

# Local Expression of Transgene Encoded TNF $\alpha$ in Islets Prevents Autoimmune Diabetes in Nonobese Diabetic (NOD) Mice by Preventing the Development of Auto-reactive Islet-specific T Cells

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## Summary

Lately, TNF $\alpha$  has been the focus of studies of autoimmunity; its role in the progression of autoimmune diabetes is, however, still unclear. To analyze the effects of TNF $\alpha$  in insulin-dependent diabetes mellitus (IDDM), we have generated nonobese diabetic (NOD) transgenic mice expressing TNF $\alpha$  under the control of the rat insulin II promoter (RIP). In transgenic mice, TNF $\alpha$  expression on the islets resulted in massive insulinitis, composed of CD4+ T cells, CD8+ T cells, and B cells. Despite infiltration of considerable number of lymphoid cells in islets, expression of TNF $\alpha$  protected NOD mice from IDDM. To determine the mechanism of TNF $\alpha$  action, splenic cells from control NOD and RIP-TNF $\alpha$  mice were adoptively transferred to NOD-SCID recipients. In contrast to the induction of diabetes by splenic cells from control NOD mice, splenic cells from RIP-TNF $\alpha$  transgenic mice did not induce diabetes in NOD-SCID recipients. Diabetes was induced however, in the RIP-TNF $\alpha$  transgenic mice when CD8+ diabetogenic cloned T cells or splenic cells from diabetic NOD mice were adoptively transferred to these mice. Furthermore, expression of TNF $\alpha$  in islets also downregulated splenic cell responses to autoantigens. These data establish a mechanism of TNF $\alpha$  action and provide evidence that local expression of TNF $\alpha$  protects NOD mice from autoimmune diabetes by preventing the development of autoreactive islet-specific T cells.

Insulin-dependent diabetes mellitus (IDDM)<sup>1</sup> is an autoimmune disease in which self-reactive lymphocytes mediate the complete destruction of pancreatic  $\beta$  cells (reviewed in reference 1). A considerable body of evidence suggests that IDDM is determined by both genetic and environmental factors. Of the genes involved, the strongest association between IDDM is found between genes of the major histocompatibility complex (MHC), and specifically HLA-DQ in humans (2). However, a multitude of studies show that IDDM is a polygenic disease and in addition, studies with identical twins show a concordance on the order of 30–50%, suggesting that environmental factors play a key role in the development of diabetes (3).

The study of IDDM has been greatly facilitated by the availability of good animal models (4–7). The Biobreeding (BB) rat and the nonobese diabetic (NOD) mouse develop a form of IDDM that is broadly similar to the human disease. The NOD mouse has been the focus of considerable

attention since it shows a reproducibly high frequency of disease (80–90% of females and 50–60% in males) (4). In the NOD mouse, the islets of Langerhans become infiltrated with lymphoid cells, first around the islet (peri-insulinitis) around 3–4 wk, which gradually intensifies to insulinitis.  $\beta$  cell destruction follows and results in the loss of detectable insulin and, the onset of overt diabetes, which plateaus at  $\sim$ 4–6 mo of age. Genetic analysis shows that NOD diabetes is also a polygenic disease (8, 9). Of the genes involved in diabetes, the MHC (Idd-1), plays a key role (10–12). Like diabetes-susceptible humans, the NOD mouse expresses an unusual MHC class II I-A allele (the mouse homologue of HLA-DQ), which carries a small amino acid residue (S) at position 57; this parallel structure between these two susceptibility alleles has further supported the relevance of the NOD model to human disease (7, 13). The use of modern genetic methods has led to the identification of other recessive loci which predispose to IDDM. Thus, genes have been identified on chromosomes 3, 7, 11, and 14 which appear to play a cumulative role in increasing the frequency of IDDM. Several of these genes, notably Idd-3 and Idd-10 on chromosome 3, predispose to insulinitis and diabetes, whereas Idd-5 seems to predispose predominantly

<sup>1</sup>Abbreviations used in this paper: BB, biobreeding; DLN, draining lymph nodes; GAD, glutamic acid decarboxylase; IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; PLN, peripheral lymph nodes; RIP, rat insulin II promoter.

to insulinitis (8, 14). Insulinitis and diabetes are not 100% correlated, there are certain strains of NOD mice such as NOD/WEHI which seem to show insulinitis but little diabetes (15). These results are broadly consistent with the concept that insulinitis may be necessary, but possibly not sufficient for the induction of diabetes. However, the interpretation of these kinds of results is complex, given that the frequency of diabetes in NOD mice is notoriously variable from location to location, and strongly influenced by numerous factors even including diet (4).

A considerable amount of data associates cytokines with the pathogenesis of IDDM, some of which is focussed on the cytotoxic effects of cytokines on islet  $\beta$  cells (16–20). In contrast to the cytotoxic effects of cytokines, a protective role of cytokines in diabetes in genetically diabetic prone NOD mice and BB rats has also been postulated (20). TNF $\alpha$  has been implicated in many pathological and physiological processes and has been the focus of many studies for past few years; its role in exacerbation or protection of IDDM is however not clear. TNF $\alpha$  is a potent inflammatory mediator which activates endothelial cells *in vitro* to express a number of leukocyte adhesion molecules, including E-selectin, VCAM-1, and ICAM-1 (21). It also strongly upregulates the level of MHC class I on a variety of different cell types, including islets (22). Although TNF $\alpha$  does not increase the level of MHC class II, it can synergize with IFN- $\gamma$  to cause upregulation of MHC class II on islets (23). The proinflammatory properties of TNF $\alpha$  *in vitro* are reflected *in vivo*. Thus, transgenic mice expressing TNF in a multitude of tissues, including synovia and the islets of Langerhans, exhibit a pronounced infiltration of inflammatory cells (24–27). In fact, detailed studies of transgenic mice in which the rat insulin promoter (RIP) directed the synthesis of TNF $\alpha$  or TNF $\beta$  showed that these molecules direct the recruitment of CD4 and CD8 T cells as well as B cells and cause upregulation of MHC class I but not MHC class II (25–27). These transgenic mice appear to express the transgene locally since circulating levels of cytokines are not detectable, and cultured islets from transgene positive, but not from transgene negative mice, express significant levels of TNF $\alpha$ . Nonetheless, the infiltrate induced by TNF $\alpha$  does not lead to diabetes in these transgenic mice.

Despite the proinflammatory properties of TNF $\alpha$  *in vitro* and *in vivo* in transgenic animals, Jacob et al. showed, surprisingly, that TNF $\alpha$  retarded the development of IDDM in adult NOD mice, when administered in pharmacologic doses (28). This striking result suggested that the inflammatory properties of TNF $\alpha$  in fact have an inhibitory rather than a stimulatory effect on diabetes development. However, the fact that this cytokine was presented systemically confounds the interpretation of these studies. Specifically, it could be argued that the cytokine effects are distant rather than local at the islets of Langerhans, and could for example result in the recruitment of leukocytes away from that tissue, because of the activation of endothelia elsewhere. Systemic administration of cytokines can also act indirectly on the immune system, for example IL-1 and TNF $\alpha$  can increase the secretion of adrenocorticotrophic hormone by

stimulating the hypothalamic-pituitary axis which suppresses inflammatory cells and cytokines (29). Alternatively, the effects of systemic TNF $\alpha$  could nonetheless be mediated through direct events in the islets of Langerhans. In addition, a recent demonstration of exacerbation of development of diabetes, when TNF $\alpha$  administered early starting with neonatal NOD mice, indicates the complex effects of TNF $\alpha$  on IDDM (30). Results showing a controversial role of TNF $\alpha$  by pharmacological administration of TNF $\alpha$  were further complicated by finding that when pharmacologic doses of monoclonal anti-TNF $\alpha$  antibodies were administered for 4 wk, the incidence of diabetes in NOD mice was significantly reduced. On the other hand, when anti-TNF $\alpha$  antibodies were administered for a period of 8 wk, the incidence of diabetes was significantly increased (31), and diabetes were completely prevented when anti-TNF $\alpha$  monoclonal antibodies were administered neonatally (30). To address the effect of local TNF $\alpha$  in islets of NOD mice and determine the mechanism of action of TNF $\alpha$  in IDDM, we generated transgenic mice in which the TNF $\alpha$  gene was directed by the RIP and injected directly into NOD mice. To our surprise, the resulting NOD mice show pronounced resistance to spontaneous IDDM. However, they remain susceptible to adoptive transfer of IDDM with mature effector cells. These results therefore support the notion that locally produced TNF $\alpha$  protects islets from diabetes by inhibiting the development of auto-aggressive T cells.

## Materials and Methods

**Mice.** NOD mice originally obtained from Jackson Laboratories (Bar Harbor, ME) were bred in our colony. All mice used in this project were housed in specific pathogen-free conditions. In our colony, female NOD mice develop diabetes from 12 wk of age reaching 90% by 24 wk. NOD-SCID mice were obtained from the Jackson Laboratories. RIP-TNF $\alpha$  transgenic mice were generated by us, and both transgenic positive and non-transgenic litter mates were housed in specific pathogen-free conditions in our NOD colony.

**Generation of Transgenic Mice.** RIP-TNF $\alpha$  transgenic mice were generated as described previously (27). In brief, the locus encoding murine TNF $\alpha$  were derived from EMBL 7 clone 13 subcloned into pUC-12. The TNF $\alpha$  gene was isolated as a 2.7-kb *Nar*I/*Sal*I fragment and subcloned into *Cl*aI/*Sal*I sites in pSK-RIP. This construct was used previously to generate RIP-TNF $\alpha$  transgenic mice in (C57BL/B  $\times$  CBA)F2 mice (27); in these mice TNF $\alpha$  was faithfully expressed under the control of RIP as reported earlier (27). For this project transgenic mice were directly constructed in NOD background by injecting fertilized NOD eggs with the same construct. Several transgene positive founder lines were established by screening mice for expression of RIP-TNF $\alpha$  by Southern analysis of DNA extracted from tails. One of the founder lines (V 93.3) was crossed with NOD and offsprings were monitored for RIP-TNF $\alpha$  expression. Both transgene positive and negative progeny were housed under the same conditions and were monitored for the phenotypic effects.

**Measurement of TNF $\alpha$  Secretion by Islets.** Islets were isolated by collagenase digestion of whole pancreata. 100–150 islets were cultured in 0.5 ml of DMEM containing 20 mM glucose and

10% fetal calf serum for 24 h, as previously described (27). Supernatants were collected and analyzed for presence of TNF $\alpha$  by ELISA methods. ELISA procedures were performed by using antibodies obtained from PharMingen (San Diego, CA) and their recommended protocols were followed.

**Immunohistochemistry and Assessment of Insulinitis.** Insulinitis was assessed by histology. Pancreata were prepared for histological analysis by either immediate freezing of the tissue in OCT compound over dry ice or by fixing in 10% formalin and then embedding in paraffin as described previously (26). Paraffin embedded blocks were sectioned and stained with hematoxylin and eosin. The slides were viewed by light microscopy for presence and pattern of insulinitis. Frozen sections were prepared for evaluating presence of CD4 and CD8 T cells, B cells and macrophages in islet infiltrates. Monoclonal antibodies (conjugated with biotin) against CD4, CD8, macrophage(F/480) and B220 markers were used to stain frozen sections and slides were developed and processed as described previously (26).

**Diabetes Measurements.** Diabetes was assessed by weekly measurement of urine glucose by using Diastix (Ames, Elkhart, IN), and diabetes was confirmed by blood glucose measurements. Mice were considered to be diabetic if three consecutive measurements of blood glucose were greater than 250 g/dl. To eliminate possible artifacts caused by environmental components such as food, transgene positive and transgene negative mice were housed together; we reasoned that any artefact of this nature would be localized to individual cages, and therefore would randomize across transgene positive and transgene negative mice.

**Flow Cytometric Analysis.** Single cell suspensions of spleen, peripheral lymph nodes, blood, and thymus tissue were prepared from 6–8-wk-old NOD or RIP-TNF $\alpha$  transgenic mice. Cells were stained by incubating for 45 min at 4°C with anti-CD4-PE, anti-CD8-FITC, and B220-Biotin. Cells were subsequently washed and fixed in 1% formaldehyde. Samples were analyzed on a Becton Dickinson FACS® star plus Flowcytometer. A minimum of 10,000 cells were collected for each sample.

**Leukocyte Isolation and Counts.** Single cell suspensions from spleen, thymus, peripheral lymph nodes (cervical, axillary, inguinal, and periaortic), and mesenteric lymph nodes were prepared and the number of cells were determined using a hemocytometer. Blood samples were obtained by puncture of the retro orbital plexus of anesthetized mice.

**T Cell Proliferation Assay.** For polyclonal stimulation of T cells,  $5 \times 10^5$  splenic cells were cultured in presence of 2.5  $\mu$ g/ml ConA or 3  $\mu$ g/ml anti-CD3 in 96-well plates in Bruffs medium supplemented with 5% FCS, 2 mM glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and different concentrations of the antigen. Proliferation was measured by addition of 1  $\mu$ Ci of [ $^3$ H]thymidine (International Chemical and Nuclear, Irvine, CA) for the last 18 h of a 5-d culture, and incorporation of radioactivity was assayed by liquid scintillation counting. For anti-KLH responses, mice were immunized with 100  $\mu$ g KLH in a 1:1 emulsion with CFA, containing 1 mg/ml *Mycobacterium tuberculosis* strain H37Ra, (Difco Laboratories, Detroit, MI) in the hind footpads. After 9 d, the popliteal lymph nodes were removed and cell suspensions were prepared. The lymph node cells were cultured ( $5 \times 10^5$  per well) in various concentration of KLH in bruffs medium supplemented with 5% FCS. Proliferation was measured by uptake of [ $^3$ H]thymidine as stated above.

**T Cell Proliferation in Response to Islet Extracts and GADp524-543.** Spleen cell proliferation in response to islet extracts or GADp524-543 (SRLSKVAPVIKARMMGYGTT) were measured by culturing spleen cells ( $1 \times 10^6$ /well) obtained from RIP-

TNF $\alpha$  transgenic or non-transgenic mice in HL-1 serum-free medium as described (32). Islet cell extracts were prepared by isolating islets by collagenase digestion of whole pancreata, as previously described (27) followed by sonication of single cell preparation of islets and five cycles of freeze thaw. Proliferation of spleen cells was measured in 5-d culture by uptake of [ $^3$ H]thymidine as mentioned above.

**Cytokine Assays.** Assays for cytokine production by spleen cells from RIP-TNF $\alpha$  transgenic or non-transgenic mice were done by culturing  $1 \times 10^5$  purified CD4+ T cells with  $5 \times 10^5$  irradiated (3,000 R) splenic cells from unimmunized NOD mice and different amounts of the GADp524-543 in Bruffs medium, supplemented as described above. After 24 h of culture, 100  $\mu$ l of culture supernatant was removed from each well for IL-2 measurements. For IL-4 and IFN $\gamma$  measurements, supernatants were removed after 4 d of culture. ELISA method was used to determine cytokine levels in supernatants, using antibodies from PharMingen, and using their recommended protocols.

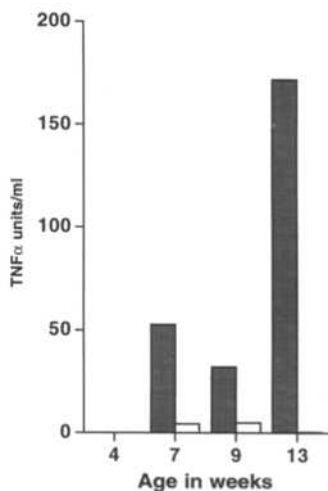
**Anti-GADp524-543 Antibodies Analysis by ELISA.** Anti-GADp524-543 levels were determined by specific ELISA method. Briefly, 96-well plates were coated with 10  $\mu$ g/ml GADp524-543 for 24 h. Plates were then washed and blocked with 1% FCS in PBS. Diluted serum samples were then added to triplicate wells and plates were incubated for 1 h at 37°C. Plates were washed and the antigen specific antibody titers determined with the biotin conjugated goat anti-mouse-Ig detection antibody (Southern Biotechnology, Birmingham, AL). ELISA was developed by using streptavidin conjugated horse radish peroxidase and Elisa T-Turbo developing reagent substrate (ICN Biomedicals, Inc., Costa Mesa, CA). Plates were analyzed on a Dynatech MR700 ELISA plate reader (Dynatech Labs., Inc., Chantilly, VA).

**Generation and Propagation of CD8 Diabetogenic T Cell Clones.** The CD8 diabetogenic T cell clones used in this project are well characterized (33) and cause rapid diabetes upon adoptive transfer to NOD-SCID mice. These clones were generated from islet infiltrates of 7-wk-old females as described by us previously, and were routinely maintained by our laboratory (33).

**Adoptive Transfer of Diabetes.** Adult female RIP-TNF $\alpha$  transgenic and non-transgenic litter mates and NOD-SCID mice were used as recipients. In some experiments, recipient mice were irradiated with 725 rad from a caesium source. Spleen cells from diabetic NOD or non-diabetic NOD or from RIP-TNF $\alpha$  mice ( $6-10 \times 10^6$  cells/mouse) or CD8 cloned T cells  $6 \times 10^6$  were adoptively transferred intravenously to recipient mice. Non-irradiated recipients were examined for diabetes on a weekly basis and irradiated recipient mice were monitored for diabetes daily after adoptive transfer.

## Results

**Generation of Transgenic NOD Mice Expressing RIP-TNF $\alpha$ .** We used the same construct that we have described previously that directs the expression of substantial levels of TNF $\alpha$  in (C57BL/6  $\times$  CBA)F2 mice (27). In our previous study we constructed several lines of transgenic mice in which the expression of this gene showed reproducible production of TNF $\alpha$  from the pancreas but not from other tissues tested. Specifically, this transgene was expressed at the level of RNA and protein in pancreatic tissue, and as biologically active TNF $\alpha$  in supernatants from cultured islets. In situ hybridization in those studies showed that the

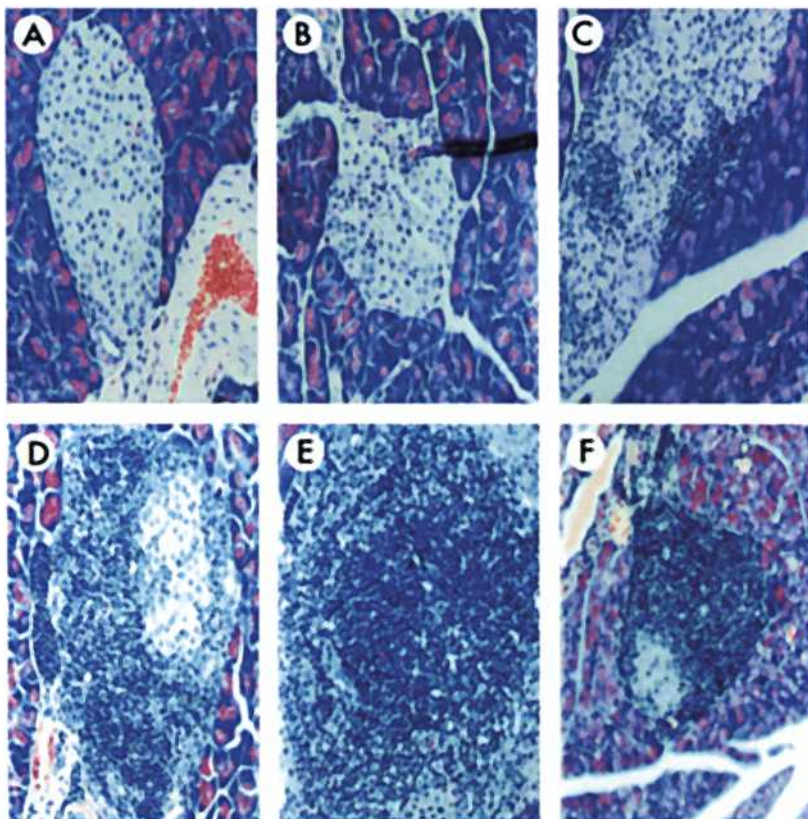


**Figure 1.** Production of TNF $\alpha$  by islets from RIP-TNF $\alpha$  transgenic mice. Islets from RIP-TNF $\alpha$  transgenic mice (■) or from negative litter mate control NOD mice (□) ranging in age from 4 wk to 13 wk were examined for TNF $\alpha$  secretion. Islets pooled from four mice were cultured for 48 h in 0.5 ml of culture medium containing 20 mM glucose. Amount of TNF $\alpha$  secreted in supernatants at a dilution of 1:10 were determined by TNF $\alpha$ -specific ELISA.

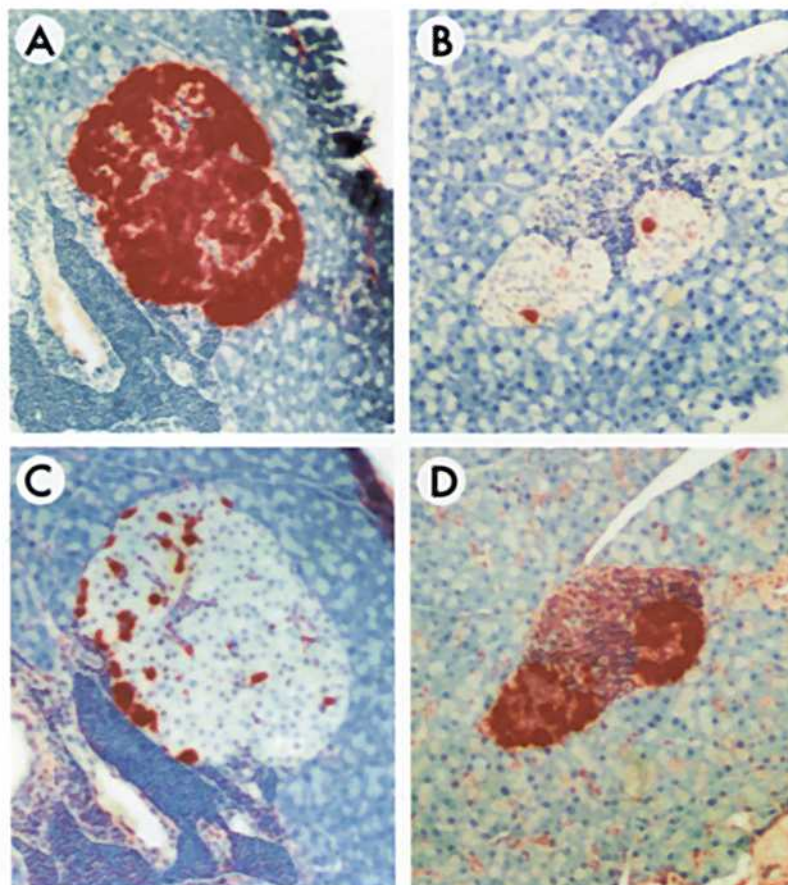
TNF $\alpha$  expression in the islets was localized to the islets and not to the infiltrating mononuclear cells which developed in these animals. Fertilized NOD eggs were injected with this validated construct, and several transgene positive founder lines were obtained. Transgene founders were crossed with NOD mice and their progeny was examined for expression of RIP-TNF $\alpha$  by Southern analysis (27). After preliminary characterization, line V 93.3 was selected and further bred with NOD mice to monitor the pheno-

typic effects. Expression of TNF $\alpha$  protein by islets was determined by analyzing supernatants from cultures containing islets from transgene positive or negative litter mates in the presence of glucose. A considerable amount of TNF $\alpha$  was secreted by islets obtained from RIP-TNF $\alpha$  transgenic mice after 48 h of culture, whereas no TNF $\alpha$  was produced by the islets of control mice cultured under similar conditions (Fig. 1). These results indicate that TNF $\alpha$  is expressed and secreted by islets of transgene positive mice. To determine at what age TNF $\alpha$  is first expressed in RIP-TNF $\alpha$  mice, islets obtained from mice of various ages were tested for production of TNF $\alpha$  in vitro. TNF $\alpha$  could not be detected in mice of 4 wk old, whereas measurable amounts of TNF $\alpha$  was detected at 7 wk of age and at later time points (Fig. 1). In addition, serum from these mice was also tested to determine levels of TNF $\alpha$ . No TNF $\alpha$  could be detected in serum of transgenic mice at any age (data not shown). This result indicates that TNF $\alpha$  is only locally expressed in islets of Langerhans.

*Expression of TNF $\alpha$  in the Pancreas of Transgenic NOD Mice Results in Insulinitis.* Our previous studies indicated that the RIP-TNF $\alpha$  transgene mediates dramatic infiltration of lymphocytes in the islets of Langerhans in mice generated on the (C57BL/6  $\times$  CBA)F2 background that had been back crossed to C57BL/6 (27). We therefore monitored the effects of RIP-TNF $\alpha$  transgene on insulinitis as a function of age in NOD mice. Histological examination of pancreata from RIP-TNF $\alpha$  transgenic mice reveals that mononuclear



**Figure 2.** Histological analysis of insulinitis in RIP-TNF $\alpha$  transgenic mice. Paraffin-embedded pancreas sections from RIP-TNF $\alpha$  transgenic mice were stained with hematoxylin and eosin ranging in age from 4 wk to 48 wk; (A) 4 wk old, (B) 7 wk old, (C) 9 wk old, (D) 13 wk old, (E) 26 wk old, and (F) 48 wk old. Unique patterns of insulinitis was discovered in islets of RIP-TNF $\alpha$  mice.



**Figure 3.** Insulin and glucagon staining of pancreas from RIP-TNF $\alpha$  transgenic mice (A and C) and negative transgenic littermate control mice (B and D). Paraffin-embedded sections were stained with antibodies against insulin (A and B) and with glucagon (C and D).

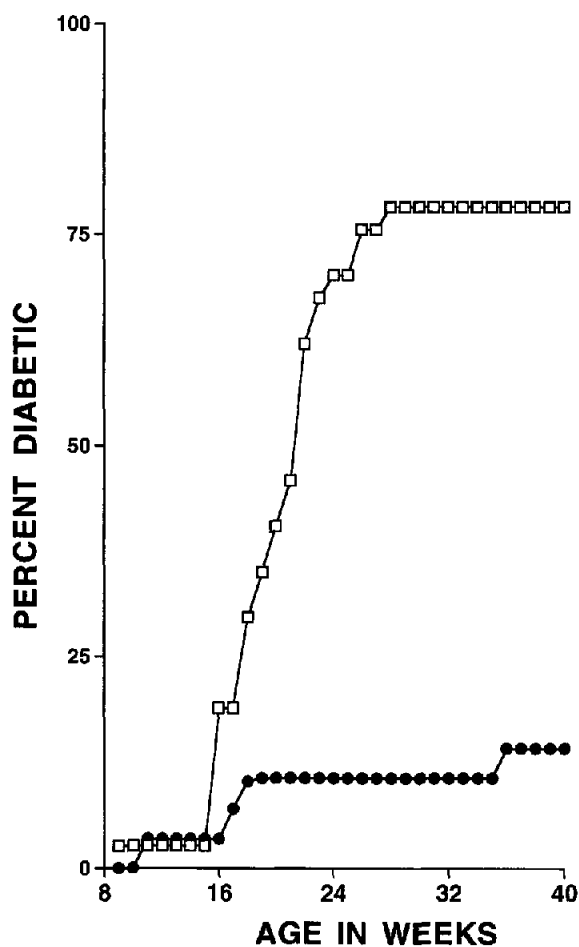
cells begin to infiltrate into islets at  $\sim 7$  wk old. This infiltration (insulinitis) was restricted to islets and it was not observed in surrounding acinar tissues (Fig. 2). In contrast to non-transgenic NOD mice, the immune cell infiltrate in RIP-TNF $\alpha$  transgenic mice progresses to massive infiltration, almost completely obscuring the islets. The nature of the infiltrating cells was determined by immunohistochemical staining of frozen pancreata. Immunohistochemical staining shows the presence of numerous CD4 and CD8 cells as well as B220+ cells in the infiltrates of RIP-TNF $\alpha$  transgenic mice (data not shown).

*Infiltration of Mononuclear Cells into Islets in RIP-TNF $\alpha$  Transgenic Mice Does Not Prevent Insulin Production.* To ascertain whether the massive infiltration of mononuclear cells in islets of RIP-TNF $\alpha$  transgenic mice affects functioning of the beta cells in RIP-TNF $\alpha$  transgenic mice, we analyzed paraffin embedded sections of pancreata from RIP-TNF $\alpha$  mice for insulin and glucagon by immunocytochemistry (Fig. 3). Examination of the sections from control NOD mice showed the presence of glucagon-producing  $\alpha$  cells aligned along the perimeter of the islet capsule, and considerable damage to insulin-producing  $\beta$  cells were predominantly in the central portion of the islet, indicating normal organization of  $\alpha$  and  $\beta$  cells in these sections. By contrast, sections from RIP-TNF $\alpha$  transgenic mice revealed no damage to  $\beta$  cells or loss of insulin content in is-

lets; however the organization of the glucagon producing cells was disrupted (with  $\alpha$  cells located within islets) as we described previously for the RIP-TNF $\alpha$  transgenic mice on the B6 background (27). Thus, in spite of massive mononuclear infiltration of islets in RIP-TNF $\alpha$  mice, their  $\beta$  cell physiology remains normal.

*Expression of TNF $\alpha$  in Islets Prevents the Development of Spontaneous Diabetes in NOD Mice.* RIP-TNF $\alpha$  transgene positive and non-transgenic litter mates were then housed under the specific pathogen-free conditions used for our NOD colony and monitored for the development of diabetes. Since female mice develop diabetes at a significantly greater frequency than male NOD mice, we monitored only females in this study. Expression of TNF- $\alpha$  in islets dramatically retarded the development of IDDM. Whereas  $\sim 80\%$  of female NOD transgene-negative mice developed diabetes by 25 wk, at this time only  $\sim 15\%$  of transgene positive animals developed disease. This study was performed with two separate groups of animals, both of which are summarized in Fig. 4. Thus, whereas transgenic TNF $\alpha$  mediated substantial infiltration into the islets of Langerhans, this did not lead to autoimmune diabetes.

*Expression of TNF $\alpha$  in the Pancreas of Transgenic NOD Mice Has No Effects on the Number of Lymphocytes in Lymphoid Organs or on In Vitro Proliferation of T Cells.* To rule out the influence of TNF $\alpha$  expression in islets on lymphocyte devel-



**Figure 4.** Onset of diabetes in RIP-TNF $\alpha$  mice. Transgene positive mice (●) and negative litter mates (□) were housed randomly (both positive [ $n = 28$ ] and negative [ $n = 37$ ] mice in the same cages) in filter frame units. Diabetes was measured by monitoring of urinary glucose followed by blood glucose. Mice were considered to be diabetic if three consecutive measurements of blood glucose greater than 250 mg/dl were observed.

opment, we carefully examined the various subpopulations of lymphocytes in lymphoid organs in RIP-TNF $\alpha$  transgenic mice. Both RIP-TNF $\alpha$  transgenic and non-transgenic mice were examined for the number and ratios of CD4, CD8, and B220 positive cells in spleen, peripheral lymph nodes (PLN), thymus and blood by Flow cytometry. The frequency of various subsets of lymphocytes in RIP-TNF $\alpha$  and non-transgenic litter mates were indistinguishable, indicating that local expression of TNF $\alpha$  in islets does not influence the development of lymphoid tissues in NOD mice (Fig. 5). In addition, *in vitro* proliferative responses of PLN cells to CD3 and ConA were measured, and the data presented in Table 1 indicates that *in vitro* T cell proliferation in response to polyclonal stimulators is normal in RIP-TNF $\alpha$  transgenic mice.

*Expression of TNF $\alpha$  in the Pancreas of Transgenic NOD Mice Does Not Induce Generalized Immunosuppression.* Since expression of TNF $\alpha$  in islets prevents the development of spontaneous diabetes in transgenic mice, one could argue

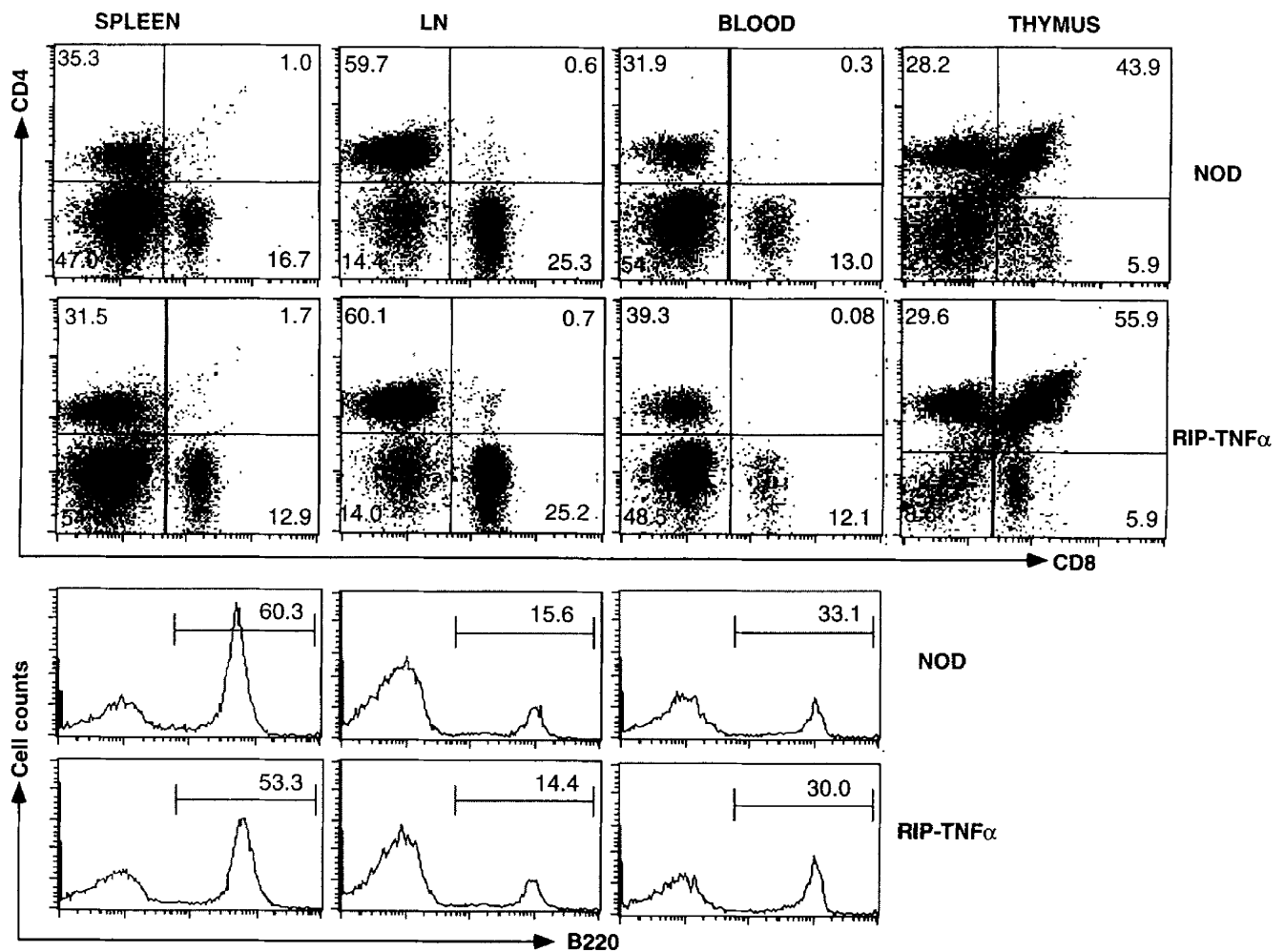
that the protection offered by the RIP encoded transgene (TNF $\alpha$ ) may be due to general suppression of the immune system. To rule out this possibility, we used an antigen-specific system to measure T cell responses of RIP-TNF $\alpha$  transgenic mice to a well-characterized foreign protein antigen, keyhole limpet hemocyanin (KLH). Mice were immunized with KLH in complete Freund's adjuvant (CFA) and 9 d later their draining lymph nodes (DLN) were tested for *in vitro* recall proliferative responses. RIP-TNF $\alpha$  transgenic mice exhibited recall proliferative responses similar to that of non-transgenic litter mates, indicating that local expression of TNF $\alpha$  in islets does not lead to the induction of suppression of immune responses to foreign protein antigens (Fig. 6). Thus, these data suggest that expression of TNF $\alpha$  in islets does not cause general suppression of the immune system.

*Expression of TNF $\alpha$  in Islets Inhibits the Development of Immune Responses to Islets and GAD Peptide.* Although the studies presented above show that responses to foreign protein antigens in RIP-TNF $\alpha$  transgenic mice are not significantly different from negative litter mate controls, the possibility that specific regulatory mechanisms could suppress islet specific autoimmune responses remained an explanation for suppression of IDDM by RIP-TNF $\alpha$  transgene. To address this we examined the spontaneous T cell responses and serum antibody levels to a synthetic peptide derived from glutamic acid decarboxylase (GAD), containing amino acid sequence 524-543 (GADp524-543). Spontaneous immune responses to this peptide in young NOD mice has been shown previously (32). To determine whether spontaneous responses to GADp524-543 are inhibited by local expression of TNF $\alpha$  in islets, splenic cells from both RIP-TNF $\alpha$  and non-transgenic litter mates were examined for proliferation and cytokine production in response to *in vitro* challenge with this peptide. Splenic cells from RIP-TNF $\alpha$  mice showed a complete lack of proliferative responses to GADp524-543, whereas a signifi-

**Table 1.** *In Vitro* Proliferation of Naive T Cells in the Presence of Polyclonal Stimuli

	Spleen cell proliferation							
	Con-A ( $\mu\text{g/ml}$ )				$\alpha\text{CD3}$ ( $\mu\text{g/ml}$ )			
	0.01	0.1	2.0	5.0	0.1	1.0	5.0	10.0
NOD	4.03	5.99	10.18	16.03	3.4	5.17	10.40	14.53
RIP-TNF	4.67	6.68	15.89	27.73	4.33	7.35	15.54	18.78

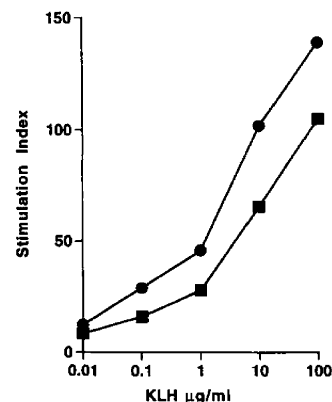
Lymph node T cells purified from unimmunized RIP-TNF $\alpha$  transgenic mice or negative transgenic litter mates were cultured in the presence of media alone or anti-CD3 (A) and ConA. Proliferation was determined after 4 d of culture by incorporation of [ $^3\text{H}$ ]thymidine and data are presented as stimulation index.



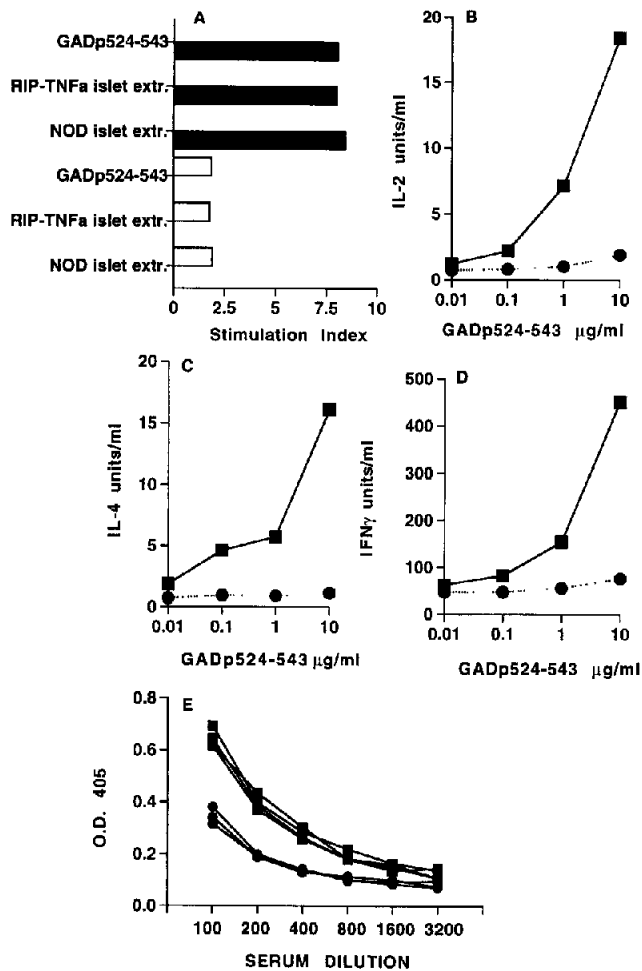
**Figure 5.** Flow cytometry analysis of various subpopulations of lymphocytes in lymphoid organs. Both RIP-TNF $\alpha$  transgenic mice and negative litter mates were examined for CD4, CD8, and B220 expressing cells in spleen, lymph node, blood, and thymus by staining them with monoclonal antibodies against these markers. Values given in the quadrants are the percent events within that quadrant. Values given in histograms are percent gated events indicated by a bar.

cant response was apparent in the spleen of non-transgenic litter mates (Fig. 7 A). Splenic cells from non-transgenic NOD mice, showed significant production of IL-2, IL-4, and IFN $\gamma$ , whereas no such production of cytokines could be detected from spleen cells from RIP-TNF $\alpha$  transgenic mice (Fig. 7, B-D). In addition, serum from both non-transgenic and RIP-TNF $\alpha$  transgenic mice were tested for the presence of anti-GAD antibodies. The levels of anti-GADp524-543 antibodies were significantly lower in RIP-TNF $\alpha$  transgenic mice when compared with non-transgenic litter mates (Fig. 7 e). In addition, proliferative responses of splenic cells from RIP-TNF $\alpha$  and non transgenic litter mates were measured against islet extracts obtained from NOD mice or from RIP-TNF $\alpha$  transgenic mice. Again a lack of proliferative response was seen in RIP-TNF $\alpha$  transgenic mice, whereas non transgenic mice responded well (Fig. 7 a). These results demonstrated that local expression of TNF $\alpha$  had profound effect on the development of autoantigen specific immune responses in both the B and T cell compartments. Moreover, since splenic cells from

non transgenic mice were able to proliferate in response to islets from RIP-TNF $\alpha$  transgenic mice, it can be concluded that expression of TNF $\alpha$  does not change the antigenic moieties present on or in the islets.



**Figure 6.** Recall proliferative responses of KLH-primed draining lymph node cells. In vitro proliferative recall responses of RIP-TNF $\alpha$  transgenic mice (●) and negative litter mate control mice (■) to KLH. Draining lymph node cells from mice immunized with KLH/CFA 9 days earlier were cultured in the presence of media alone or indicated concentrations of KLH. Proliferation was determined after 4 d of culture by incorporation of [ $^3$ H]thymidine and data are presented as stimulation index.



**Figure 7.** In vitro proliferation of naive T cells in presence of various antigens. T cells purified from unimmunized RIP-TNF $\alpha$  transgenic mice (open bars) or negative transgenic litter mates (filled bars) were cultured in the presence of media alone or indicated antigens (A). Proliferation was determined after 4 days of culture by incorporation of [ $^3$ H]thymidine and data are presented as stimulation index. Cytokine production by T cells purified from spleens of unimmunized RIP-TNF $\alpha$  transgenic mice (●) or negative transgenic litter mates (■) cultured in the presence of media alone or GADp524-543, IL-2 production (B), IL-4 production (C), and IFN $\gamma$  production (D). (E) Presence of serum antibodies against GADp524-543 in RIP-TNF $\alpha$  transgenic mice (●) or negative transgenic litter mates (■) from individual mice as determined by ELISA.

*Adoptively Transferred Splenic Cells from RIP-TNF $\alpha$  Transgenic Mice Fail to Cause Diabetes in NOD-SCID Mice.* To understand the mechanism of protection offered by local expression of TNF $\alpha$  in islets, we adoptively transferred splenic cells from diabetic NOD mice or from age matched RIP-TNF $\alpha$  transgenic mice to NOD-SCID recipients. When diabetic splenic cells from NOD mice were transferred into NOD-SCID recipients, diabetes developed within 2–3 wk, whereas age matched splenic cells from RIP-TNF $\alpha$  mice failed to cause diabetes in NOD-SCID recipients (Table 2). We also adoptively transferred splenic cells from age matched non-diabetic, non-transgenic mice to NOD-SCID mice, and again diabetes developed in recipient mice, although, the development of diabetes was slower than that induced by splenic cells from diabetic NOD mice. These results suggest that sensitized autoreactive T cells having the potential to cause diabetes were present in the spleens of NOD mice while the splenic cell population from RIP-TNF $\alpha$  transgenic mice lacked this capability. The inability of spleen cells from RIP-TNF $\alpha$  transgenic mice to cause diabetes in NOD-SCID recipients on adoptive transfer could be explained at least by two possible mechanisms: either autoreactive cells with the potential to cause diabetes fail to develop in RIP-TNF $\alpha$  transgenic mice, or autoreactive cells are present in the spleens of RIP-TNF $\alpha$  transgenic mice but these cells are prevented from attack in the islets of the recipient mice by some kind of regulatory mechanism. In addition, these results also suggested that TNF $\alpha$  expression in islets is not required to protect islets from a destructive immune response.

To distinguish these possibilities, we adoptively transferred a mixture of diabetic spleen cells and spleen cells obtained from RIP-TNF $\alpha$  mice to NOD-SCID recipients, and examined the development of diabetes in recipient mice. Admixture of spleen cells from diabetic mice with spleen cells from RIP-TNF $\alpha$  mice did not prevent the development of diabetes (Table 2), but delayed the development of diabetes by a few weeks. Although these results indicate that protection offered by local expression of TNF $\alpha$  is not due to generation of potent specific regulatory suppressor cells, we cannot rule out the possibility of such regulation by these simple adoptive transfer experiments. To

**Table 2.** Transfer of Diabetes by Adoptive Transfer of Splenic Cells

Source of Cells	Recipients	Incidence of diabetes (weeks post-transfer)														
		3	4	5	6	7	8	9	10	11	12	13	14	23	40	
NOD (diabetic)	SCID	3/6	5/6	6/6												
NOD (Non-diabetic)	SCID	1/5	1/5	1/5	1/5	1/5	2/5	2/5	3/5	3/5	3/5	4/5	4/5	5/5		
RIP-TNF $\alpha$	SCID	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	0/9	1/9	1/9	1/9	1/9	
RIP-TNF $\alpha$ + NOD (diabetic)	SCID	2/10	2/10	4/10	8/10	8/10	8/10	9/10	10/10							
NOD (Non-diabetic) + NOD (diabetic)	SCID	2/4	4/4													

6–10  $\times$  10<sup>6</sup> splenic cells depleted of RBC from NOD (diabetic within two weeks) mice were transferred intravenously either alone or in the presence of the same amount of splenic cells from RIP-TNF $\alpha$  transgenic mice. Diabetes were monitored by urine glucose analysis at weekly intervals.



**Table 3.** *Transfer of Diabetes by Adoptive Transfer of CD8+ Cells into Irradiated Recipients*

Source of Cells	Recipients	Incidence of diabetes (days post-transfer)											
		5	6	7	8	9	10	15	16	19	20		
NOD (diabetic)	NOD	0/3	0/3	0/3	0/3	0/3	0/3	0/3	1/3	2/3	3/3		
G9-C8	NOD	1/4	1/4	3/4	3/4	4/4							
NOD (diabetic)	RIP-TNF $\alpha$	0/3	0/3	0/3	0/3	0/3	0/3	1/3	2/3	3/3			
G9-C8	RIP-TNF $\alpha$	1/4	3/4	4/4									

$6 \times 10^6$  diabetogenic cloned CD8+ T cells (G9-C8) or  $10 \times 10^6$  splenic cells depleted RBC from NOD (diabetic within two weeks) mice were transferred intravenously to irradiated (725 Rads) RIP-TNF $\alpha$  transgenic mice or negative litter mates. Diabetes was monitored by urine glucose analysis at daily intervals.

rule out the existence of specific regulatory cell mediated mechanism of protection by TNF $\alpha$ , further studies including a detailed kinetics analysis by using various ratios of cells will be needed.

*Local Expression of TNF $\alpha$  Does Not Protect Islets against Effector Cells.* To determine if protection offered by local expression of TNF $\alpha$  in islets was mediated by the direct effects of TNF $\alpha$  on the functioning of effector cells, we used an adoptive transfer system in which spleen cells from diabetic NOD mice, or cloned diabetogenic CD8 T cells (33) were used to transfer diabetes. Irradiated NOD or RIP-TNF $\alpha$  transgenic NOD mice were adoptively transferred with either cloned CD8 T cells or with diabetic splenic cells, and the development of diabetes was monitored (Table 3). In addition, non-irradiated recipients were also adoptively transferred with cloned CD8 T cells. Rapid development of diabetes occurred in all cases, indicating that TNF $\alpha$  does not block the functions of effector cells which are already primed in vivo (Table 4).

## Discussion

Since TNF $\alpha$  is a powerful mediator of inflammation with a pleiotropic range of actions, it is not surprising that this molecule has been found to be involved in autoimmunity. It is therefore of considerable interest that a few years ago Jacob et al. (28) and Satoh et al. (34) demonstrated that

TNF $\alpha$ , when administered in pharmacologic doses to adult NOD mice and BB rats (35) completely prevented the development of IDDM. However, the molecular and cellular basis for protection offered by TNF $\alpha$  has not been explained. Recently, Yang et al. have shown that this situation is more complex, since administration of TNF $\alpha$  neonatally exacerbates the development of diabetes (30). These studies implied that when TNF $\alpha$  is administered early it potentiates the initiating events in the development of IDDM, but if administered late, it inhibits an effector phase of the disease which normally leads to destruction of islets. However, from the above studies it was not clear whether the effects of the systemic administration of TNF $\alpha$  were fundamentally pharmacologic in activity and regulated the autoimmune response at sites distal from islets or whether TNF $\alpha$  is mediating a local effect and playing a direct role in pathology of IDDM. We therefore generated transgenic mice directly in NOD mice by injection of the RIP-TNF $\alpha$  construct that we have successfully used previously in a non-autoimmune predisposed strain. After initial characterization, we have studied these mice for the development of autoimmune diabetes. We show here that the RIP-TNF $\alpha$  transgene prevents the development of diabetes in NOD mice and that TNF $\alpha$  itself is not required to be present to prevent the destruction of islets by autoreactive T cells. Rather, protection is mediated by intervention at earlier steps in the development of anti-islet specific autoimmu-

**Table 4.** *Transfer of Diabetes by Adoptive Transfer of Diabetogenic CD8+ Cloned T Cells into Non-irradiated Recipients*

Source of Cells	Recipients	Incidence of diabetes (weeks post-transfer)												
		5	6	7	8	9	10	11	12	13	14	15	20	
G9-C8	RIP-TNF $\alpha$	3/5	3/5	3/5	3/5	3/5	3/5	3/5	3/5	3/5	3/5	4/5	4/5	4/5
G9-C8	NOD	4/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5	4/5	5/5		

$6 \times 10^6$  diabetogenic cloned CD8+ T cells (G9-C8) were transferred intravenously to RIP-TNF $\alpha$  transgenic mice. Diabetes were monitored by urine glucose analysis at weekly intervals.

nity and not at the final effector phase. Our data are consistent with those of McDevitt and colleagues (28, 30), who have shown that administration of TNF $\alpha$  to adult NOD mice leads to protection of diabetes. Our RIP-TNF $\alpha$  transgenic mice fail to express TNF $\alpha$  in the neonatal periods where TNF $\alpha$  exacerbates disease and only start to express TNF $\alpha$  as adults (7 wk) at which time we also start to see infiltration in islets.

There are a number of possible explanations for the absence of diabetes in the RIP-TNF $\alpha$  transgenic NOD mice. First, local expression of TNF $\alpha$  may inhibit the homing of autoreactive cells to the pancreatic islets; second, TNF $\alpha$  may not prevent the migration of autoreactive cells to islets but rather inhibit their ability to initiate or sustain islet specific destructive response; third, auto-reactive cells may fail to develop in the RIP-TNF $\alpha$  transgenic mice; fourth, TNF $\alpha$  may alter the properties of islets and make them resistant for destructive attack by autoreactive cells; fifth, TNF $\alpha$  may down regulate expression of autoantigens on islets required for the recognition for successful destructive response; finally, expression of TNF $\alpha$  under the control of the RIP might lead to general suppression of the immune system or may directly alter the regulatory mechanism of the immune system.

Our data presented here provide evidence to rule out several of the possibilities mentioned above; they also further provide evidence for the mechanism whereby TNF $\alpha$  inhibits development of diabetes. The lack of development of diabetes in RIP-TNF $\alpha$  transgenic mice is not due to inhibition of the migration of leukocytes to the islets, since massive islet infiltration is seen in these mice, which is even more dramatic than in the control non-transgenic NOD mice. Moreover, infiltrating cells include CD4, CD8, and B220 positive cells, and the cellularity of this infiltrate is not different from the typical insulinitis in control NOD mice (27). Despite massive infiltration, immunohistochemistry indicates normal insulin levels and a lack of destruction of  $\beta$  cells in RIP-TNF $\alpha$  transgenic mice. These observations suggested that infiltrating cells in islets of RIP-TNF $\alpha$  mice were unable to initiate or sustain an autoimmune destructive response.

Local expression of TNF $\alpha$  in islets does not lead to the general suppression of immune responses, since *in vitro* proliferative responses of T cells from RIP-TNF $\alpha$  are comparable to those of NOD mice. In contrast, whereas responses to KLH are normal in RIP-TNF $\alpha$  transgenic mice, specific autoimmune responses to the  $\beta$  cell autoantigen GAD and autoantigens contained in islet extracts were inhibited. RIP-TNF $\alpha$  transgenic mice showed reduced immune responses to islet auto-antigens since the antibody response, spleen cell proliferation and cytokine production to a peptide fragment of GAD is substantially reduced in these animals along with lack of response to islet extracts. GADp524-543, GAD65, and islet extracts have been used previously to measure autoantigen specific responses of non-diabetes prone mice such as Balb/c, B10.GD, F1(NOD  $\times$  Balb/c), and other inbred strains, and little to no response to these antigens was discovered when compared to the NOD strain

(32, 36). Also, GAD reactivity has been only shown to correlate with insulinitis in NOD mice. Thus, our result suggests that TNF $\alpha$  abrogates, at least in part, the anti-islet immune response.

To determine if local expression of TNF $\alpha$  conferred a long-term protective effect by altering the development of autoaggressive cells, we adoptively transferred lymphocytes from age matched diabetic NOD, non-diabetic NOD or RIP-TNF $\alpha$  transgenic NOD mice to recipient NOD-SCID mice. Spleen cells from diabetic NOD mice induced diabetes in NOD-SCID mice rapidly,  $\sim$ 3 wk after transfer. However, spleen cells from age matched RIP-TNF $\alpha$  transgenic mice did not induce diabetes in these animals. These results indicate that sensitized autoreactive lymphocytes with the potential to cause diabetes were absent in RIP-TNF $\alpha$  transgenic mice, suggesting that local expression of TNF $\alpha$  prevents the development of autoreactive cells.

These studies provide a potential explanation of the fascinating results of McDevitt's group (28). Our data suggest first that the TNF $\alpha$  mechanism is mediated by local events in the islets of Langerhans since RIP-TNF $\alpha$  transgenic mice do not have detectable levels of systemic TNF $\alpha$ . We further show that the effect of TNF $\alpha$  on the inhibition of development of disease is a long-lasting effect on the lymphocytes, rather than a short-term inhibition of effector function, since transfer of spleen cells from TNF $\alpha$  transgenic mice to NOD-SCID recipients does not provoke diabetes. Indeed there is no apparent inhibition of effector cell function since active effector cells can transfer diabetes to RIP-TNF $\alpha$  NOD mice (see below).

The possibility that a regulatory cell population controls the function of autoreactive cells present in the spleen of RIP-TNF $\alpha$  transgenic mice is unlikely but cannot be conclusively ruled out by our studies. A more detailed study of the kinetics of adoptively transferred mixtures of splenic cells from diabetic mice and splenic cells obtained from RIP-TNF $\alpha$  mice to NOD-SCID recipients, using different ratios and perhaps different subsets of T cells is required before making definitive conclusions. We considered the possibility that the protective effect mediated by TNF $\alpha$  in RIP-TNF $\alpha$  transgenic mice might require the continuous presence of TNF $\alpha$ , for example, to inhibit the action of autoaggressive T cells. To test this possibility, we adoptively transferred diabetic NOD splenic cells or cloned diabetogenic CD8 T cells to irradiated NOD or RIP-TNF $\alpha$  transgenic mice recipients, and cloned CD8 T cells were also adoptively transferred to non-irradiated recipients. Our data indicated that local expression of TNF $\alpha$  does not protect against effector cells which are already primed *in vivo*. Taken together, therefore, our results show that local production of TNF $\alpha$  in the islets of Langerhans alters the immune response to this tissue, thereby preventing the development of disease. The protective effect of TNF $\alpha$ , does not appear to require the presence of this cytokine; instead this cytokine confers a long-term protective effect by altering the autoaggressive potential of cells from these animals. Adoptive transfer experiments suggest a role for TNF $\alpha$  significantly earlier than the delivery of effector function.

The dramatic results that we have seen with TNF $\alpha$  confirm our recent observations using a second proinflammatory cytokine IL-6 (37). Transgenic NOD mice expressing IL-6, under the control of the RIP, also show reduction in the frequency and rate with which they develop IDDM. The effect of TNF $\alpha$  is, however, much more pronounced than the approximately two- to threefold effect seen in RIP-IL-6 transgenic mice (37). An inflammatory infiltrate is induced in both IL-6 and TNF $\alpha$  transgene mice, but in contrast to TNF $\alpha$ , the inflammatory infiltrate in IL-6 transgene is found on the NOD genetic background, but not on the diabetes resistant C57BL/6 background. Nonetheless, these results support the concept that a lymphocytic infiltrate mediated by these inflammatory cytokines might play a protective role. We would like to propose that these cytokines recruit cells to the islets, where they encounter islet

auto-antigens expressed at enhanced levels because of the effects of the cytokines. Since islets do not express costimulatory molecules, this encounter between lymphocytes and islet cells expressing MHC-peptide, might receive stimulation only through the antigenic receptor which, under certain circumstances, has been shown to mediate anergy *in vitro*; in other cases, such encounters have been suggested to cause apoptosis of T cells, perhaps mediated by the stimulation of IFN $\alpha$  production (38). An alternative hypothesis must be considered, however, and that is that the cytokines TNF $\alpha$  and IL-6 have some direct additional protective effects, for example, either on the islets or on the infiltrating cells. Further experiments using mice doubly transgenic mice for TNF $\alpha$  and a diabetogenic TCR are in progress to resolve these issues.

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