2R. To test this possibility, NOD and BALB/c thymocytes were cultured in the presence of 2.5 μg/ml Con A for 72 h, and were then analyzed by flow cytometry for their surface expression of HIL-2R. The percentages of these thymocytes that express HIL-2R were very similar (Table 1), and their levels of surface expression of HIL-2R were equivalent (our unpublished data).

Binding of IL-2 to the HIL-2R generally results in an increased level of expression of the HIL-2R (23). To determine whether the post-HIL-2R part of the IL-2 pathway that enhances HIL-2R membrane expression is intact, the level of HIL-2R surface expression in NOD and BALB/c thymocytes stimulated by Con A plus 800 U/ml rIL-2 was assayed. The latter saturating concentration of rIL-2 was chosen since NOD thymocytes proliferate rather poorly in response to lower concentrations of rIL-2 (7). The addition of 800 U/ml rIL-2 increased both the proportions of T cells bearing HIL-2R (Table 1) and the surface densities of these receptors on NOD and BALB/c thymocytes (our unpublished data).

Results obtained by Scatchard analysis of [^{125}I]rIL-2 binding to thymocytes supported our estimates of the relative levels of expression of HIL-2R on these cells observed by flow cytometry. The number per cell and affinity of HIL-2R were very similar in both young (<7 wk) and old (>7 wk) Con A–activated NOD and BALB/c thymocytes (Table 2). An age-dependent effect was noted since a twofold decrease in the number of HIL-2R molecules per cell on old vs. young activated thymocytes was observed. No significant difference was observed between the affinity and number per cell of HIL-2R on NOD and BALB/c quiescent thymocytes (data not shown). Thus, the surface density and binding capacity of HIL-2R on activated NOD thymic T cells appears to be normal (i.e., equivalent to that of activated BALB/c thymocytes), and presumably is not responsible for the Con A–mediated proliferative defect of >7-wk-old NOD thymocytes.

NOD Thymic T Cells Secrete Reduced Amounts of IL-2 after Stimulation by Anti-TCR/CD3 but Not Con A. Since the level of HIL-2R expression on Con A–activated NOD thymocytes is normal, we analyzed whether the unresponsiveness of these T cells results from a defect in IL-2 production after stimulation through the TCR, as has been observed in Th1 clones (3, 4, 9, 26, 27). The capacity of NOD thymocytes to secrete IL-2 after activation by either anti-TCR α/β, anti-CD3, or Con A was compared. Anti-TCR α/β (Fig. 1 *A*) and anti-CD3 (Fig. 1 *B*) each stimulated ~10-fold less IL-2 secretion by thymocytes from 8–12-wk-old NOD mice than from age- and sex-matched control BALB/c thymocytes. However, the amounts of IL-2 secreted by NOD and BALB/c Con A–activated thymic T cells were virtually identical (Fig. 1 *C*). Thus, mitogen stimulation of NOD thymocytes overcomes the defect in IL-2 secretion that is observed after crosslinking the TCR with an anti-TCR mAb.

NOD Spleen T Cells Secrete Reduced Amounts of IL-2 after Stimulation by Either Anti-TCR/CD3 or Con A. To determine whether the reduced IL-2 secretion observed in stimulated NOD thymic T cells is also manifested in activated NOD peripheral T cells, we compared the levels of IL-2 secretion by activated spleen T cells from NOD and BALB/c mice. The level of IL-2 secreted by activated NOD splenic T cells is significantly lower than that noted for activated thymic T cells. This was observed not only for after T cell stimulation by anti-TCR α/β (Fig. 1 *D*) and anti-CD3 (Fig. 1 *E*) but also by Con A (Fig. 1 *F*). Thus, while Con A normalizes the defect in IL-2 secretion by NOD thymic T cells, this is not the case for Con A–activated NOD spleen T cells.

NOD Thymic and Splenic T Cell Proliferative Unresponsiveness Is Associated with Diminished IL-4 Secretion and Is Reversed by Exogenous rIL-4. Interaction between the IL-2- and IL-4-stimulated signaling pathways plays an important role in

Table 1. *Frequency of HIL-2R-bearing NOD and BALB/c Con A–activated T Cell Blasts Is Equivalent*

Strain	Stimulus: Con A	Stimulus: Con A + IL-2
	%	%
NOD	25	56
BALB/c	18	49

Thymocytes from 8–12-wk-old NOD or control BALB/c mice were incubated for 72 h at 37°C with Con A (2.5 μg/ml) in the absence or presence of rIL-2 (800 U/ml). The recovered viable cells were stained with an anti-HIL-2R mAb and FITC-labeled goat anti-rat Ig (H + L), and the large thymocyte blasts were gated and analyzed. The mean percentages of positively stained thymocytes obtained in three independent experiments are indicated, and the SD of the means were ≤10%.

Table 2. *Expression of HIL-2R by Con A–activated NOD Thymocytes Is Normal*

Strain	Age	Affinity K_d	Molecules/cell
		pM	
NOD	Young	38	2,025
BALB/c	Young	67	2,145
NOD	Old	52	1,015
BALB/c	Old	57	1,215

Assays of binding of [^{125}I]rIL-2 to Con A–activated NOD thymocytes were performed as described in Materials and Methods. Young mice were 4–6 wk of age and old mice were 8–12 wk of age. The number of binding sites per cell and the dissociation constants (K_d) of the HIL-2R were calculated by Scatchard analysis. Each result was obtained using a group of two to five mice in three independent experiments.

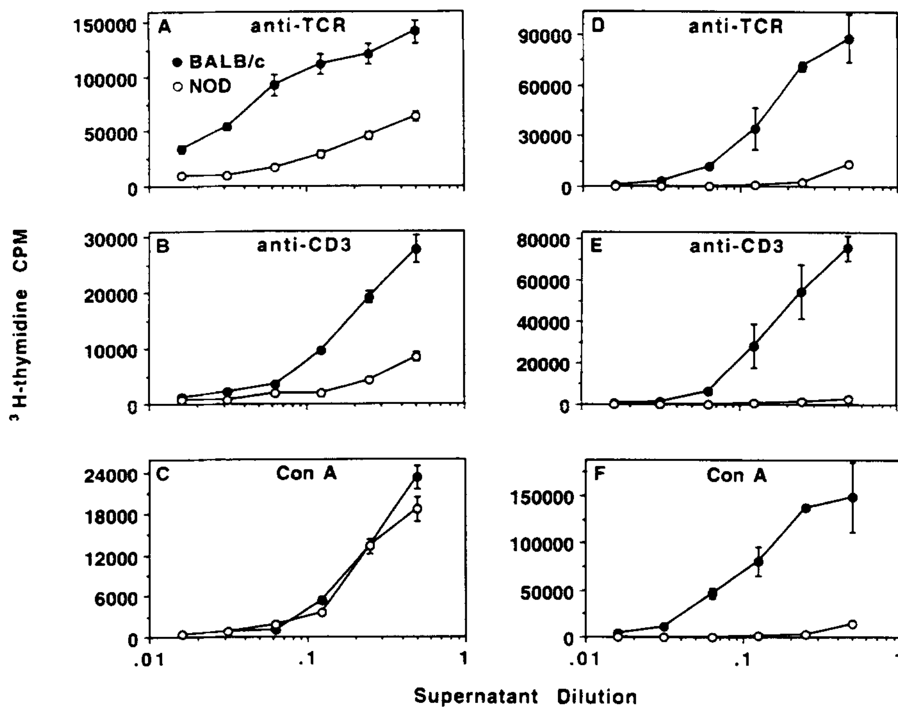

Figure 1. IL-2 secretion by activated thymic and splenic T cells. Thymic (*A–C*) and splenic (*D–F*) T cells from 8–12-wk-old NOD and BALB/c mice were activated for 48 h at 37°C by either plate-bound anti-TCR α/β (*A* and *D*) and anti-CD3 (*B* and *E*) mAbs, or by 2.5 μg/ml Con A (*C* and *F*). Culture supernatants were removed and assayed for their IL-2 activity by stimulation of proliferation of CTLL IL-2-dependent T cells. The results of triplicate cultures are expressed as the mean values ± SD, and are representative of three different experiments.

the regulation of T cell proliferation and maturation (28–30). In addition, Th1 and Th2 cells differ in their function and production of autocrine growth factors; IL-2 is produced by Th1 and IL-4 is produced by Th2, respectively (31). We therefore examined the level of secretion of IL-4 by activated NOD thymic and splenic T cells. NOD thymocytes activated by either anti-TCR α/β (Fig. 2 *A*) or anti-CD3 (Fig. 2 *B*) did not secrete detectable amounts of IL-4 in comparison with similarly activated thymocytes from age- and sex-matched BALB/c control mice. Con A–activated NOD thymocytes secreted considerably less IL-4 than did similarly activated BALB/c thymocytes (Fig. 2 *C*). Similar results were obtained

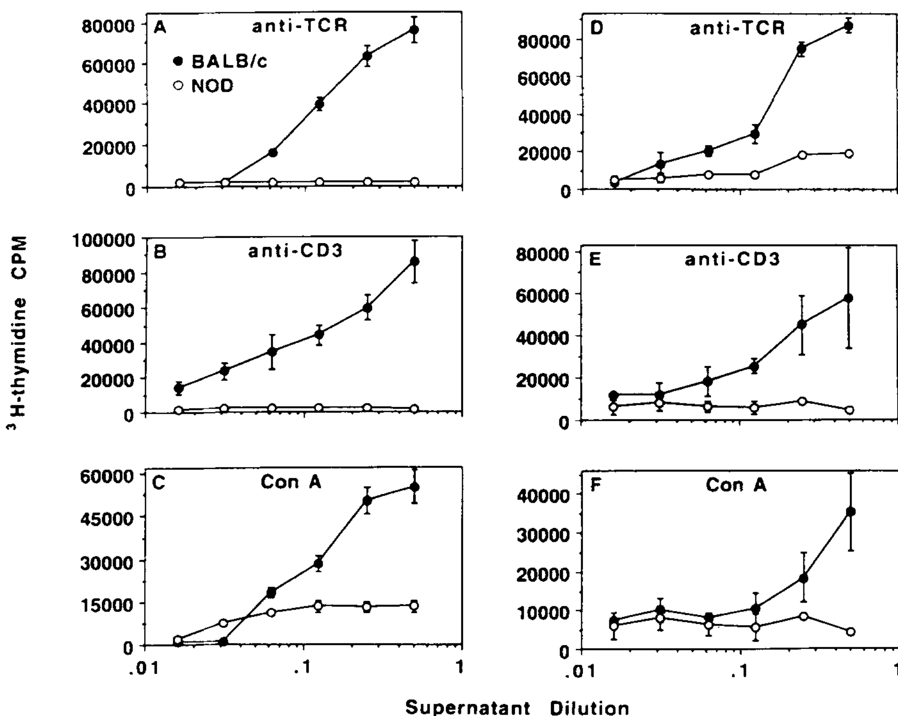

Figure 2. IL-4 secretion by activated thymic and splenic T cells. Thymic (*A–C*) and splenic T cells (*D–F*) from 8–12-wk-old NOD and BALB/c mice were activated for 48 h at 37°C by either plate-bound anti-TCR α/β (*A* and *D*) or anti-CD3 (*B* and *E*) mAbs, or by 2.5 μg/ml Con A (*C* and *F*). Culture supernatants were removed and assayed for their IL-4 activity by stimulation of proliferation of CT.4S IL-4-dependent T cells. The results of triplicate cultures are expressed as the mean values ± SD, and are representative of three different experiments.

upon activation of NOD splenic T cells activated by either anti-TCR α/β (Fig. 2 *D*), anti-CD3 (Fig. 2 *E*), or Con A (Fig. 2 *F*). Therefore, NOD thymic and peripheral T cells both display a relative inability to secrete IL-4 after activation through either the TCR or by mitogen.

To establish whether this defect in IL-4 secretion is causally related to T cell hyporesponsiveness, the ability of rIL-4 plus either anti-TCR α/β, anti-CD3, or Con A to stimulate NOD T cell proliferation was assayed. Addition of exogenous rIL-4 restored to normal the proliferative capacity of NOD thymocytes stimulated by anti-TCR α/β (Fig. 3 *A*), anti-CD3 (Fig. 3 *B*), or Con A (Fig. 3 *C*). Similarly, exogenous rIL-4 also restored the proliferative response of anti-TCR α/β-activated NOD spleen T cells to that of activated control BALB/c spleen T cells (Fig. 3 *D*). In contrast, supraphysiological amounts of rIL-2 only partially correct the thymic T cell unresponsiveness to anti-CD3 (Fig. 4) or Con A (7). Therefore, NOD thymic and peripheral T cell proliferative unresponsiveness may be due to diminished IL-4 secretion as a consequence of a defect in the Th2 T cell subset.

Exogenous rIL-4 Completely Restores Normal IL-2 Secretion by NOD Thymic T Cells, whereas Exogenous rIL-2 Only Partially Restores IL-4 Secretion by NOD Thymic T Cells. To test whether an increased level of IL-2 secretion is associated with the capacity of exogenous rIL-4 to restore the proliferative responsiveness of NOD T cells, the ability of anti-TCR α/β plus exogenously added rIL-4 to stimulate IL-2 secretion by BALB/c and NOD thymocytes was assayed. The amount of rIL-4 added, i.e., 125 U/ml, promotes essentially a maximal proliferative response of anti-TCR α/β-stimulated NOD thymic T cells (Fig. 3 *A*). Equivalent levels of IL-2 secretion by anti-TCR α/β + rIL-4–activated thymocytes from both strains were noted (Fig. 5 *A*). Since the IL-2-dependent CTLL cell line used here may be partially responsive to IL-4, we verified whether CTLL proliferation observed in Fig. 5 *A* was at all due to the presence of residual exogenous rIL-4 present in the supernatants of the activated T cells. The ability of the 11B11 anti-IL-4 mAb to block CTLL and CT.4S cell proliferation was compared. This mAb (10 μg/ml) reduced the CTLL proliferative response to BALB/c- and NOD-activated thymic T cell culture supernatants by only 33 and 26%, respectively (Fig. 5 *B*). In contrast, this mAb inhibited virtually 100% of the IL-4-dependent CT.4S proliferative response, demonstrating that the CTLL proliferative response obtained in Fig. 5 *A* was stimulated predominantly by IL-2. Thus, the ability of IL-4 to restore the proliferative responsiveness of NOD T cells activated via the TCR is associated with its capacity to restore to normal (i.e., to the BALB/c T cell level) the level of IL-2 secretion by these cells.

To test whether deficient IL-4 secretion mediates the partial restoration of the NOD thymic T cell proliferative response by exogenous rIL-2, the ability of anti-TCR α/β plus exogenously added rIL-2 to stimulate IL-4 secretion by BALB/c and NOD thymocytes was assayed. The amount of rIL-2 added, i.e., 250 U/ml, promotes essentially a maximal proliferative response of anti-TCR α/β-stimulated NOD thymic T cells (Fig. 4). NOD thymocytes secreted considerably less IL-4 than did BALB/c thymocytes after stimulation with anti-TCR α/β + rIL-2 (Fig. 5 *C*). Since the IL-4-dependent CT.4S cell line is partially responsive to IL-2 at a concentration of $\geq$50 U/ml (19), we verified whether any CT.4S proliferation observed in Fig. 5 *C* was stimulated by residual exogenous rIL-2 present in the supernatants of the activated T cells. The ability of the S4B6 anti-IL-2 mAb to block CT.4S and CTLL cell proliferation was compared. This mAb (25% of hybridoma culture supernatant) reduced the CT.4S proliferative response to BALB/c- and NOD-activated thymic T cell culture supernatants by only 27 and 33%, respectively. In contrast, this mAb inhibited the IL-2-dependent CTLL proliferative response to BALB/c- and NOD-activated thymic T cell culture supernatants by 86 and 85%, respectively (Fig. 5 *D*). Thus, the inability of IL-2 to completely restore the proliferative responsiveness of TCR-activated NOD thymic T cells is associated with the partial restoration of IL-4 secretion by these cells.

NOD Thymic T Cells Require a Continuous IL-4 or IL-2 Stimulus to Secrete Normal Levels of IL-2 or Partial Levels of IL-4, Respectively. Our data described above raise the possi-

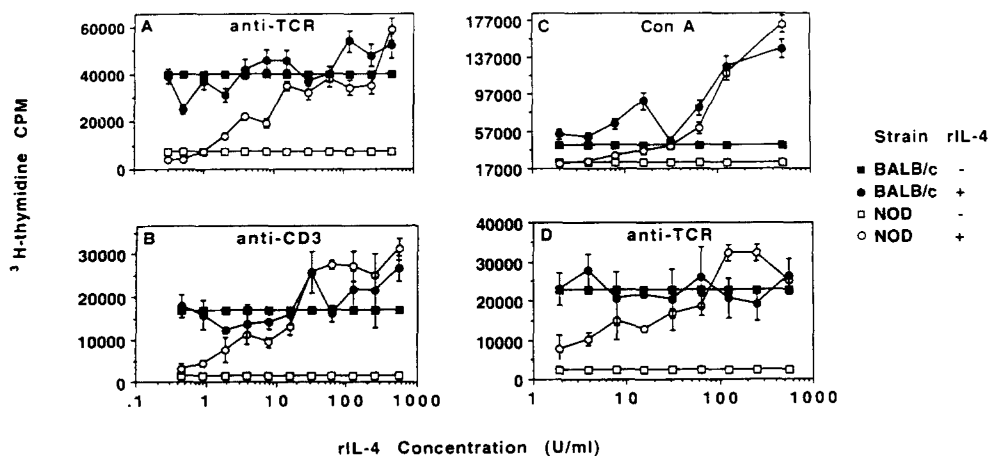

Figure 3. Exogenous rIL-4 corrects the proliferative unresponsiveness of activated NOD thymic and splenic T cells. Thymocytes from 8–12-wk-old NOD and BALB/c mice were activated by either plate-bound anti-TCR α/β (*A*) and anti-CD3 (*B*) mAbs or by Con A (*C*) in the presence of varying amounts of rIL-4. Splenic T cells were activated by plate-bound anti-TCR α/β mAb in the presence of varying amounts of rIL-4 (*D*). Cell proliferation was determined by [^{3}H]thymidine incorporation. The results of triplicate cultures are expressed as the mean values ± SD, and are representative of three different experiments.

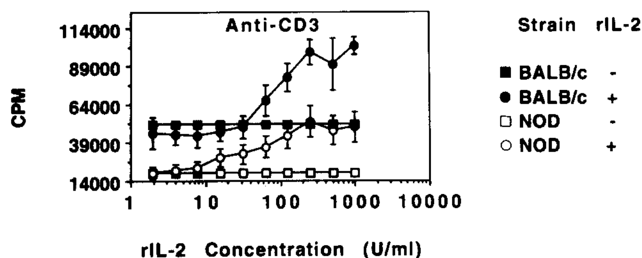

Figure 4. Exogenous rIL-2 partially corrects the proliferative unresponsiveness of activated NOD thymic T cells. Thymocytes from 8–12-wk-old NOD and BALB/c mice were activated by anti-CD3 mAb in the presence of varying amounts of rIL-2. Cell proliferation was determined by [^{3}H]thymidine incorporation. The results of triplicate cultures are expressed as the mean values ± SD, and are representative of three different experiments.

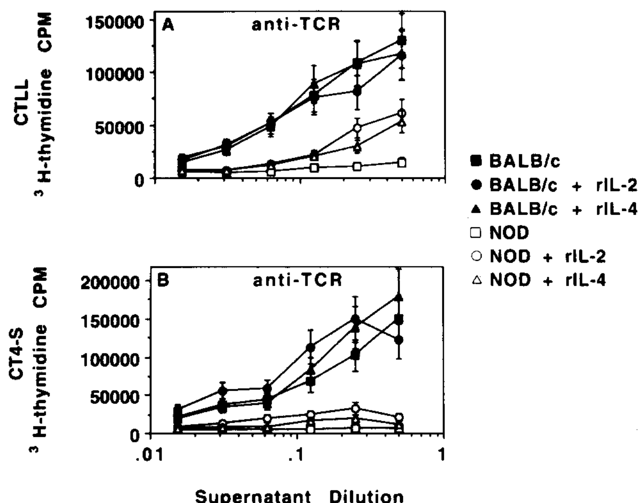

Figure 6. NOD thymic T cells require a continuous IL-4 or IL-2 stimulus to secrete normal levels of IL-2 or partial levels of IL-4, respectively. Thymic T cells from 8–12-wk-old NOD and BALB/c mice were activated by anti-TCR α/β in the presence of rIL-2 (250 U/ml) or rIL-4 (125 U/ml). After 24 h of culture, cells were harvested, layered above 30% Percoll, and centrifuged at 800 *g* for 20 min to remove dead cells. After washing, viable T cell blasts were incubated for an additional 48 h in 96-well plates coated with anti-TCR. (*A*) Culture supernatants were harvested and assayed for their IL-2 activity. (*B*) Culture supernatants were harvested and assayed for their IL-4 activity. The results of triplicate cultures are expressed as the mean ± SD, and are representative of three different experiments.

bility that NOD T cell proliferative unresponsiveness may be due primarily to a defect in the Th2 T cell subset. Interestingly, Ben-Sasson et al. (18) previously reported that Th2 cells may actually consist of two distinct subsets: one that requires IL-2 for IL-4 production and a second that can produce IL-4 without the requirement of IL-2. Based on this report, we considered that the following two possibilities may explain our findings. First, only the IL-2-responsive NOD Th2 subset produces IL-4 upon stimulation with anti-TCR + rIL-2, and this would account for the partial restoration of endogenous IL-4 production. Second, this IL-2-responsive Th2 subset does not produce sufficient amounts of endogenous IL-4 upon stimulation with anti-TCR + rIL-2, and therefore IL-4 may not be available in the amounts required for complete restoration of the response to be achieved.

To further examine why rIL-2 only partially restores the level of IL-4 secretion and proliferative responsiveness of NOD thymic T cells, we investigated the autocrine effect of rIL-4 on these responses of NOD Th2 cells and compared it with the effect of rIL-2 on these cells. If there is a defect only in the IL-2-dependent pathway of IL-4 production, the addition of sufficient amounts of exogenous rIL-4 would be expected to restore the production of endogenous IL-4 because all Th2 T cells are responsive to high concentrations of IL-4.

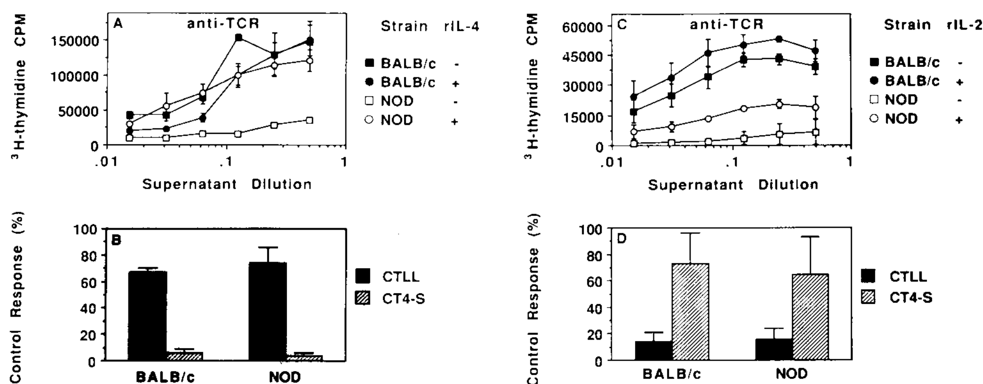

Figure 5. Exogenous rIL-4 completely restores IL-2 secretion by NOD thymic T cells whereas exogenous rIL-2 only partially restores IL-4 secretion by NOD thymocytes. (*A*) Thymic T cells from 8–12-wk-old NOD and BALB/c mice were activated by anti-TCR α/β in the presence of rIL-4 (125 U/ml). Culture supernatants were harvested and assayed for their IL-2 activity, as in Fig. 1, in the presence of the 11B11 anti-IL-4 mAb. The results of triplicate cultures are expressed as the mean ± SD, and are representative of three different experiments. (*B*) NOD- and BALB/c-stimulated T cell culture supernatants were assayed for their IL-2 and IL-4 activities in the presence or absence (control) of the 11B11 anti-IL-4 mAb. The results of triplicate cultures are expressed as the mean ± SD, and are representative of two different experiments. (*C*) Thymic T cells from 8–12-wk-old NOD and BALB/c mice were activated by anti-TCR α/β in the presence of rIL-2 (250 U/ml). Culture supernatants were harvested and assayed for their IL-4 activity, as in Fig. 2, in the presence of the S4B6 anti-IL-2 mAb. The results of triplicate cultures are expressed as the mean ± SD, and are representative of two different experiments. (*D*) NOD- and BALB/c-stimulated T cell culture supernatants were assayed for their IL-2 and IL-4 activities in the presence or absence (control) of the S4B6 anti-IL-2 mAb. The results of triplicate cultures are expressed as the mean ± SD, and are representative of two different experiments.

To test this possibility, BALB/c and NOD thymic T cell blasts were generated during an initial 24-h culture in the presence of anti-TCR α/β plus exogenous rIL-4 or rIL-2, and after washing and selection of viable T cell blasts, their profiles of IL-2 and IL-4 secretion were determined after a further 48 h of stimulation in the presence of only anti-TCR α/β. NOD T cell blasts secreted significantly lower levels of IL-2 than did BALB/c T cell blasts (Fig. 6 *A*). Interestingly, BALB/c T blasts pretreated with rIL-2 or rIL-4 secreted equivalent levels of IL-2, and similar results were obtained for NOD T blasts. However, the levels of IL-4 secretion by NOD T blasts pretreated with rIL-2 or rIL-4 were considerably lower than those of BALB/c T cell blasts, and were equivalent to those observed in untreated control cultures (Fig. 6 *B*). Thus, although stimulation by anti-TCR plus rIL-4 restores the proliferative responsiveness of NOD T cells (Fig. 3) by enhancement of IL-2 secretion by these cells (Fig. 5 *A*), these data indicate that rIL-4 must be present continuously throughout the culture period so that normal IL-2 secretion by activated NOD T cell blasts can be achieved. In addition, these observations demonstrate that Th2 cells are significantly more unresponsive to both IL-2 and IL-4 than Th1 cells. Presumably, the continuous presence of IL-4 is required for NOD Th1 cell blasts to synthesize a sufficient amount of IL-2 that will enable these cells to progress through the cell cycle and proliferate. These results may also account for our previous findings that NOD thymic T cell blasts, generated after 3 d of culture in the continuous presence of rIL-2, are deficient in their TCR-mediated activation of $p21^{ras}$ and tyrosine phosphorylation of $p42^{mapk}$ (32). Because reduced $p21^{ras}$ and $p42^{mapk}$ activities inhibit progression to S phase of the cell cycle, these deficiencies likely mediate the proliferative unresponsiveness of these cells (32).

In Vivo Administration of rIL-4 Protects NOD Mice against Diabetes. Inasmuch as rIL-4 corrects the proliferative hyporesponsiveness of prediabetic NOD mice thymic T cells in vitro, we examined whether administration of murine rIL-4 in vivo prevents diabetes in NOD mice. As shown in Fig. 7, chronic treatment with the rIL-4 preparation supplied by Sterling Drug markedly suppressed diabetes development in NOD/Lt females (1/12 diabetic at the end of 20 wk vs. 9/12 receiving vehicle control). At 21 wk of age, widespread, severe insulitis was present in the pancreas of one of the two normoglycemic rIL-4-treated females examined. Severe insulitis was present in the pancreases of both of the normoglycemic vehicle-treated controls necropsied. Four of the nine rIL-4-treated mice remaining normoglycemic at the cessation of rIL-4 treatment at 20 wk subsequently developed diabetes by 52 wk of age. Of the five normoglycemic rIL-4-treated mice surviving to 52 wk, pancreases of three were free of insulitis, while insulitis was present in the other two. The single control mouse remaining normoglycemic at the end of 20 wk remained normoglycemic to 52 wk of age. However, only a single islet with granulated β cells was found in the pancreas of this mouse, suggesting that diabetes was incipient. Heavy sialitis in submandibular glands and focal nephritis in the kidney was observed at 52 wk of age in all mice regardless of treatment.

A similar protective effect from diabetes was obtained in a second experiment in which another source of murine rIL-4 derived from a baculovirus expression system (18) was administered to NOD/Jd female mice beginning at 3 wk of age. This preparation, injected intraperitoneally for 15 wk in a similar amount (50 ng, twice weekly) to the rIL-4 used above in the first experiment, protected eight of eight NOD/Jd mice from diabetes upon analysis at 28 wk of age. The incidence of diabetes in untreated female NOD/Jd mice at this age was ~92%.

Effect of In Vivo Administration of rIL-4 on Various Immune Parameters of NOD Mice. The unusually high percentage of T cells present in the spleens of NOD/Lt mice (33) was not altered by treatment with rIL-4 (Sterling Drug preparation) (Table 3). In addition, cervical and pancreatic lymph nodes

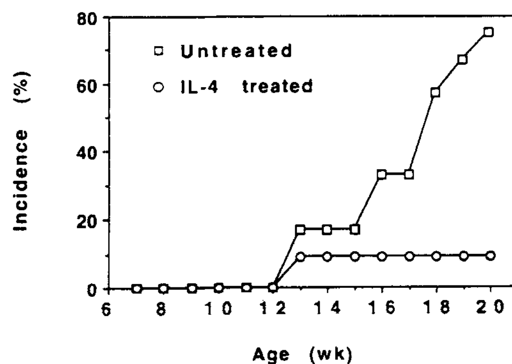

Figure 7. Decreased incidence of diabetes in female NOD/Lt mice treated in vivo with rIL-4. 12 female NOD/Lt prediabetic mice (randomized from four different litters) were injected twice weekly, from 6 to 20 wk of age, with 500 U (equivalent to 50 ng) murine rIL-4 or with vehicle (PBS + 1% serum from 6-wk-old prediabetic female NOD/Lt mice) only. Mice were screened weekly for the presence of glycosuria starting at 7 wk of age. Diabetes was diagnosed when mice were glycosuric for a consecutive 2 wk.

Table 3. *rIL-4 Treatment In Vivo Does Not Reduce the High Percentage of T Cells in NOD/Lt Spleen*

Treatment	B cells	T cells	CD4 cells*	CD8 cells*	T/CD8 ratio
	%	%	%	%	
Vehicle	31.5	52.4*	35.4	17.0	3.0
IL-4	36.3	53.8	38.1	15.7	3.4

Pooled splenic leukocytes from two normoglycemic females per group were enumerated by FACS® at the end of the 14-wk treatment period (i.e., at 20 wk of age).

* The percentage of T cells in the spleens of mouse strains at The Jackson Laboratory generally ranges from 25 to 30%. Thus, the presence of ~50% T cells in the spleens of NOD/Lt mice represents an unusually high percentage.

were enlarged in both rIL-4-treated and vehicle control-treated NOD/Lt mice (our unpublished data).

NOD/Lt macrophages secrete little IL-1 after LPS stimulation, and due to a stimulator cell defect, NOD/Lt T cells neither proliferate in response to self-MHC class II in an SMLR nor acquire immunoregulatory function (22). Since protection from diabetes in NOD/Lt mice treated with IL-2 in vivo was associated with increased IL-1 secretion from LPS-stimulated macrophages and a reversal of the SMLR defect (34), we examined whether these defects were also reversed by rIL-4 treatment. T cells from NOD/Lt female mice treated with rIL-4 from 6 to 20 wk of age failed to respond in an SMLR and did not acquire immunoregulatory function (Table 4). Treatment of NOD/Lt mice with rIL-4 in vivo also failed to increase appreciably the ability of macrophages from these mice to secrete IL-1 after LPS stimulation (Table 5). This suggests that the mechanism(s) by which the in vivo administration of rIL-4 protects prediabetic NOD mice from diabetes differs from that associated with rIL-2 therapy.

Discussion

We have demonstrated that after activation by either anti-CD3 or anti-TCR α/β mAbs, IL-2 and IL-4 secretion by thymic and peripheral (splenic) T cells from NOD mice (>7 wk old) are significantly reduced. This suggests that NOD T cell proliferative unresponsiveness is mediated by a defect in the signaling pathway that links the TCR to IL-2 and IL-4 production. However, based on our studies of Con A activation of NOD thymocytes, a defect in IL-2-mediated signal transduction may not necessarily be causal to decreased thymic T cell proliferation for several reasons. First, equivalent percentages of NOD and BALB/c Con A–activated T cell blasts express HIL-2R both in the absence and presence of exogenous rIL-2. Second, the relative affinity (K_d) of HIL-2R for IL-2 and the number of HIL-2R molecules per cell on activated NOD and BALB/c thymic T cells are similar. This result differs from the previous report that HIL-2R expression is reduced about two- to threefold in mitogen-stimulated $CD4^+$ and $CD8^+$ spleen T cells from 10-wk-old NOD/ShiKbe mice, and that this reduction might mediate the pathogenesis of type I diabetes in these mice (25). T cell responsiveness in the thymus of NOD/Del mice therefore does not appear to be due to a decrease in either the level of expression or binding affinity of HIL-2R on $CD4^+$ or $CD8^+$ T cells. Third, Con A–activated NOD thymocytes secrete normal levels of IL-2. Fourth, although we did not formally test if IL-2-induced signal transduction is normal in thymic T cells from >7-wk-old NOD mice, we found that Con A plus exogenous rIL-2 induces an increase in HIL-2R expression. Hence, it seems that the post-HIL-2R part of the IL-2-mediated signaling pathway that results in this enhanced expression is intact.

Various modalities (anti-TCR mAbs vs. Con A) of NOD thymic and splenic T cell activation stimulated different levels of IL-2 secretion. The higher level of IL-2 secretion observed for Con A–activated thymic T cells may result from the ability of Con A to bind to and signal through several T cell surface molecules in addition to the TCR (35). This may not occur to the same extent for Con A–activated NOD splenic T cells, which yielded a low level of IL-2 secretion. There may exist different requirements of activation by NOD thymic and splenic T cells. Alternatively, Con A may activate a thymic T cell subpopulation that is absent from the spleen. Nonetheless, we observed that NOD thymic T cell unresponsiveness is maintained after activation by Con A even in the presence of normal levels of IL-2 secretion, HIL-2R expression, and IL-2/HIL-2R binding. This unresponsiveness is not restored

Table 4. *Treatment of NOD/Lt Mice with rIL-4 In Vivo Does Not Restore Their Ability to Activate Immunoregulatory T Cells in an SMLR*

Strain	Treatment	SMLR response*	Suppression of MLC response‡
		cpm ± SEM	*%*
NOD	Control	285 ± 165	0
NOD	IL-4	524 ± 132	5
SWR/Bm	Control	3,320 ± 466	56§

Nylon wool–enriched T cells (SMLR responders) and irradiated splenic leukocytes (SMLR stimulators) were pooled from two mice each.
* SMLR blastogenic responses (5×10^5 T cells cultured in triplicate with 5×10^5 stimulators) represent the mean [^{3}H]thymidine uptake over the final 8 h of a 6-d culture.
‡ Viable blast cells recovered on day 6 from bulk SMLR cultures (5×10^6 T cells plus 5×10^6 stimulators) were added in triplicate at 2.5×10^5/well to an allogeneic MLC consisting of either 5×10^5 NOD or SWR T cells responding to 5×10^5 irradiated CBA/J splenocytes.
§ Significant suppression ($p < 0.05$ by Student's t test) from allogeneic MLC response in the absence of SMLR-activated T cells. The mean [^{3}H]thymidine uptake on day 4 for the unsuppressed MLC response of NOD T cells to CBA stimulators was 27,116 ± 3,329 cpm, whereas the response of SWR/Bm T cells to CBA stimulators was 69,064 ± 6,061 cpm.

Table 5. *Treatment of NOD/Lt Mice with rIL-4 In Vivo Does Not Increase the IL-1 Secretory Capacity of Macrophages*

Macrophage source	Treatment	Endogenous IL-1 secretion	LPS-stimulated IL-1 secretion
		U/ml	*U/ml*
NOD	Control	0	1.3
NOD	IL-4	0	2.0
SWR/Bm	Control	0	4.2

Peritoneal macrophages were pooled from two mice each and cultured at 10^6/ml for 24 h in the presence and absence of 10 μg/ml LPS. IL-1 content of culture supernatants was determined by comparing their ability to support C3H/HeJ thymocyte proliferation stimulated by a murine rIL-1 standard (Hoffmann-La Roche, Inc., Nutley, NJ).

to normal by addition of physiological concentrations of exogenous rIL-2, and is at best only partially corrected by addition of supraphysiological amounts of rIL-2 (7; this report). A similar finding was reported for the inability of IL-2 to promote normal proliferative responses of Con A–stimulated NOD spleen T cells from 3- and 10-wk-old NOD/ShiKbe mice (25). These data are compatible with the observation that anergy can be induced in Th1 cells as a consequence of TCR occupancy by Ag in the absence of cell division (9). This can be achieved either because the Ag is presented by an APC that cannot provide the costimulatory signal(s) necessary for IL-2 production or because the T cell cannot respond to IL-2. The latter explanation likely accounts for NOD thymic T cell proliferative unresponsiveness, since we previously showed that NOD thymic APCs are capable of providing a costimulatory signal(s) and that, in comparison with control BALB/c thymocytes, NOD thymocytes proliferate relatively poorly in response to exogenous rIL-2 (7; this report).

Why then do NOD thymic T cells not respond well to IL-2? Is this due to the requirement of another proliferative stimulus? Since a determining factor in the control of T cell proliferation is the crosstalk between the IL-2 and IL-4 signaling pathways (28–30), and T cell production of IL-2 and IL-4 can be regulated by anergy induction (10), we considered that this additional stimulus might be IL-4, an autocrine growth factor for Th2 cells (31). We found that in vitro activated NOD thymic and splenic T cells fail to produce sufficient IL-4 to support their proliferation. Relatively little if any IL-4 secretion was detectable after stimulation of NOD thymic and splenic T cells by an anti-TCR α/β or anti-CD3 mAb. In addition, the level of IL-4 secretion by NOD thymic T cell blasts stimulated by anti-TCR plus either rIL-4 or rIL-2 for 24 h and then further activated for 48 h by anti-TCR was as low as that of control NOD T blasts. In contrast, their level of IL-2 secretion was slightly enhanced compared with that of control NOD T blasts. These findings indicate that IL-4 secretion by NOD thymic T cells is compromised to a greater extent than IL-2 secretion. Unlike rIL-2, exogenous rIL-4 completely restored the in vitro proliferative capacity of NOD thymic and splenic T cells. IL-4-induced restoration of the thymic T cell response stimulated by anti-TCR was associated with a normalization of the level of IL-2 secretion by these cells. This result agrees closely with the previous observation that IL-4 has a critical role in the stimulation of IL-2 production by mouse T cells in response to accessory cell–independent stimuli (plate-bound anti-CD3) (36). Similar results were also previously reported for the activation of human T cells by PHA (37) and anti-CD2 (38). Since IL-2 also potentiates the production of IL-4 by anti-CD3-activated T cells (18, 39), partial restoration of the in vitro proliferative response of NOD thymocytes by exogenous rIL-2 may arise from the inability of IL-2 to potentiate sufficient IL-4 secretion (Fig. 5 *C*). In addition, NOD thymocytes exhibit significantly reduced IL-4 secretion (Fig. 2 *B*) and diminished proliferative responsiveness after stimulation with anti-CD3 even in the presence of normal amounts of IL-2 (Fig. 4). For complete restoration of the NOD thymocyte proliferative response, IL-4 therefore needs to be present in addition to either exogenous rIL-2 or Con A–induced endogenous IL-2. Thus, decreased IL-4 secretion by activated NOD thymic and peripheral T cells appears to be a primary defect that elicits the proliferative unresponsiveness of these cells.

The in vivo administration of either rIL-2 (34) or two different preparations of rIL-4 (this report) protects prediabetic NOD mice against diabetes. Protection against diabetes in NOD mice by in vivo treatment with rIL-2 is associated with a reversal of the decreased splenic T cell SMLR of these mice and an increase in the LPS-induced IL-1 secretion by peritoneal exudate macrophages (34). In contrast, we report here that protection from diabetes by in vivo rIL-4 therapy is not associated with similar changes in the SMLR and/or IL-1 secretion. Thus, IL-2 and IL-4 appear to protect NOD mice from diabetes by different mechanisms. Unlike rIL-2, rIL-1, rIFN-γ, and rTNF-α (7), rIL-4 completely restores the in vitro NOD thymic T cell proliferative response and may achieve close to 100% protection from diabetes in vivo by a similar mechanism(s).

Reconstitution of lymphopenic prediabetic BB rats with the IL-4-producing CD4$^+$CD45RC^{low} subset of Th cells but not with the IL-2-producing CD4$^+$CD45RC^{high} Th subset protects these rats against autoimmune diabetes (40). In addition, diabetes and insulitis may be completely prevented by injection of the IL-2- and IL–4-producing subset of CD4$^+$CD45RC^{low}TCR α/β^+RT6$^+$ thoracic duct T cells from healthy donors into a normal nonautoimmune rat strain that may be induced to become lymphopenic and diabetic by adult thymectomy and sublethal γ irradiation (41). Altered ratios of CD4$^+$CD45RA to CD4$^+$CD45RO of PBL T cells together with decreased proliferative responses in the AMLR occur in human prediabetic patients, and may be diagnostic indicators of rapid progression to overt disease (42, 43). Immunohistochemical analyses performed using fluorescent mAbs indicate that IFN-γ predominates in situ in the relative absence of IL-4 at the time of diabetes onset in NOD pancreatic islets previously transplanted beneath the kidney capsule of syngeneic female NOD mice at 4.5 mo of age. In contrast, IL-4 predominates and IFN-γ is present in significantly lower amounts in NOD islets transplanted beneath the kidney capsule of control age-matched female NOD mice that were protected from diabetes by the previous administration (at 1 mo of age) of CFA (44). The in vivo administration of anti-IFN-γ mAbs to NOD mice also prevents the onset of diabetes (45). Moreover, during the induction of tolerance, immunization with antigen and adjuvant induces an expansion of IL-4-producing Th2 cells (46). IL-4 inhibits the secretion of IFN-γ by tolerant Th1 cells that retain the ability to secrete IFN-γ. In addition, Th2 cells that are expanded by IL-4 may secrete other cytokines, such as IL-10, which has also been shown to regulate Th1-dependent immune responses (46). Taken together, these observations suggest that T cell proliferative unresponsiveness in vitro and onset of diabetes in vivo may arise by the anergy and/or deletion of CD4$^+$ IL-4-producing Th2 cells in NOD mice, thereby enhancing the expansion of IFN-γ producing potentially diabetogenic Th1 clones.

Mature CD4$^+$CD8$^-$ thymocytes are the predominant

IL-4 producers during primary immune responses (47). These IL-4-producing thymocytes are exported to the periphery and provide the IL-4 necessary for the generation of peripheral IL-4-secreting Th2 cells. Hence, the choice of an immature thymocyte to differentiate into a Th1 or Th2 mature T cell is likely dependent on the relative abundancy of IL-4 (47). In support of this notion is the observation that low doses of IL-4 are insufficient to promote IL-2-supported growth of thymic T cell precursors (27). A failure of NOD thymocytes to produce sufficient IL-4 for the differentiation of and export from the thymus of certain regulatory $CD4^+CD8^-$ Th2 clones may potentiate their unresponsiveness and eventual deletion. In this case, the balance between self-tolerance and autoimmunity would be disrupted and could result in type I diabetes.

Thus, IL-4 therapy may prevent the onset of diabetes in NOD mice by promoting the differentiation and exit from the thymus of "protective" regulatory $CD4^+CD8^-$ T cells. This protection afforded by IL-4 may also be mediated by its ability to perturb the development of $CD4^-CD8^+$ thymocytes, which was shown to occur in IL-4 transgenic mice in which the intrathymic expression of IL-4 was constitutively increased (48). Only peripheral $CD4^+$ T cells were found in significant numbers in these transgenic mice, while $CD4^-CD8^+$ thymocytes bearing high levels of TCR α/β were present in increased numbers, apparently because of their failure to emigrate to the periphery. Since these results indicate that IL-4 can regulate thymocyte maturation, they may explain in part how IL-4 can protect from diabetes by exerting reciprocal effects on the maturation of $CD4^+CD8^-$ (positive regulation) and $CD4^-CD8^+$ (negative regulation) thymic T cells.

In conclusion, our findings further document the therapeutic value of immunostimulation protocols for the prevention of autoimmune type I diabetes. In addition to IL-2 (34) and TNF-α (49, 50), we now report that IL-4 may also be used efficaciously for this purpose. Since IL-4 is required for the production of IL-2, this might explain in part why IL-2 deficiencies have been noted in both NOD (22, 51) and other strains of autoimmune mice (52), as well as in human type I diabetic patients (53). Further experimentation is required to test the possibility that prevention of type I diabetes in NOD mice by IL-4 is mediated by correction of an IL-2 deficiency.

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