

Effect of Tumor Necrosis Factor α on Insulin-dependent Diabetes Mellitus in NOD Mice. I. The Early Development of Autoimmunity and the Diabetogenic Process

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Summary

Tumor necrosis factor (TNF) α is a cytokine that has potent immune regulatory functions. To assess the potential role of this cytokine in the early development of autoimmunity, we investigated the effect of TNF on the development of insulin-dependent diabetes mellitus (IDDM) in nonobese diabetic (NOD) mice, a spontaneous murine model for autoimmune, insulin-dependent type I diabetes. Treatment of newborn female NOD mice with TNF every other day for 3 wk, led to an earlier onset of disease (10 versus 15 wk of age in control mice) and 100% incidence before 20 wk of age (compared to 45% at 20 wk of age in control phosphate-buffered saline treated female mice). In contrast, administration of an anti-TNF monoclonal antibody, TN3.19.12, resulted in complete prevention of IDDM. In vitro proliferation assays demonstrated that mice treated with TNF developed an increased T cell response to a panel of β cell autoantigens, whereas anti-TNF treatment resulted in unresponsiveness to the autoantigens. In addition, autoantibody responses to the panel of β cell antigens paralleled the T cell responses. The effects mediated by TNF appear to be highly age dependent. Treatment of animals either from birth or from 2 wk of age had a similar effect. However, if treatment was initiated at 4 wk of age, TNF delayed disease onset. These data suggest that TNF has a critical role in the early development of autoimmunity towards β -islet cells.

TNF has been the focus of intensive study because of its involvement in many physiological and pathological processes. It is now clear that TNF is produced predominantly by activated macrophages and occasionally by other cell types such as CD4⁺ T cells, predominantly of the Th1 subset (1–3). Furthermore, the broad range of expression of its receptors suggests that TNF has a number of biological activities in a wide variety of cells and tissues (4). Importantly, a role for TNF has been found in virtually all inflammatory diseases or lesions and it is therefore believed that TNF is a major mediator of inflammation.

Insulin-dependent diabetes mellitus (IDDM)¹ is a T cell-mediated chronic autoimmune disease that is character-

ized by lymphocytic infiltration of the pancreatic islets of Langerhans and by the selective destruction of insulin-producing β cells in the islets. Since TNF is a proinflammatory cytokine and its gene is localized within the MHC, which has been shown to have a strong genetic linkage to several autoimmune disorders including IDDM, TNF has been considered to be a possible candidate cytokine mediating the pathogenic destruction of β -islet cells. Earlier studies have provided two sets of conflicting evidence regarding the function(s) of TNF in IDDM. The first set of evidence suggests that TNF has an active role in the pathogenesis of IDDM. This is based on the finding that TNF mRNA is produced by CD4⁺ T cells in situ in inflamed islets of nonobese diabetic (NOD) mice (5) and that in the presence of other inflammatory cytokines such as IL-1 or IFN- γ , TNF in vitro mediates cytotoxicity to pancreatic islets (6–9). Recently, it has been shown that transgenic mice expressing TNF in the pancreatic β cells under the control of the rat-insulin promoter (RIP-TNF) exhibit massive lymphocytic infiltration of the islets (but were free of overt diabetes) indicating that TNF is an important

¹ Abbreviations used in this paper: CPH, carboxypeptidase-H; GAD, glutamic acid decarboxylase; HEL, hen egg lysozyme; HSP60, murine 60-kD heat-shock protein; IDDM, insulin-dependent diabetes mellitus; MNC, mononuclear cell; NOD, non-obese diabetic; TNF, tumor necrosis factor α .

cytokine for lymphocyte invasion and for causing local inflammation (10, 11). In contrast, the second set of evidence shows that strains of autoimmune-prone mice, such as NOD and New Zealand black/white (NZB/NZW), appear in some *in vitro* assays to have a reduced ability to produce TNF. It has been shown that peritoneal macrophages from NOD mice and bio-breeding (BB) rats, both of which develop spontaneous IDDM, and from NZB/NZW mice, a murine model of autoimmune lupus, produce less TNF upon *in vitro* stimulation with LPS than other nonautoimmune-prone inbred strains (12–14). Thus, it is believed that this reduced production of TNF may play a role in the diabetogenic process (12). Further studies (12–16) have demonstrated that treatment of adult animals with subtoxic doses of recombinant TNF can lead to prevention of spontaneous IDDM or to prolonged survival of mice with murine lupus.

Several recent studies have provided some interesting insights into the function of TNF in the development of the immune system. Giroir et al. (17) have shown that in transgenic mice which carry the reporter gene chloramphenicol acetyl transferase (CAT) coupled to the TNF promoter, expression of the CAT gene is detected very early during thymic development and for the first 3–4 wk of neonatal life. Furthermore, the thymus seems to be the only organ constitutively producing TNF. Using an anti-TNF polyclonal antiserum, De Kossodo et al. (18) have demonstrated that blocking TNF function during early development results in thymic and splenic atrophy and lymphopenia. Together, these data strongly suggest that TNF is an important cytokine for thymic development and it may be at this level that TNF influences the development of autoimmunity.

In an attempt to clarify these controversial findings and to gain a better understanding of the role of TNF in the development of autoimmunity and in the regulation of autoimmune responses to target organs, we have studied the effect of TNF and anti-TNF treatment in newborn and young NOD mice on the pathogenesis of IDDM (19).

Materials and Methods

Mice. NOD/McD (K^d, I-A^{g7}, D^b) mice were initially obtained from Dr. E. Leiter at The Jackson Laboratory (Bar Harbor, ME) and were subsequently bred and raised at the Stanford University Animal Facility under barrier isolation conditions. The spontaneous incidence of diabetes in the colony is currently 70–80% in females and 10–20% in males by 30 wk of age.

Cytokines and mAbs. Recombinant murine TNF and IFN- γ were kindly provided by Genentech Inc. (South San Francisco, CA). The activity and concentration of TNF was 1.2×10^7 U/ml and 0.98 mg/ml; IFN- γ was 5.2×10^6 U/ml and 1 mg/ml. TN3.19.12 (hamster IgG1), mAb specific for murine TNF, was prepared as described (20). L2.3D9 is an Ig-isotype-matched control mAb (hamster IgG1) for TN3.19.12. Ascites containing L2.3D9 was provided by Dr. R. Schreiber and antibody was purified by a protein-A chromatography. DB-1, an IgG1 mouse anti-rat IFN- γ mAb which neutralizes the bioactivity of both rat and mouse species, was prepared and purified using DEAE chromatography from ascites produced by the DB-1 hybridoma provided by Dr. P. H. M. van der Meide (The Primate Center, TNO, Rijswijk, the Nether-

lands) (21). An Ig-isotype-matched control mAb 4E8/E1 (mouse anti-human idiotype mAb) was purified utilizing a protein-A column from ascites provided by Dr. R. Levy (Stanford University). The bioactivity of these cytokines and mAbs were confirmed by *in vitro* assays before application *in vivo*.

In Vivo Treatment with Cytokines and Anticytokine mAbs. NOD female mice from at least two separate litters were randomized and regrouped before treatment. Body weight was measured every other day for 3 wk. Four different treatment regimens were employed: (a) Newborn females were treated intraperitoneally every other day with 20 μ l PBS only, 1 μ g murine TNF (10^4 U) in 20 μ l PBS, 4.8 μ g murine IFN- γ (2.5×10^4 U) in 20 μ l PBS, 20 μ g/g bodyweight of TN3.19.12 or L2.3D9 or 100 μ g/g bodyweight of DB-1 or 4E8/E1 from the first day after birth for 24 d. (b) 2-wk-old female NOD mice received intraperitoneal injections of PBS, 1 μ g TNF or 20 μ g/g bodyweight TN3.19.12 every other day for 3 wk. (c) 4-wk-old NOD females received intraperitoneal injections of PBS, 1 μ g TNF, or a constant dose (100 μ g/injection) of TN3.19.12 every other day for 3 wk. (d) 10-wk-old NOD females received intraperitoneal injections of PBS or 3 μ g TNF for 4 mo as described previously (12).

Histology and Hematology. Tissues, including salivary gland, thymus, spleen, adrenal gland, and stomach, were removed and embedded in OCT compound (Tissue-Tek, Miles, Elkhart, IN) and frozen in dry ice. Cryostat sections were cut and sections were stained with hematoxylin and eosin. Mononuclear cell counts were carried out on Giemsa-stained blood smears.

Assessment of Insulinitis and Diabetes. Insulinitis was assessed by histology. Pancreata were prepared for histology by either (a) immediate freezing of tissue in OCT compound over dry ice or in liquid nitrogen or (b) fixing in neutral buffered formalin and then embedding in paraffin. The fixed blocks were sectioned and stained with hematoxylin and eosin. The slides were viewed by light microscopy. The severity of insulinitis was assessed as peri-insulinitis (islets surrounded by few lymphocytes) and intra-islet insulinitis (lymphocytic infiltration into the interior of islets).

To assess diabetes, mice were monitored weekly for glycosuria using Chemstrip (Boehringer Mannheim, Indianapolis, IN). Diabetes was diagnosed when mice were glycosuric for at least three consecutive weeks. Diabetes incidence is expressed as a percentage (mice with diabetes divided by total mice treated in the group).

Cloning and Preparation of Murine β -islet Cell Antigens. cDNAs encoding the 65- and 67-kD isoform of GAD, carboxypeptidase-H (CPH), and peripherin were cloned from murine brain, murine pituitary, and murine insulinoma libraries, respectively (22). The cDNA of murine 60-kD heat-shock protein (HSP60) was kindly provided by Drs. S. Ikawa and R. Weinberg (Massachusetts Institute of Technology, Cambridge, MA). The proteins were prepared as previously described (22). The cDNAs were engineered to encode six histidine residues at the COOH terminus of each protein. Recombinant proteins were expressed in a baculovirus expression system for GAD67, GAD65, CPH, and peripherin, and affinity purified using a Ni²⁺-conjugated resin (Invitrogen, San Diego, CA). HSP60 was produced in an *Escherichia coli* expression system and similarly purified.

Immunization of Mice with Hen Egg Lysozyme or OVA. Mice were injected subcutaneously in the hind footpads and at the base of the tail with 100 μ g of hen egg lysozyme (HEL) or OVA emulsified with CFA.

Lymphocyte Proliferation Assay. Spleen mononuclear cells (MNC) prepared from individual mice were incubated in triplicate at 5×10^6 /ml for 72 h in 0.1 ml culture medium in the presence of 20 μ g/ml of each of the panel of β cell antigens. [³H]thymidine (1

$\mu\text{Ci}/\text{well}$) (Du Pont, Boston, MA) was added 18 h before termination of culture. Cultures were harvested using a 24-well cell harvester (Cambridge Technology, Inc., Watertown, MA). The extent of cell proliferation was determined by assay of the amount of [^3H]thymidine incorporation using a beta counter (Beckman Instruments, Inc., Irvine, CA) and expressed as stimulation index (cpm in the presence of antigen divided by cpm in the presence of medium only). The culture medium contained RPMI-1640 supplemented with 1% Nutridoma-SP (Boehringer Mannheim), 0.05 mM 2-ME (Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin.

ELISA for Detection of Antigen-specific Autoantibody. Mice were bled and sera of individual mice were collected. 96-well plates (Dynatech, Chantilly, VA) were coated with 10 $\mu\text{g}/\text{ml}$ of the recombinant β cell antigens in 0.1 M NaHCO_3 , pH 8.5, overnight at 4°C. Wells were then blocked with 0.1% gelatin in Tris-buffered saline, pH 8.0, for 4 h at room temperature. Sera were added at a dilution of 1:100 in blocking buffer. Bound antibody was detected with biotin-conjugated goat anti-mouse Ig and avidin-conjugated alkaline-phosphatase (Sigma Chemical Co.). Reactions were developed with nitrophenylphosphate for 30 min and absorbance at 410 nm was read with a plate reader (model MR700; Dynatech). The standard error of the mean for each set of duplicates was <10%.

Adoptive Transfer of Diabetes and Passive Transfer of Protection by Spleen MNC. The acute diabetes-transfer experiments were carried out according to Wicker et al. (23). 10^7 spleen MNC from diabetic mice were injected intravenously into young (about 9–10-wk-old) male prediabetic NOD recipients. These recipient mice were irradiated with gamma radiation (650 rad; 1 rad = 0.01 Gy) from an x-ray source before cell injection. To assess whether mAb treatment generated regulatory cells that mediate the protective effect, a passive transfer of protection by spleen MNC was performed as described by Boitard et al. (24). Briefly, spleen MNC cells were prepared from TN3.19.12-treated and diabetes-protected NOD mice at 32 wk of age. 2×10^7 MNC from these mice were injected intravenously into irradiated recipients 24 h before transfer of 10^7 diabetogenic MNC.

Statistical Method. Significance between data obtained from various groups was analyzed by using the χ^2 test. Probability values <0.05 were considered significant.

Results

Neonatal Administration of TNF Accelerates IDDM Onset and Enhances Disease Frequency, while Inhibiting TNF Activity with Anti-TNF mAb Prevents IDDM. To study the early effects of TNF on the diabetogenic process, NOD female mice were injected intraperitoneally with PBS, 1 μg recombinant murine TNF, 20 $\mu\text{g}/\text{g}$ body weight of anti-TNF mAb TN3.19.12, or 20 $\mu\text{g}/\text{g}$ body weight of an Ig-isotype matched hamster mAb L2.3D9 every other day from the first day after birth to 24 d of age. Fig. 1 *A* shows that mice treated with TNF began developing overt diabetes ~ 4 wk earlier (at 10 wk of age) than control mice treated with PBS, and that all of the TNF-treated mice (27 out of 27) became diabetic before 20 wk of age. In contrast, none of the TN3.19.12-treated mice developed diabetes even after 30 wk of age. The protective effect of anti-TNF treatment is specific since the control mAb L2.3D9 had no effect on the disease process (Fig. 1 *A*). Furthermore, treatment with IFN- γ , the anti-IFN- γ mAb DB-1, or a control mAb for DB-1, 4E8/E1, failed to influence the

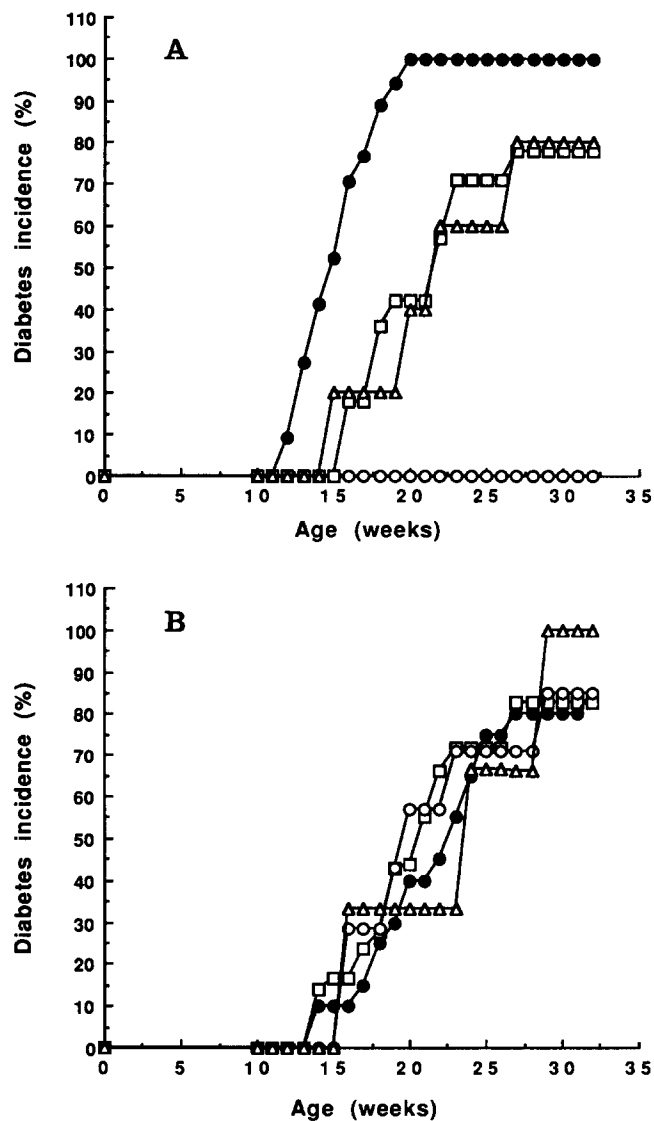


Figure 1. Administration of TNF to neonatal female mice accelerates IDDM onset and enhances disease incidence whereas anti-TNF prevents IDDM. (*A*) Newborn female NOD mice were injected intraperitoneally every other day from birth for 3 wk with PBS (\square , $n = 22$), 1 μg TNF (\bullet , $n = 27$), 20 $\mu\text{g}/\text{g}$ body weight of TN3.19.12 (\circ , $n = 19$), or L2.3D9 (Δ , $n = 8$). (*B*) Newborn female NOD mice were injected intraperitoneally every other day from birth for 3 wk with PBS (\square , $n = 18$), 4.8 μg IFN- γ (\bullet , $n = 16$) or 100 $\mu\text{g}/\text{g}$ body weight of DB-1 (\circ , $n = 7$), or 4E8/E1 (Δ , $n = 5$). Diabetes incidence was monitored weekly over the following 30 wk.

course of disease (Fig. 1 *B*). In addition, the nonautoimmune-prone BALB/c strain of mice, when treated neonatally with TNF in the same regimen, exhibited no signs of insulinitis or diabetes (data not shown).

Since it has been shown that TNF can mediate cachexia and that neutralization of TNF activity in vivo with a polyclonal anti-TNF rabbit serum can lead to retardation of mouse development, thymic and splenic atrophy, and lymphopenia (1, 18), we carefully monitored mouse development by measuring the body weight and the mortality of the mice during

and after TNF or TN3.19.12 treatment. As shown in Fig. 2, neonatal treatment with either TNF (2 μg /injection) or TN3.19.12 (20 $\mu\text{g}/\text{g}$ body weight) for 3 wk had no significant effect on mouse development and growth, or mortality.

To determine whether anti-TNF treatment with TN 3.19.12 induces lymphoid atrophy and lymphopenia, several mice were killed after 3 wk of treatment with TN3.19.12, and the weight of the thymus and the spleen, and lymphocyte numbers in the blood were determined. No significant difference in thymic and splenic weight or size (data not shown) was observed between PBS- (thymus: 62.8 ± 5.1 g; spleen: 53.6 ± 2.8 g, $n = 9$) and TN3.19.12-treated (thymus: 57.2 ± 3.8 g; spleen: 61.4 ± 6.2 g, $n = 12$) groups. No reduction of lymphocyte numbers in the blood (lymphopenia) was found in TN3.19.12-treated mice, both in terms of relative ($40 \pm 16\%$ vs. $37.5 \pm 12\%$ in PBS controls, $n = 5$) and absolute (29.1 ± 4.6 vs. $28 \pm 5.9 \times 10^3$ lymphocytes/ mm^3 in PBS controls, $n = 5$) numbers. Flow cytometry analysis of thymocytes and splenic MNC demonstrated that the frequency of $\text{CD4}^+/\text{CD8}^+$ vs. $\text{CD4}^-/\text{CD8}^-$, $\text{CD4}^+/\text{CD8}^-$ vs. $\text{CD4}^-/\text{CD8}^+$ were comparable in TNF-, TN3.19.12-, and PBS-treated mice (data not shown).

These results indicate that the administration of TNF or this particular anti-TNF mAb in neonates has no significant cachectic or lethal effects, or any dramatic influence on the development of lymphoid tissues in NOD mice. In addition, histological analysis of mice treated either with TNF or TN3.19.12 revealed no detectable morphological changes or pathological alterations in thymus, spleen, adrenal gland, and stomach (data not shown).

Age-dependent Effect of TNF on IDDM. To further investigate the apparent age-dependent effect of TNF treatment

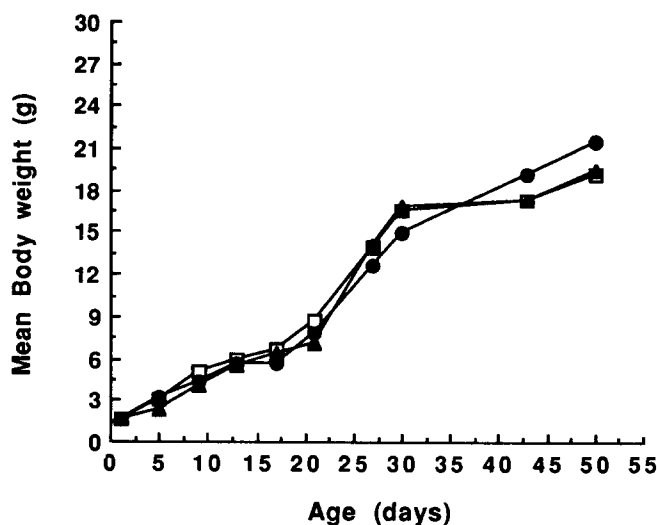


Figure 2. Treatment of neonatal mice with TNF or TN3.19.12 does not effect mouse development. Female NOD mice were injected intraperitoneally every other day from birth for 3 wk with PBS (\square , $n = 12$), 2 μg TNF (\bullet , $n = 10$), or 20 $\mu\text{g}/\text{g}$ body weight of TN3.19.12 (\blacktriangle , $n = 7$). Mouse bodyweight was monitored every other day over the first 3 wk and biweekly thereafter.

on IDDM, female NOD mice were treated with TNF or TN3.19.12 beginning at 2 or 4 wk of age. Treatment initiated at 2 wk of age resulted in enhanced disease incidence with TNF (100%, nine out of nine mice at 27 wk of age) and prevention of disease with TN3.19.12 (0%, zero out of nine mice at 34 wk of age) (Fig. 3 A). In contrast, when TNF treatment was initiated at 4 wk of age, a delay in the onset of IDDM was observed. Administering a constant dose (100 μg) of TN3.19.12 only partly prevented disease (about 50%, four out of nine mice) (Fig. 3 B). However, when animals were treated with 3 μg of TNF for 4 mo beginning at the age of 10 wk as done previously (12), the disease was reduced from 80 to 20% showing that TNF mediates distinct effects on IDDM (data not shown) that are clearly age dependent.

TNF Potentiates the Development of Autoreactive T Lymphocytes. To elucidate the mechanisms by which neonatal administration of TNF induces IDDM and anti-TNF treatment prevents disease onset, we examined autoimmune responses to a panel of murine pancreatic β cell antigens including GAD67, GAD65, peripherin, CPH, and HSP60. To measure T cell responses, mice treated with PBS, TNF, or TN3.19.12 from birth for 3 wk, were killed at 10 wk of age, and splenic MNC were cultured in the presence of the β cell antigens. Fig. 4 shows that TNF-treated mice exhibited an enhancement of T cell responses to the β cell antigens relative to the PBS-treated animals. In contrast, T cells from mice treated with TN3.19.12 did not respond to these antigens (Fig. 4), and this unresponsiveness induced by TN3.19.12 persisted for more than 6 mo (data not shown). Antibody responses to the panel of antigens were also measured using an antigen-specific ELISA. As demonstrated in Fig. 5, TNF treatment led to increased antibody responses, whereas mice treated with anti-TNF mAb exhibited absent or very significantly decreased responses to the panel of antigens. These results indicate that TNF is capable of promoting a diabetogenic autoimmune response. Moreover, the fact that anti- β cell responses can be inhibited by blocking TNF activity further suggests that TNF is involved in the sensitization and/or triggering of diabetogenic lymphocytes.

Anti-TNF Treatment Downregulates Autoimmune Responses but Does Not Induce Immune Suppression. The protective effect of TN3.19.12 may be mediated through induction of a state of immune unresponsiveness, possibly through the generation of regulatory T cells. To rule out this possibility, we measured the immune responses in TN3.19.12-treated animals to two foreign antigens, HEL and OVA. 1 wk after immunization with HEL or OVA, lymphocytes from lymph nodes were assayed in vitro for their reactivities to HEL or OVA. Fig. 6 shows that mice treated with TN3.19.12 had enhanced responses to HEL and OVA relative to the PBS-treated mice. These findings suggest that the neonatal neutralization of TNF activity in vivo by mAb TN3.19.12 does not deplete immune competent cells or suppress their functions. If anything, the immune response to foreign antigens appears to be enhanced.

It has been demonstrated that treatment of NOD mice with mAbs specific for MHC class II antigen can induce suppressor cells which mediate the prevention of IDDM (24).

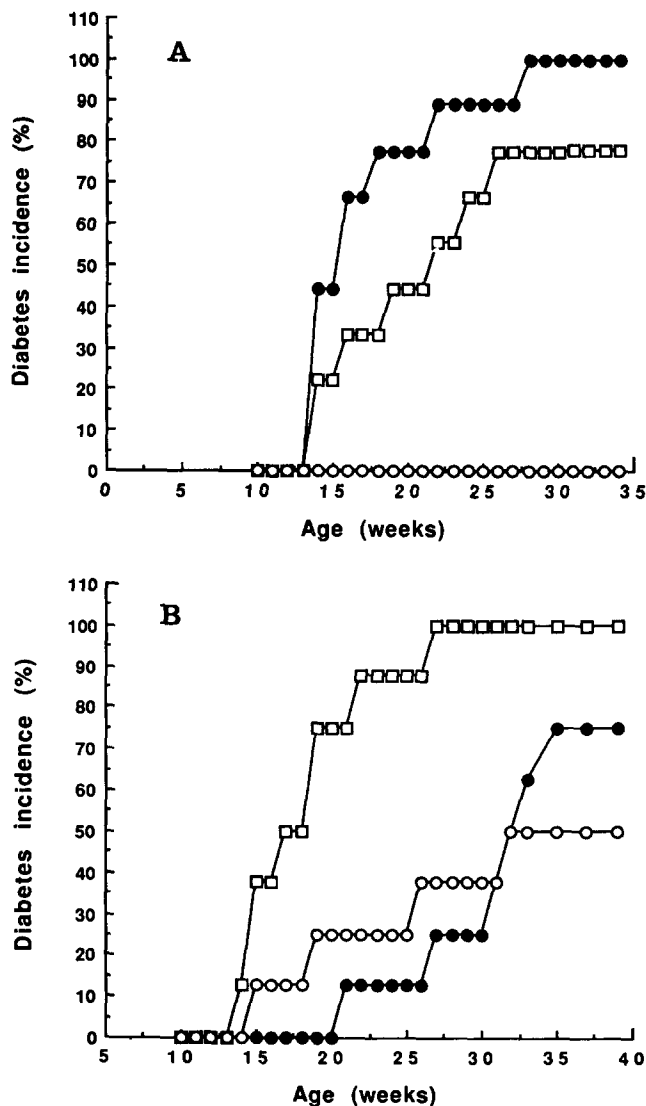


Figure 3. The effect mediated by TNF on IDDM is age dependent. (A) Groups of nine female NOD mice at 2 wk of age were injected intraperitoneally with PBS (□), 1 μ g TNF (●) or 20 μ g/g body weight of TN3.19.12 (○) every other day for 3 wk. (B) Groups of eight female NOD mice at 4 wk of age were injected intraperitoneally with PBS (□), 1 μ g TNF (●), or 100 μ g TN3.19.12 (○) every other day for 3 wk. Diabetes incidence was monitored weekly over the following 30 wk.

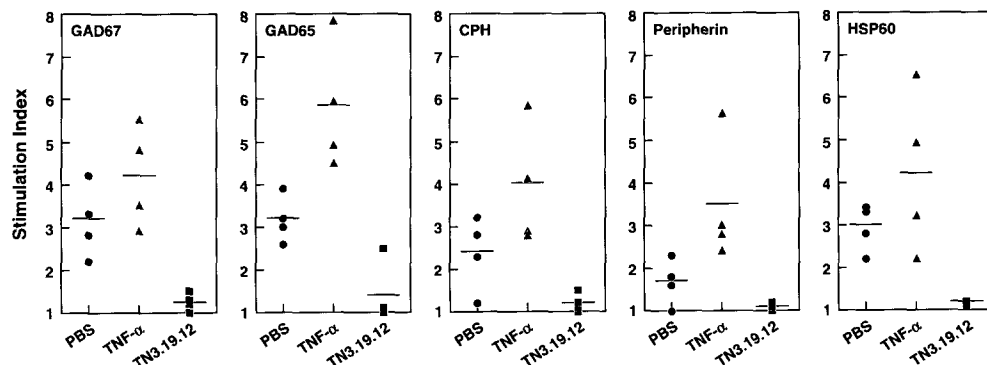


Figure 4. Spontaneous T cell responses to β cell autoantigens in TNF- and anti-TNF-treated mice. Splenic MNC from mice treated with PBS, TNF, or TN3.19.12 as described in the legend to Fig. 1 were examined for their ability to respond to: GAD67, GAD65, CPH, peripherin, and HSP60 in vitro. The range of cpm obtained in medium controls was 286–1,692. The proliferative response is presented as a stimulation index.

To determine whether a similar form of prevention was being induced by TN3.19.12, MNC from TN3.19.12-treated mice were adoptively transferred into irradiated young male NOD recipients before the transfer of spleen cells from diabetic donors. As shown in Table 1, MNC from TN3.19.12-treated mice failed to suppress the pathogenic effect of the transferred diabetogenic spleen MNC, suggesting that the observed protective effect in the anti-TNF-treated mice is not due to the induction of regulatory or suppressor cells.

Blocking TNF Activity Inhibits Insulinitis. To gain further insight into the mechanism of protection induced by anti-TNF treatment, we examined histological sections of pancreata and salivary glands of TN3.19.12-treated mice (at 32 wk of age). As shown in Table 2, only 27% (3 out of 11) of the TN3.19.12-treated mice developed a low or moderate degree of insulinitis compared to PBS-treated mice (100% insulinitis, and 56% with severe insulinitis). These data suggest that removal of TNF in vivo can inhibit lymphocyte migration into the islets. Thus, the TN3.19.12-induced protection of diabetes may result from its inhibitory effect on lymphocytic trafficking into the islets. It is interesting to note that the inflammation found in the salivary glands (sialadenitis) which normally develops after 6 mo of age in NOD mice, remained unaffected by neonatal anti-TNF treatment (Table 2). This may be due to the differences in the expression pattern of different cell adhesion molecules found in the inflamed islets (mainly expressing mucosal addressin cell adhesion molecule 1 [MAdCAM-1] and little peripheral vascular addressin, [PNAd]) and salivary gland (mainly expressing PNAd but no MAdCAM-1) (25, and Yang, X.-D., S. Michie, R. Tisch, N. Karin, L. Steinman, and H. O. McDevitt, manuscript submitted for publication).

Discussion

In this study, we have demonstrated that neonatal treatment of NOD mice with TNF results in an earlier onset and an increased incidence of IDDM, whereas blocking TNF activity by administering an anti-TNF mAb prevents these mice from developing IDDM. It is intriguing that the TNF-mediated effects on IDDM appear to be age dependent. These

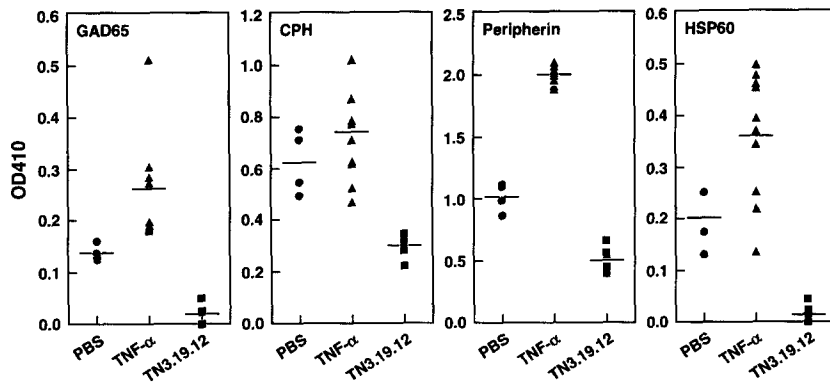


Figure 5. Antibody response to β cell autoantigens in TNF- and anti-TNF-treated mice. Mice were treated with PBS, TNF, or TN3.19.12 as described in Fig. 1. Individual mice at 20 wk of age were assessed for antibody responses to: GAD65, CPH, peripherin, and HSP60 using an ELISA assay. Antibody detection is represented as units of optical density at 410 nm.

data strongly suggest that the first 3 wk after birth are a critical period for the initiation of the diabetogenic process in NOD mice and that interference with immune function during this period can significantly alter disease outcome. This notion is further supported by additional lines of evidence. First, NOD mice thymectomized at birth fail to develop IDDM, whereas disease incidence is enhanced if animals are thymectomized after 3 wk of age (26–28). Second, our recent study (22) has shown that autoimmune responses to the panel of β cell antigens in NOD mice appear temporally and sequentially. No response to the panel of antigens can be detected before 3 wk of age. However, responses to GAD65 can be initially detected at 4 wk of age, suggesting that during the first 3 wk after birth, autoreactive lymphocytes become sensitized and activated in vivo. Third, in NOD mice, no

lymphocytic infiltration in the islets can be found before 3 wk of age and is first detected to a minimal degree in mice at ~ 4 wk of age (29). In addition, in a transgenic NOD mouse expressing a TCR specific for an unknown β -islet cell antigen, massive lymphocytic invasion of the islets begins abruptly at 3 wk of age (30). Taken together, these results suggest that up to 3 wk of age, events occur that are critical in the diabetogenic process and that some of these events may be associated with the development of T cells in the thymus. Thus, the thymus may be one of the primary target organs of TNF action in the newborn mouse.

Emerging evidence implies that TNF is indeed a critical factor for thymic development and may also be required for the development of other lymphoid tissues. Early studies have demonstrated that TNF may be involved in thymocyte activation and proliferation (31, 32), and that together with IFN- γ , TNF promotes the interaction between thymic epithelial cells and thymocytes (33), suggesting that TNF may play a role in thymocyte growth and intrathymic cell differentiation. Consistent with this assumption, Giroir et al. (17) have been able to demonstrate that TNF is constitutively produced in the thymus during development. It is interesting to note that it has been shown that blocking TNF (and possibly lymphotoxin- α) activity by administering a polyclonal anti-TNF antibody in utero or within the first 3 wk of life induces thymic and splenic atrophy, lymphopenia, and growth retardation in an outbred strain of NMRI mice (18). Similarly, transgenic mice expressing high levels of soluble TNF receptor p55, which neutralizes TNF and lymphotoxin- α activity, have defects comparable to those seen in anti-TNF-treated mice (Garcia, I., and P. Vassalli, unpublished data). Furthermore, it has been found that lymphotoxin- α mutant mice have a striking absence of peripheral lymph nodes and Peyer's patches, and have disordered splenic morphology (34). A study on TNF receptor p55 mutant mice has revealed that in the absence of p55, lymphoid tissues can develop, although these mice have an impaired ability to clear intracellular *L. monocytogenes* infection (35). Therefore, if TNF is involved in the fetal development of lymphoid tissues, its action should be mediated by the TNF receptor p75. Together, these data clearly suggest that TNF and lymphotoxin- α are among the essential cytokines for the development of lymphoid tissues.

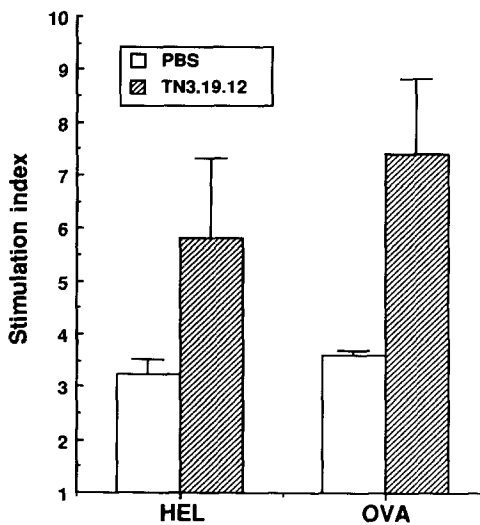


Figure 6. Immune responses to the antigens HEL or OVA in anti-TNF-treated mice. Groups of five to seven mice were injected intraperitoneally every other day from birth for 3 wk with PBS or 20 μ g/g body weight of TN3.19.12. Mice were immunized with 100 μ g s.c. of either HEL or OVA in CFA. 1 wk later, lymphocytes from draining lymph nodes were harvested and tested for their ability to respond to HEL (20 μ g/ml) or OVA (100 μ g/ml) in an in vitro proliferation assay. Specific responses are presented as a stimulation index.

Table 1. Treatment with TN3.19.12 Failed to Generate Suppressor Cells

Treatment of donors	Incidence of transfer diabetes			
	Day 10	Day 15	Day 25	Day 35
TN3.19.12	0/10 (0%)	2/10 (20%)	8/10 (80%)	10/10 (100%)
PBS	0/10 (0%)	1/10 (10%)	8/10 (80%)	10/10 (100%)
None	0/10 (0%)	3/10 (30%)	10/10 (100%)	10/10 (100%)

No protection from diabetes was passively transferred by MNC from TN3.19.12-treated mice. Groups of 10 male NOD mice at the age of 9–10 wk were irradiated and subsequently received 2×10^7 i.v. spleen MNC from PBS or TN3.19.12-treated mice at the age of 32 wk, or no cells as control. The following day, 10^7 diabetogenic spleen MNC were transferred intravenously into all the recipients. Diabetes incidence was monitored every other day for 5 wk.

Table 2. Inhibition of Insulinitis by Anti-TNF Treatment

Treatment	Insulinitis incidence	Number of islets with infiltration			Sialadenitis incidence
		No infiltration	Peri-insulinitis	Intra-islet-insulinitis	
PBS	4/4 (100%)	33/111 (29.7%)	17/111 (15.3%)	61/111 (56.0%)	4/4 (100%)
TN3.19.12*	3/11 (27.3%)	394/419 (94.0%)	19/419 (4.6%)	6/419 (1.4%)	7/7 (100%)

Female NOD mice were injected intraperitoneally every other day from birth for 3 wk with PBS or 20 $\mu\text{g/g}$ body weight of TN3.19.12. At 32 wk of age, mice were killed and pancreata and salivary glands were assessed histologically for insulinitis or sialadenitis.

* A highly significant difference of insulinitis severity between mice treated with PBS and TN3.19.12: $\chi^2 = 258.9$, $p < 0.0001$.

The reason that we failed to observe an obvious developmental defect of lymphoid tissues in TN3.19.12-treated animals in the present study may be due to the fact that TN3.19.12 predominantly neutralizes TNF although TN3.19.12 recognizes both TNF and lymphotoxin- α (20). This suggests that these two cytokines may have distinct functions in thymocyte development.

The fact that blocking TNF activity before 3 wk of age with the neutralizing antibody TN3.19.12 prevents insulinitis and diabetes, clearly suggests that TNF is an essential mediator for the generation and/or activation of autoreactive lymphocytes. What could be the primary target cells of TNF administered in vivo, and via which pathways does TNF elicit its effects? Based upon the present study and previous findings, several possibilities may be considered. First, the primary target of TNF could be lymphocytes. Our data have shown that TNF enhances autoimmune responses to a panel of β cell antigens (Fig. 5). In this scenario, TNF could be either (a) modulating T cell development in the thymus, thereby leading to more autoreactive T cells in the periphery or (b) activating peripheral lymphocytes and overriding mechanisms of peripheral tolerance. Second, TNF may function by activating macrophages. As a consequence, activated macrophages may (a) enter the islets and begin to recruit autoreactive lymphocytes to the islets (36, 37); (b) potentiate processing and presenta-

tion of β cell autoantigens (38, 39); or (c) release cytokines such as IL-12 which promotes Th1 responses that are believed to mediate the diabetogenic process in IDDM (40, 41). Third, TNF may trigger and activate the expression of adhesion molecules on both lymphocytes and/or endothelial cells of the venules found in the islets (42, 43), thereby facilitating the process of lymphocyte homing to the islets. We have recently shown that this homing process is essential for the progression of insulinitis and diabetes in NOD mice (44).

Why is the effect of TNF age dependent? It is very likely that the target tissues or cells that respond to TNF do so in a developmentally stage-specific manner. As mentioned above, a qualitative change occurs in NOD mice regarding the diabetogenic potential of the T cells leaving the thymus. Therefore, activation and enhancement of thymic function(s) by TNF may lead to distinct consequences depending upon the developmental stage of the thymus.

It is still puzzling how administration of TNF in adult animals could lead to prevention of IDDM in rodents (12, 14, 15). TNF has been shown to be a major mediator of many inflammatory diseases and blocking TNF action has proved to be an efficient means of treatment for some inflammatory diseases, including experimental allergic encephalomyelitis (EAE) in mice, an animal model of human multiple sclerosis, collagen-induced arthritis in mice, and rheumatoid arthritis

in patients (45–47). It is possible that repeated injection of TNF into adult animals could result in a phenomenon referred to as “TNF tolerance” (48, 49). TNF tolerance may result from the establishment of an autoantibody response specific for TNF and/or the stimulation of release of soluble TNF receptors after injection of large doses of TNF for an extended period of time (e.g., 3 μg /injection every 2 d for 4 mo) (12). Both anti-TNF antibody and soluble TNF receptors may block TNF activity in vivo and in turn prevent disease. It has been reported that anti-cytokine autoantibodies such as anti-TNF, anti-IL-1 α , and anti-IL-6 can be detected in patients with immunoinflammatory and infectious diseases, as well as in normal individuals (50–52). It is interesting to note that some of these autoantibodies have been shown to be able to specifically inhibit bioactivities of the respective cytokines in vitro (53). Recently, Bemelmans et al. (54) have reported that injection of recombinant TNF can result in the release of the extracellular parts of both 55- and 75-kD TNF receptors, namely the soluble TNF receptors in vivo. These potential mechanisms are currently under investigation. In addition, it has been shown in the EAE model, that treatment of mice with TN3.19.12 did not appear to deplete cells expressing TNF on the surface, suggesting that

TN3.19.12 does not function by depleting TNF expressing lymphocytes (45).

The partial protective effect observed when TN3.19.12 is initially injected into 4-wk-old mice may be due to insufficient amounts of antibody (100 μg /injection, which was not based on the body weight) (Fig. 3). The treatment with TN3.19.12 in adult animals has been shown to enhance insulinitis severity (55). However, in this previous study (55), the effect of TN3.19.12 was investigated on insulinitis but not on spontaneous diabetes. The degree of insulinitis severity is not necessarily correlated with the incidence of diabetes. For instance, male NOD mice develop very severe insulinitis but only 10–20% become diabetic.

The present study on the neonatal effect of TNF on IDDM in mice clearly demonstrates that TNF is a proinflammatory cytokine that is involved in the immunopathogenic process of IDDM. TNF appears to have distinct effects on diabetogenic process depending upon the developmental stages of the target tissue or cell. The protective effect of anti-TNF treatment may have implications for the development of a cytokine or cytokine receptor-based prevention of human IDDM.

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