Local Expression of Transgene Encoded TNFα 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, TNFα 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 TNFα in insulin-dependent diabetes mellitus (IDDM), we have generated nonobese diabetic (NOD) transgenic mice expressing TNFα under the control of the rat insulin II promoter (RIP). In transgenic mice, TNFα expression on the islets resulted in massive insulitis, composed of CD4+ T cells, CD8+ T cells, and B cells. Despite infiltration of considerable number of lymphoid cells in islets, expression of TNFα protected NOD mice from IDDM. To determine the mechanism of TNFα action, splenic cells from control NOD and RIP-TNFα 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-TNFα transgenic mice did not induce diabetes in NOD-SCID recipients. Diabetes was induced however, in the RIP-TNFα transgenic mice when CD8+ diabetogenic cloned T cells or splenic cells from diabetic NOD mice were adoptively transferred to these mice. Furthermore, expression of TNFα in islets also downregulated splenic cell responses to autoantigens. These data establish a mechanism of TNFα action and provide evidence that local expression of TNFα protects NOD mice from autoimmune diabetes by preventing the development of autoreactive islet-specific T cells.

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease in which self-reactive lymphocytes mediate the complete destruction of pancreatic β 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 and 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-insulitis) around 3–4 wk, which gradually intensifies to insulitis. β cell destruction follows and results in the loss of detectable insulin and, the onset of overt diabetes, which plateaus at ~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 insulitis and diabetes, whereas Idd-5 seems to predispose predominantly...
to insulinitis (8, 14). Insulitis and diabetes are not 100% correlated, there are certain strains of NOD mice such as NOD/WEHI which seem to show insulitis 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 focused on the cytotoxic effects of cytokines on islet β 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). TNFα 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. TNFα 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 TNFα does not increase the level of MHC class II, it can synergize with IFN-γ to cause upregulation of MHC class II on islets (23). The proinflammatory properties of TNFα in vitro are reflected in vivo. Thus, transgenic mice expressing TNFα 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 TNFα or TNFβ showed that these molecules directly recruit 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 TNFα. Nonetheless, the infiltrate induced by TNFα does not lead to diabetes in these transgenic mice.

Despite the proinflammatory properties of TNFα in vitro and in vivo in transgenic animals, Jacob et al. showed, surprisingly, that TNFα retarded the development of IDDM in adult NOD mice, when administered in pharmacologic doses (28). This striking result suggested that the inflammatory properties of TNFα 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 cytokines 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 TNFα can increase the secretion of adrenocorticotropic hormone by stimulating the hypothalamic-pituitary axis which suppresses inflammatory cells and cytokines (29). Alternatively, the effects of systemic TNFα could nonetheless be mediated through direct events in the islets of Langerhans. In addition, a recent demonstration of exacerbation of development of diabetes, when TNFα administered early starting with neonatal NOD mice, indicates the complex effects of TNFα on IDDM (30). Results showing a controversial role of TNFα by pharmacologic administration of TNFα were further complicated by finding that when pharmacologic doses of monoclonal anti-TNFα antibodies were administered for 4 wk, the incidence of diabetes in NOD mice was significantly reduced. On the other hand, when anti-TNFα antibodies were administered for a period of 8 wk, the incidence of diabetes was significantly increased (31), and diabetes were completely prevented when anti-TNFα monoclonal antibodies were administered neonatally (30). To address the effect of local TNFα in islets of NOD mice and determine the mechanism of action of TNFα in IDDM, we generated transgenic mice in which the TNFα 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 TNFα 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-TNFα 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-TNFα transgenic mice were generated as described previously (27). In brief, the locus encoding murine TNFα were derived from EMBL 7 clone 13 subcloned into pUC-12. The TNFα gene was isolated as a 2.7-kb NarI/Sal fragment and subcloned into ClaI/SalI sites in pSK-RIP. This construct was used previously to generate RIP-TNFα transgenic mice in (C57BL/6 × CBA)F2 mice (27); in these mice TNFα 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-TNFα by Southern analysis of DNA extracted from tails. One of the founder lines (V 93.3) was crossed with NOD and offspring were monitored for RIP-TNFα expression. Both transgene positive and negative progeny were housed under the same conditions and were monitored for the phenotypic effects.

Measurement of TNFα 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 TNFα 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 Insulitis.** Insulitis was assessed by histology. Pancreata were prepared for histological analysis by either immediate freezing of the tissue in OCT compound (Miles, Elkhart, IN), 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 insulitis. 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 Dastix (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-TNFα transgenic mice. Cells were stained by incubating for 45 mm 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 FACScan® 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 × 10⁶ splenic cells were cultured in presence of 2.5 µg/ml ConA or 3 µg/ml anti-CD3 in 96-well plates in Brui↵ medium supplemented with 5% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and different concentrations of the antigen. Proliferation was measured by addition of 1 µCi of [³H]thymidine (International Chemical and Nuclear, Irvine, CA) for the last 18 h of 5-d culture, and incorporation of radioactivity was assayed by liquid scintillation counting. For anti-KLH responses, mice were immunized with 100 µg 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 × 10⁶ per well) in various concentration of KLH in brui↵ medium supplemented with 5% FCS. Proliferation was measured by uptake of [³H]thymidine as stated above.

**T Cell Proliferation in Response to Eel Extracts and GADp524-543.** Spleen cell proliferation in response to eel extracts or GADp524-543 (SR1SKVAPVIKRMMEPYGT) were measured by culturing spleen cells (1 × 10⁶/well) obtained from RIP-TNFα 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 [³H]thymidine as mentioned above.

**Cytokine Assays.** Assays for cytokine production by spleen cells from RIP-TNFα transgenic or non-transgenic mice were done by culturing 1 × 10⁶ purified CD4+ T cells with 5 × 10⁵ irradiated (3,000 R) splenic cells from immunonodulated NOD mice and different amounts of the GADp524-543 in Brui↵ medium, supplemented as described above. After 24 h of culture, 100 µl of culture supernatant was removed from each well for IL-2 measurements. For IL-2 and IFN-γ 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 µg/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 previously, and were routinely maintained by our laboratory (33).

**Adoptive Transfer of Diabetes.** Adult female RIP-TNFα 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-TNFα mice (6-10 × 10⁶ cells/mouse) or CD8 cloned T cells 6 × 10⁶ 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-TNFα.** We used the same construct that we have described previously that directs the expression of substantial levels of TNFα in (C57BL/6 × 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 TNFα 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 TNFα in supernatants from cultured islets. In situ hybridization in those studies showed that the
TNFα 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-TNFα by Southern analysis (27). After preliminary characterization, line V 93.3 was selected and further bred with NOD mice to monitor the phenotypic effects. Expression of TNFα 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 TNFα was secreted by islets obtained from RIP-TNFα transgenic mice after 48 h of culture, whereas no TNFα was produced by the islets of control mice cultured under similar conditions (Fig. 1). These results indicate that TNFα is expressed and secreted by islets of transgene positive mice. To determine at what age TNFα is first expressed in RIP-TNFα mice, islets obtained from mice of various ages were tested for production of TNFα in vitro. TNFα could not be detected in mice of 4 wk old, whereas measurable amounts of TNFα 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 TNFα. No TNFα could be detected in serum of transgenic mice at any age (data not shown). This result indicates that TNFα is only locally expressed in islets of Langherhans.

Expression of TNFα in the Pancreas of Transgenic NOD Mice Results in Insulitis. Our previous studies indicated that the RIP-TNFα transgene mediates dramatic infiltration of lymphocytes in the islets of Langerhans in mice generated on the (C57BL/6 × CBA)F2 background that had been backcrossed to C57BL/6 (27). We therefore monitored the effects of RIP-TNFα transgene on insulitis as a function of age in NOD mice. Histological examination of pancreata from RIP-TNFα transgenic mice reveals that mononuclear...
Figure 3. Insulin and glucagon staining of pancreas from RIP-TNFα transgenic mice (A and C) and negative transgenic litter mate 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 ~7 wk old. This infiltration (insulitis) 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-TNFα 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-TNFα transgenic mice (data not shown).

Expression of TNFα in Islets Prevents the Development of Spontaneous Diabetes in NOD Mice. RIP-TNFα 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 TNFα in islets dramatically retarded the development of IDDM. Whereas ~80% of female NOD transgene-negative mice developed diabetes by 25 wk, at this time only ~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 TNFα mediated substantial infiltration into the islets of Langerhans, this did not lead to autoimmune diabetes.

Expression of TNFα 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 TNFα expression in islets on lymphocyte devel-
that the protection offered by the RIP encoded transgene (TNFα) 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-TNFα 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-TNFα transgenic mice exhibited recall proliferative responses similar to that of non-transgenic litter mates, indicating that local expression of TNFα 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 TNFα in islets does not cause general suppression of the immune system.

Expression of TNFα 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-TNFα 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-TNFα transgene. To address this we examined the spontaneous T cell responses and serum antibody levels to a well-characterized 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 TNFα in islets, splenic cells from both RIP-TNFα 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-TNFα 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

<table>
<thead>
<tr>
<th>Spleen cell proliferation</th>
<th>αCD3 (µg/ml)</th>
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<tr>
<td>Con-A (µg/ml)</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>0.1</td>
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<tr>
<td>2.0</td>
<td>5.0</td>
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<tr>
<td>10.0</td>
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<table>
<thead>
<tr>
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</tr>
<tr>
<td>4.03</td>
</tr>
<tr>
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<td>10.18</td>
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<td>3.4</td>
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<tr>
<td>5.17</td>
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<tr>
<td>10.40</td>
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<tr>
<td>14.53</td>
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<tr>
<td>RJP-TNF</td>
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<tr>
<td>4.67</td>
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<td>15.89</td>
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<td>15.54</td>
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<td>18.78</td>
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Lymph node T cells purified from unimmunized RIP-TNFα 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 [3H]thymidine and data are presented as stimulation index.

development, we carefully examined the various subpopulations of lymphocytes in lymphoid organs in RIP-TNFα transgenic mice. Both RIP-TNFα 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-TNFα and non-transgenic litter mates were indistinguishable, indicating that local expression of TNFα 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-TNFα transgenic mice.

Expression of TNFα in the Pancreas of Transgenic NOD Mouse Does Not Induce Generalized Immunosuppression. Since expression of TNFα in islets prevents the development of spontaneous diabetes in transgenic mice, one could argue
Figure 5. Flow cytometry analysis of various subpopulations of lymphocytes in lymphoid organs. Both RIP-TNFα transgenic mice and non-transgenic 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.

Significant 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 IFNγ, whereas no such production of cytokines could be detected from spleen cells from RIP-TNFα transgenic mice (Fig. 7, B–D). In addition, serum from both non-transgenic and RIP-TNFα transgenic mice were tested for the presence of anti-GAD antibodies. The levels of anti-GADp524-543 antibodies were significantly lower in RIP-TNFα transgenic mice when compared with non-transgenic litter mates (Fig. 7 e). In addition, proliferative responses of splenic cells from RIP-TNFα and non-transgenic litter mates were measured against islet extracts obtained from NOD mice or from RIP-TNFα transgenic mice. Again a lack of proliferative response was seen in RIP-TNFα transgenic mice, whereas non-transgenic mice responded well (Fig. 7 a). These results demonstrated that local expression of TNFα had profound effect on the development of autotantigen 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-TNFα transgenic mice, it can be concluded that expression of TNFα 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-TNFα transgenic mouse (○) and negative litter mate control mouse (□) 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 [3H]thymidine and data are presented as stimulation index.
Adaptively Transferred Splenic Cells from RIP-TNFα Transgenic Mice Fail to Cause Diabetes in NOD-SCID Mice. To understand the mechanism of protection offered by local expression of TNFα in islets, we adaptively transferred splenic cells from diabetic NOD mice or from age matched RIP-TNFα 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-TNFα mice failed to cause diabetes in NOD-SCID recipients (Table 2). We also adaptively 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-TNFα transgenic mice lacked this capability. The inability of spleen cells from RIP-TNFα 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-TNFα transgenic mice, or autoreactive cells are present in the spleens of RIP-TNFα 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 TNFα expression in islets is not required to protect islets from a destructive immune response.

To distinguish these possibilities, we adaptively transferred a mixture of diabetic spleen cells and spleen cells obtained from RIP-TNFα transgenic 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-TNFα 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 TNFα 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.

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

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<tr>
<th>Source of Cells</th>
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<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
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<th>23</th>
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<tr>
<td>NOD (diabetic)</td>
<td>SCID</td>
<td>3/6</td>
<td>5/6</td>
<td>6/6</td>
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<tr>
<td>NOD (Non-diabetic)</td>
<td>SCID</td>
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</tr>
<tr>
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</tr>
<tr>
<td>RIP-TNFα + NOD (diabetic)</td>
<td>SCID</td>
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<td>2/10</td>
<td>4/10</td>
<td>8/10</td>
<td>8/10</td>
<td>8/10</td>
<td>9/10</td>
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<tr>
<td>NOD (Non-diabetic) + NOD (diabetic)</td>
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6–10 × 10⁶ 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-TNFα transgenic mice. Diabetes were monitored by urine glucose analysis at weekly intervals.

1970  Prevention of Diabetes by Transgene Encoded TNFα in Islets
Table 3. Transfer of Diabetes by Adoptive Transfer of CD8+ Cells into Irradiated Recipients

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>Recipients</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>15</th>
<th>16</th>
<th>19</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD (diabetic)</td>
<td>NOD</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
<td>2/3</td>
<td>3/3</td>
<td></td>
</tr>
<tr>
<td>G9-C8</td>
<td>NOD</td>
<td>1/4</td>
<td>1/4</td>
<td>3/4</td>
<td>3/4</td>
<td>4/4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD (diabetic)</td>
<td>RIP-TNFα</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
<td>2/3</td>
<td>3/3</td>
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<td></td>
</tr>
<tr>
<td>G9-C8</td>
<td>RIP-TNFα</td>
<td>1/4</td>
<td>3/4</td>
<td>4/4</td>
<td></td>
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</tr>
</tbody>
</table>

6 × 10⁶ diabetogenic cloned CD8+ T cells (G9-C8) or 10 × 10⁶ splenic cells depleted RBC from NOD (diabetic within two weeks) mice were transferred intravenously to irradiated (725 Rads) RIP-TNFα 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 TNFα, further studies including a detailed kinetics analysis by using various ratios of cells will be needed.

Local Expression of TNFα Does Not Protect Islets against Effector Cells. To determine if protection offered by local expression of TNFα in islets was mediated by the direct effects of TNFα 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-TNFα 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 TNFα does not block the functions of effector cells which are already primed in vivo (Table 4).

Discussion

Since TNFα 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 TNFα, 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 TNFα has not been explained. Recently, Yang et al. have shown that this situation is more complex, since administration of TNFα neonatally exacerbates the development of diabetes (30). These studies implied that when TNFα 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 TNFα were fundamentally pharmacologic in activity and regulated the autoimmune response at sites distal from islets or whether TNFα 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-TNFα 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-TNFα transgene prevents the development of diabetes in NOD mice and that TNFα 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

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>Recipients</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
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</tr>
</thead>
<tbody>
<tr>
<td>G9-C8</td>
<td>RIP-TNFα</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
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<td>4/5</td>
<td>4/5</td>
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</tr>
<tr>
<td>G9-C8</td>
<td>NOD</td>
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<td>4/5</td>
<td>5/5</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

6 × 10⁶ diabetogenic cloned CD8+ T cells (G9-C8) were transferred intravenously to RIP-TNFα transgenic mice. Diabetes were monitored by urine glucose analysis at weekly intervals.

1971 Grewal et al.
nity and not at the final effector phase. Our data are consis-
tent with those of McDevitt and colleagues (28, 30), who
have shown that administration of TNFα to adult NOD
mice leads to protection of diabetes. Our RIP-TNFα
transgenic mice fail to express TNFα in the neonatal peri-
ods where TNFα exacerbates disease and only start to ex-
press TNFα as adults (7 wk) at which time we also start to
see infiltration in islets.

There are a number of possible explanations for the ab-
sence of diabetes in the RIP-TNFα transgenic NOD mice.
First, local expression of TNFα may inhibit the homing of
autoreactive cells to the pancreatic islets; second, TNFα
cannot prevent the migration of autoreactive cells to islets
but rather inhibit their ability to initiate or sustain islet spe-
cific destructive response; third, auto-reactive cells may fail
to develop in the RIP-TNFα transgenic mice; fourth, TNFα
may alter the properties of islets and make them resis-
tant for destructive attack by autoreactive cells; fifth,
TNFα may down regulate expression of autoantigens on
islets required for the recognition for successful destructive
response; finally, expression of TNFα 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
everal of the possibilities mentioned above; they also fur-
ther provide evidence for the mechanism whereby TNFα
inhibits development of diabetes. The lack of development
of diabetes in RIP-TNFα transgenic mice is not due to in-
hibition 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 insulitis in control NOD mice
(27). Despite massive infiltration, immunohistochemistry
indicates normal insulin levels and a lack of destruction of
β cells in RIP-TNFα transgenic mice. These observations
suggested that infiltrating cells in islets of RIP-TNFα mice
were unable to initiate or sustain an autoimmune destruc-
tive response.

Local expression of TNFα in islets does not lead to the
general suppression of immune responses, since in vitro
proliferative responses of T cells from RIP-TNFα are com-
parable to those of NOD mice. In contrast, whereas re-
sponses to KLH are normal in RIP-TNFα transgenic mice,
specific autoimmune responses to the β cell autoantigen GAD
and autoantigens contained in islet extracts were inhibited.
RIP-TNFα transgenic mice showed reduced immune re-
sponses to islet auto-antigens since the antibody response,
spleen cell proliferation and cytokine production to a pep-
tide fragment of GAD is substantially reduced in these ani-
mals 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 × 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 cor-
relate with insulitis in NOD mice. Thus, our result suggests
that TNFα abrogates, at least in part, the anti-islet immune
response.

To determine if local expression of TNFα 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-TNFα transgenic NOD mice to recipient NOD-
SCID mice. Spleen cells from diabetic NOD mice induced
diabetes in NOD-SCID mice rapidly, ~3 wk after transfer.
However, spleen cells from age matched RIP-TNFα trans-
genic 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-
TNFα transgenic mice, suggesting that local expression of
TNFα prevents the development of autoreactive cells.

These studies provide a potential explanation of the fas-
cinating results of McDevitt’s group (28). Our data suggest
first that the TNFα mechanism is mediated by local events
in the islets of Langerhans since RIP-TNFα transgenic mice
do not have detectable levels of systemic TNFα. We fur-
ther show that the effect of TNFα on the inhibition of
development of disease is a long-lasting effect on the lym-
phocytes, rather than a short-term inhibition of effector
function, since transfer of spleen cells from TNFα trans-
genic mice to NOD-SCID recipients does not provoke di-
abetes. Indeed there is no apparent inhibition of effector
cell function since active effector cells can transfer diabetes
to RIP-TNFα NOD mice (see below).

The possibility that a regulatory cell population controls
the function of autoreactive cells present in the spleen of
RIP-TNFα transgenic mice is unlikely but cannot be con-
clusively 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-TNFα mice to NOD-SCID recipients, using differ-
ent ratios and perhaps different subsets of T cells is required
before making definitive conclusions. We considered the
possibility that the protective effect mediated by TNFα in
RIP-TNFα transgenic mice might require the continuous
presence of TNFα, for example, to inhibit the action of
autoaggressive T cells. To test this possibility, we adop-
tively transferred diabetic NOD spleen cells or cloned dia-
betogenic CD8 T cells to irradiated NOD or RIP-TNFα
transgenic mice recipients, and cloned CD8 T cells were
also adoptively transferred to non-irradiated recipients. Our
data indicated that local expression of TNFα does not pro-
tect against effector cells which are already primed in vivo.
Taked together, therefore, our results show that local pro-
duction of TNFα in the islets of Langerhans alters the im-
une response to this tissue, thereby preventing the devel-
opment of disease. The protective effect of TNFα, does
not appear to require the presence of this cytokine; instead
this cytokine conveys a long-term protective effect by alter-
ing the autoaggressive potential of cells from these animals.
Adoptive transfer experiments suggest a role for TNFα sig-
nificantly earlier than the delivery of effector function.
The dramatic results that we have seen with TNFα 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 both IL-6 and TNFα transgene mice, but in contrast to TNFα, the inflammatory infiltrate in IL-6 transgene mice 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 IFNα production (38). An alternative hypothesis must be considered, however, and that is that the cytokines TNFα 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 TNFα and a diabetogenic TCR are in progress to resolve these issues.

We are thankful to Thomas Taylor for FACS® analysis and Martha Aliten for isolation of pancreatic islets.

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