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Role of Transforming Growth Factor Beta Secreted from CD4+ Suppressor T Cells in the Prevention of Autoimmune Diabetes in Nonobese Diabetic Mice

by

Hye-Seung Han

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ABSTRACT

Insulin-dependent diabetes mellitus (IDDM) in the nonobese diabetic (NOD) mouse results from immune cell-mediated autoimmune processes directed against pancreatic β cells. The CD4+ T cell clone, NY4.2, isolated from lymphocytes infiltrating the pancreatic islets of NOD mice was demonstrated to block these β cell-specific autoimmune processes. This study was initiated to determine how these CD4+ suppressor T cells are able to inhibit immune cell-mediated β cell destruction in NOD mice. I firstly found that NY4.2 T cells had a significant immunosuppressive effect on the proliferative responses of splenic T cells from NOD mice *in vitro*. This suppressive activity of NY4.2 T cells was attributed to transforming growth factor β (TGFβ) secreted by them.

I, therefore, determined the effect of TGFβ on the function of immune cells such as T cells and macrophages involved in β cell destruction in NOD mice. I found that TGFβ inhibited the proliferation of NOD splenic T cells and had an inhibitory effect on the secretion of type 1 cytokines such as IFNγ and IL-2 in NOD splenocytes. In

contrast, TGFβ did not affect the secretion of type 2 cytokines such as IL-4 and IL-10. I also found that the function of macrophages from NOD mice was suppressed by TGFβ, as demonstrated by TGFβ-mediated inhibition of the production of cytotoxic molecules by them.

I subsequently investigated the molecular mechanisms for the influence of TGFβ on immune cell function. I found that TGFβ interfered with the signal transduction pathways induced by IL-2, a T cell growth factor, in T cells, resulting in the inhibition of IL-2-dependent T cell proliferation. I also found that TGFβ inhibited the signaling pathways directing the activation of transcription factors NFκB and AP-1 involved in the expression of the inducible nitric oxide synthase (iNOS) gene, leading to the suppression of nitric oxide (NO) production in LPS-stimulated macrophages.

On the basis of these findings, I conclude that TGFβ-mediated suppression of the activation of type 1 T cells and macrophages may be responsible for the prevention by TGFβ-producing CD4+ suppressor T cells of immune cell-mediated pancreatic β cell destruction in NOD mice.

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AP-1	activator protein-1
APC	antigen presenting cells
BSA	bovine serum albumin
cDNA	complementary DNA
ConA	concanavalin A
cpm	count per minute
CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
EAE	experimental allergic encephalomyelitis
EDTA	ethylenediaminetetraacetate
ELISA	enzyme-linked immunosorbent assay
EMC	encephalomyocarditis virus
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAD	glutamic acid decarboxylase
HBSS	Hank's balanced salt solution
HLA	human leukocyte antigen
h	hour(s)
IDDM	insulin-dependent diabetes mellitus
IFNγ	interferon gamma
Ig	immunoglobulin
IκB	inhibitor for NFκB
IKK	IκB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
IRF	interferon regulatory factor
JAK	*Janus* kinase
JNK	c-Jun N-terminal kinase
kDa	kilodalton
KRV	Kilham rat virus
LCM	lymphocyte complete medium
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
mAb	monoclonal antibody
MHC	major histocompatibility complex
min	minutes
mRNA	messenger RNA
NFκB	nuclear factor for κB

NO	nitric oxide
NOD	nonobese diabetic
OD	optical density
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
SCID	severe combined immunodeficiency
SD	standard deviation
SDS	sodium dodecyl sulfate
STAT	signal transducers and activators of transcription
TCR	T cell receptor
TGFβ	transforming growth factor beta
Th1	T helper 1 cells
TNF	tumor necrosis factor

1. INTRODUCTION

1.1 Insulin-dependent diabetes mellitus (IDDM)

Chronic hyperglycemia and disturbances of metabolism characterise diabetes mellitus (DM), which results from absolute or relative deficiencies in insulin secretion and/or action. Diabetes can be generally divided into two groups: insulin-dependent (type 1) and non-insulin-dependent (type 2). Patients with insulin-dependent diabetes mellitus (IDDM) require insulin injection to control hyperglycemia and prevent ketoacidosis. IDDM accounts for about 10% of diabetes patients and the mean age for the onset of the disease is about 11 years (Rossini et al., 1993). While Orientals or Native Americans have a fairly low incidence of IDDM, in northern European countries, IDDM affects many children and young adults (Bennett, 1994).

1.2 The nonobese diabetic (NOD) mouse

Since only patients with clinical symptoms can be generally studied, proper animal models are necessary to investigate the pathogenesis of human IDDM. The nonobese diabetic (NOD) mouse and the biobreeding (BB) rat develop spontaneous diabetes similar to human IDDM, allowing us to study them before the onset of the autoimmune responses

with genetic and immunologic manipulation (Bach, 1994; Yoon et al., 1995). In spite of some differences, such as higher incidence of IDDM in female NOD mice and lymphopenia in BB rats, there is indeed considerable similarity between these models and the human disease at the clinical, genetic, and immunologic levels. These include the polygenic control of IDDM by MHC class II alleles and multiple non-MHC loci, the transfer of disease by bone marrow-derived hematopoietic stem cells, the early appearance of an intra-islet inflammatory infiltrate (insulitis), the presence of antibodies and T cells directed against islets, and the blockade of disease progression by the modulation of T cell function (discussed in detail below). This thesis focuses on the NOD mouse as a model for human IDDM.

The NOD mouse was established in Japan in the late 1970s (Makino et al., 1980). It was distributed worldwide, and used to establish numerous colonies. These colonies differ in the frequency and the age of onset of IDDM, due to multiple environmental factors such as virus infections. Pancreatic β cell destruction in NOD mice is preceded by the infiltration of first dendritic cells and macrophages and then T cells and B cells into the perivascular duct and

peri-islet regions of the pancreatic islets of Langerhans (peri-insulitis) beginning at 3-4 weeks of age. This is followed by the slow, progressive, and selective destruction of insulin-producing pancreatic β cells by 4-6 months of age (Delovitch and Singh, 1997; Yoon et al., 1998). Both female and male NOD mice exhibit a nondestructive peri-insulitis. Females tend to develop a more invasive and destructive insulitis, leading to a higher incidence (80-90%) of IDDM than males (10-40%). Humans do not show this marked female gender bias. In addition to diabetes, NOD mice develop thyroiditis, sialitis, and, late in life, autoimmune hemolytic anemia. These extrapancreatic autoimmune manifestations, including thyroiditis, are also found in human diabetic patients with female predominance.

1.3 The etiology of IDDM

Although IDDM is an ancient and prevalent disease, its pathogenesis has been remained elusive. It is still not known what triggers it. Only the sequence of events during the development of IDDM and the genetic and environmental factors which influence its course have been a little

understood. It is now accepted that IDDM results from complex interactions between genetics and the environment, which eventually result in the selective destruction of insulin-producing pancreatic β cells via autoimmune processes.

1.3.1 Genetic factors of IDDM

IDDM has long been known as a hereditary disease based on the relatively high rate of familial transmission (Bach, 1994). The disease concordance rate is 35-40% in identical twins and 15-17% in HLA-identical siblings, compared to approximately 1% for HLA-discordant siblings. However, the disease concordance rate in identical twins is likely to be less than 40% since twins share more environmental factors than unrelated individuals.

Regarding the role of MHC molecules in antigen presentation to T cells, MHC genes are obvious candidate predisposition genes for autoimmune diseases, although the association of MHC genes with IDDM was discovered before their role was found (Signal and Blajchman, 1973). Epidemiological studies have revealed that the susceptibility to IDDM strongly correlated with DR3 and DR4

of MHC class II genes, while DR2 is associated with the resistance to IDDM in Caucasian population (Bach, 1994). More precise analytical techniques have demonstrated that the location of the HLA DQ subregion rather than the DR subregion determines the susceptibility to IDDM. Furthermore, a strong correlation between the residue at position 57 of the DQβ chain and susceptibility or resistance to IDDM was revealed (Tisch and McDevitt, 1996).

The importance of the MHC gene in the pathogenesis of IDDM is confirmed in NOD mice. Diabetes was prevented by the introduction of various MHC transgenes different from the NOD MHC class II I-A (Slattery et al., 1990; Miyazaki et al., 1990; Lund et al., 1990), class II I-E (Nishimoto et al., 1987; Bohme et al., 1990; Lund et al., 1990), or class I (Miyazaki et al., 1992) gene. In addition, congenic mice genetically identical to NOD mice except for the MHC gene did not develop diabetes (Wicker et al., 1992). Similar to humans, the onset of diabetes in NOD mice is closely related to the absence of Asp at the position 57 of I-Aβ chain. However, this does not entirely explain the role of the MHC, since transgenic expression of I-A genes without Asp at position 57 of the I-Aβ chain can protect NOD

mice from diabetes (Miyazaki et al., 1990; Lund et al., 1990). In addition, NOD mice do not express the I-E gene and transgenic expression of the I-E gene protected NOD mice from IDDM.

Difference in the disease concordance rate in identical twins (35-40%) and HLA-identical siblings (15-17%) suggests the involvement of non-MHC genes in the predisposition to IDDM. More than fifteen non-MHC predisposition loci were identified in NOD mice and the number is still increasing (Todd et al. 1991; Bach, 1994). The nature and expression of most of these genes are unknown. One of the genes on chromosome 1 could be *bcl2*, a proto-oncogene known to have anti-apoptotic function. Delayed T cell apoptosis shown in NOD mice could induce the survival and activation of autoreactive T cells. The other gene on chromosome 3 is close to the IL-2 gene.

1.3.2 Environmental factors of IDDM

Several lines of evidence point to the role of environmental factors in the pathogenesis of IDDM (Bach 1994; Yoon et al., 1995). First, more than 60% of identical twins are discordant for the development of IDDM. Second, the frequency of IDDM varies from country to country, not

due to ethnic genetic differences. Third, the incidence of IDDM is increasing in most countries, strongly pointing to an environmental influence. Finally, the incidence of diabetes can be affected in animal models by a number of nonimmunological interventions including viruses and diets.

The development of IDDM was inhibited in NOD mice by infections with the mouse lymphocytic choriomeningitis virus (Oldstone 1990), or murine hepatitis virus (Wilberz et al., 1991). The mechanisms of this virus-associated protection are not clear, but these viral infections probably explain the difference in disease frequency between NOD colonies. In contrast, some viruses were shown to induce diabetes (Yoon et al., 1995). The induction of diabetes by viruses could result from either by a direct cytolytic effect or by triggering β-cell-specific autoimmunity. Several ways in which viruses might trigger β-cell-specific autoimmunity include through T cell responses to viral neoantigens expressed on β cells, stimulation or suppression of specific subpopulations of lymphocytes, or crossreactivity of the immune response to the neoantigens and β-cell autoantigens.

Low essential fatty acid (Lefkowith et al., 1990) and protein diets (Issa-Chergui et al., 1988), independent of their direct glycemic effects, were shown to delay the onset of IDDM in NOD mice and BB rats, probably by interfering with immune responses directed against islets. By contrast, cow's milk accelerates the course of diabetes in BB rats (Elliott and Martin 1984).

1.4 IDDM as an autoimmune disease

Autoimmune diseases result from the pathogenic effect of autoantibodies or autoreactive T cells that induce inflammation, functional alterations, or anatomical lesions. There is abundant evidence that IDDM is an autoimmune disease in which insulin-producing pancreatic β cells are selectively destroyed by autoimmune responses directed against them.

1.4.1 Transfer of IDDM by T cells

Many studies using NOD mice showed that spleen cells from diabetic animals transferred IDDM into neonatal, irradiated young, and genetically immunocompromised NOD mice (Bendelac et al., 1987; Haskins and McDuffie, 1990;

Matsumoto et al., 1993; Christianson et al., 1993). Islet-infiltrating lymphocytes from prediabetic NOD mice transferred diabetes more successfully (Rohane et al., 1995). Using purified T cell populations and T cell clones derived from spleen or islets of NOD mice, it has been shown that T cells are responsible for the transfer of the disease (Shimizu et al., 1993).

Similarly, in humans, diabetes was transferred to the recipient after bone marrow transplantation with a diabetic donor (Viallettes and Maraninchi, 1993), although it cannot be ruled out that non lymphoid cells present in the donor bone marrow could be responsible for the transfer of the disease. In addition, the recurrence of diabetes was observed after pancreas transplantation between identical twins (Sibley et al., 1985), likely due to the infiltration of the transplanted pancreas by the recipient autoimmune cells.

1.4.2 Delay or prevention of IDDM by immunosuppressive therapy

The length and frequency of remissions of IDDM were increased by immunosuppressive agents such as cyclosporin A (CsA) and azathioprine plus steroids in patients with

recent onset of IDDM (Feutren et al., 1986). In NOD mice, many immunosuppressive therapies targeting T cells, such as CsA administration, neonatal thymectomy (Like and Kislaskis, 1982) and the administration of antibodies to T cells (Shizuru et al, 1988), could slow β cell destruction. The effect is better when the treatment is applied early, but some significant effect is still seen at the onset of IDDM.

1.4.3 Humoral or cellular immune responses against the pancreatic β cells

Humoral immune responses to islet cells were observed in both humans and animal models. The islet-cell antibodies (ICAs) were detected by indirect immunofluorescence on human pancreas sections (Bottazzo et al., 1974), which was the first strong evidence for the autoimmune origin of IDDM. Autoantibodies have been found against membrane and cytoplasm constituents of β cells, including insulin (Palmer et al., 1983), proinsulin (Kuglin et al., 1990), and glutamic acid decarboxylase (GAD) (Baekkeskov et al., 1990).

T cell reactivity to islet antigens in humans was shown by proliferation assays using human islets (Harrison et al., 1992) and GAD (Honeyman et al., 1993). The anti-islet T cell response was best shown in the NOD mouse, as demonstrated by the establishment of islet-specific T cell clones which can transfer autoimmune diabetes (Haskins and McDuffie, 1990; Shimizu et al., 1993).

1.4.4 Tolerance induction by the β-cell autoantigens

Since the induction of IDDM by sensitization against an autoantigen cannot be studied in humans, it has been studied only in animal models. Candidate β-cell autoantigens such as GAD65, insulin, or proinsulin are unable to induce IDDM upon active immunization of NOD mice, and only a transient hypergycemia and insulitis are elicited by a 60 kDa heat shock protein (HSP60) or its peptide (Elias et al., 1995; Delovitch and Singh 1997). Rather treatment with GAD65 (Kaufman et al., 1993; Tisch et al., 1993), insulin (Zhang et al., 1991), or HSP60 (Elias et al., 1991) protected NOD mice from IDDM by downregulating immune responses to the candidate β-cell autoantigen.

1.4.5 Indirect evidence

There is abundant indirect evidence to support the autoimmune nature of IDDM. It includes the infiltration of the pancreatic islets by mononuclear cells (insulitis), concomitant appearance with other autoimmune diseases, association of IDDM susceptibility with MHC genes, and anomalies of the immune system not directly linked to islet cell autoreactivity.

1.5 Immunopathogenesis of IDDM

Studies in the NOD mouse suggest that the diabetogenic response can be considered a series of stages culminating in massive β cell destruction and the development of overt diabetes. Peri-insulitis is first observed at 4-6 weeks of age, characterized by an accumulation of macrophages, dendritic cells, and lymphocytes that enter the peri-ductal areas but remain outside of the islet. Later, intra-insulitis dependent on the recognition of β cell autoantigens develops, characterized by the direct invasion of the islets by infiltrating cells. It was suggested that only a few β cell autoantigens are targeted in the early stages in NOD mice (Kaufman et al., 1993; Tisch et al.,

1993). As intra-insulitis progresses, additional β cell destruction occurs, leading to the sensitization and recruitment of other β cell-specific T cells with different antigen specificity. However, intra-insulitis itself does not appear to be sufficient to develop overt diabetes. This is supported by a study using transgenic NOD mice expressing a pathogenic β-cell specific T cell receptor (TCR) that exhibited intra-insulitis beginning at 3-4 weeks of age, but the time of onset (18-20 weeks) and the incidence of diabetes is only marginally enhanced (Katz et al., 1993). Thus, additional events are required for the development of overt diabetes. These events may depend on the result of interactions between effector immune cells involved in β cell destruction and regulatory T cells (discussed in detail below) or sequential targeting of specific β cell autoantigens.

1.5.1 Candidate β-cell autoantigens

The identification of β-cell autoantigens is a major challenge for pathogenesis, immunological diagnosis, and immunotherapy of IDDM. The diabetogenic response is antigen driven, as implied by the association of disease

susceptibility with the MHC genes. This is supported by the observation that the ablation of β cells by treatment with alloxan, a toxic agent specific for β cells, at an early age abrogated the ability of T cells to adoptively transfer IDDM in NOD mice (Larger et al., 1995). These results suggest that the presence of β cell autoantigens is required for the activation of diabetogenic T cells. Additional evidence for antigen-driven autoimmunity in IDDM is that immunization with antigenic self-peptides but not with adjuvant alone induced autoproliferative responses in NOD mice (Ridgway et al., 1996).

The importance of a given β cell autoantigen in IDDM is based on two criteria: correlations of the reactivity of autoantibodies and T cells with disease progression, and the modulation of the diabetogenic responses by treatment with the autoantigen or T cells specific for it.

GAD is one of the critical β cell autoantigens. GAD has two isoforms, GAD65 and GAD67, and catalyzes the biosynthesis of the neurotransmitter γ-aminobutyric acid. The presence of anti-GAD antibodies in the sera of prediabetic individuals can be used as a reliable predictive marker for progression to clinical diabetes

(Baekkeskov et al., 1990). NOD mice also exhibit antibody reactivity to GAD. Immune responses to GAD and insulin, but not to other β cell autoantigens, can be detected in NOD mice at an early age even when minimal islet inflammation is observed (Kaufman et al., 1993; Tisch et al., 1993). Some NOD mice that have extensive intra-insulitis, but are diabetes free exhibit anti-GAD reactivity (Tisch et al., 1993). These observations suggest that the recognition of GAD and insulin occurs early in the disease process, and that anti-GAD reactivity may mediate initial events associated with intra-insulitis. Treatment with GAD, either at an age preceding islet inflammation or exhibiting extensive intra-insulitis, protected NOD mice from diabetes (Kaufman et al., 1993; Tisch et al., 1993). A GAD-reactive T cell clone, which secreted type 1 cytokines, was recently shown to induce diabetes in NOD/*scid* mice (Zekzer et al., 1998), supporting the importance of anti-GAD autoimmunity in the pathogenesis of IDDM. Recently, the important role GAD plays in the development of autoimmune diabetes was corroborated by experiments using transgenic NOD mice expressing a GAD antisense gene exclusively in β cells,

resulting in the suppression of GAD expression in β cells and no diabetes (Yoon et al., 1999).

Insulin is another β-cell autoantigen that plays a role in the diabetogenic response. Anti-insulin autoantibodies are often detected in prediabetics before treatment with insulin (Palmer et al., 1983). Oral treatment of young NOD mice with whole insulin, insulin B chain or proinsulin protected animals from diabetes (Zhang et al., 1991). Furthermore, insulin B chain-specific CD4+ T cell clones accelerated diabetes in young NOD mice or adoptively transferred IDDM in NOD/*scid* mice (Daniel et al., 1995).

During the progress to diabetes in humans, additional autoantibodies to two tryptic fragments with molecular masses of 37 and 40 kDa, derived from β cells are detected. These autoantibodies have been detected in 60% of newly diagnosed individuals and appear to identify IDDM patients who rapidly progress to diabetes (Lan et al., 1996). It was recently found that the two tryptic fragments are derived from the putative tyrosine phosphatase IA-2 (Passini et al., 1995). IA-2, like GAD, is expressed primarily in pancreatic islets and brain, but its functional role remains unknown (Lan et al., 1994).

Autoantibodies and T cell reactivity against HSP60 have also been observed in NOD mice, while it remains unclear in the human. Treatment with HSP60 protected NOD mice from the disease and HSP60-specific CD4+ T cell lines were shown to accelerate or block the development of IDDM in NOD mice (Elias et al., 1991). Moreover, it was reported that treatment of diabetic NOD mice with an HSP60-specific peptide restored the normal levels of blood glucose (Elias and Cohen, 1994). Recently, transgenic NOD mice expressing HSP60 in MHC class II-positive cells were established and exhibited the reduced insulitis and diabetes by inducing a shift in immunodominant epitopes, probably resulting in the development of T cells with different cytokine profiles (Birk et al., 1996).

Thus, a number of β-cell autoantigens are recognized during the diabetogenic process and it is unlikely that the whole immune response toward a large number of β-cell autoantigens observed in diabetics is primary or pathogenic. At present, it is difficult to identify the initial diabetogenic antigen. It is proposed that the initial T cell-mediated β-cell lesions induce the release of degradation products that in turn elicit secondary immune

responses. This is suggested by the chronological appearance of T cell proliferative responses to several β-cell autoantigens in NOD mice (Tisch et al., 1993). In addition, the recent analysis of the TCR Vβ repertoire of islet-infiltrating T cells in very young NOD mice revealed that monoclonal TCR Vβ 8.2 gene product expressed at 2 weeks of age and that the progress of inflammatory responses to IDDM rapidly obscures the initial monoclonal nature of islet-infiltrating T cells (Yang et al., 1996). These results suggest that IDDM in NOD mice may be initiated by the recognition of a single autoantigen.

1.5.2 Effector immune cells

1.5.2.1 Macrophages

Macrophages have been implicated in the development of IDDM, based on their early appearance in the infiltrated islet (Jansen et al., 1994) and the ability of macrophage depletion by chronic silica treatment to prevent insulitis and diabetes (Lee et al., 1988; Ihm et al., 1990). The monoclonal antibody specific for type 3 complement receptor (CR3, CD11b/CD18 or Mac-1) on macrophages prevented intra-islet infiltration of T cells as well as macrophages, and

inhibited the development of IDDM (Hutchings et al., 1990). These results suggest that the early migration of macrophages into the islets is followed by the infiltration of T cells to the islets. The mechanism by which the macrophage promotes T cell recruitment is unclear.

It is proposed that macrophages accumulated in the pancreatic islets play a role not only in antigen processing and presentation to T cells but in the destruction of β cells by the release of β cell-toxic cytokines or free radicals. The role macrophages play in the pathogenesis of IDDM was recently reported in NOD mice (Jun et al., 1999). It was shown that macrophages are important for the development and activation of β-cell cytotoxic T cells, since T cells from macrophage-depleted NOD mice lost the ability to differentiate into β-cell cytotoxic T cells and be activated. In addition, macrophages play important roles in regulating T helper 1 (Th1) and Th2 cell balance, resulting mainly from their production of IL-12.

1.5.2.2 B lymphocytes

Although B cells or serum are not required for adoptive transfer of diabetes (Bendelac et al., 1988), it was recently suggested that B cells also play a role in the development of diabetes. The lack of B cells by a genetical manipulation (Serreze et al., 1996) or anti-B cell antibody treatment (Noorchashm et al., 1997) resulted in the prevention of insulitis and diabetes in NOD mice. It was recently demonstrated that B cells play an important role in the presentation of autoantigens such as GAD65 and HSP60 in NOD mice (Falcone et al., 1998).

1.5.2.3 T lymphocytes

T-cells can be divided into two major groups based on the expression of coreceptors for the T-cell receptor (TCR): CD4+ and CD8+ T cells. CD4+ T cells generally referred to as helper T cells (Th cells) due to their ability, upon antigen stimulation, to help other T cells to develop cytotoxic activity, and B cells to secrete antibodies. On the other hand, CD8+ T cells exert direct cellular cytotoxic effects. This classical immunologic concept implies that β-cell destruction in IDDM results from the killing of β cells by antigen-specific CD8+ T cells that

have been activated by CD4+ T-cells. However, as described below, other models are also likely to explain the mechanism for the destruction of pancreatic β cells.

Although it is known that T cells are important for the development of diabetes, how CD4+ and CD8+ T cells contribute to disease progression has remained an unresolved issue. Several groups have shown that both subsets are required to develop IDDM when spleen cells from diabetic NOD mice are transferred into neonatal, irradiated young, or genetically immunodeficient NOD recipients (Bendelac et al., 1987; Miller et al., 1988; Thivolet et al., 1991; Yagi et al., 1992; Christianson et al., 1993). It was shown that CD4+ T cells are clearly important in the development of insulitis and diabetes, as demonstrated by the ability of antibodies against CD4 to prevent both insulitis and diabetes (Shizuru et al., 1988). CD4+ T cells alone can invade the islets, but diabetes usually develops when CD8+ cells are also present, while CD8+ T cells alone do not infiltrate the islets (Thivolet et al., 1991; Yagi et al., 1992; Christianson et al., 1993). This result suggests that CD4+ T cells are the initiators of diabetes, and CD8+ T cells recruited by CD4+ T cells function as

effectors responsible for the final destruction of β cells. This proposition is supported by other findings. The infiltration of CD4+ T cells preceded CD8+ T cells into the islets when whole splenocytes from diabetic donors are transferred (O'Reilly et al., 1991). Injection of anti-MHC class I or anti-CD8 mAb into adult NOD mice reduced the development of diabetes but not insulitis (Taki et al., 1991). CD8+ T cell lines and clones with *in vitro* and *in vivo* cytotoxic activity against islet cells have been isolated (Nagata and Yoon, 1992; Nagata et al., 1994).

However, more recent results using NOD mice carrying a null mutation in the β_2-microglobulin (β_2m) gene did not support this proposition (Katz et al, 1993; Wicker et al, 1994). These animals, with almost no expression of MHC class I molecules and consequent poor development of CD8+ T cells, did not show insulitis or diabetes. In addition, treatment with anti-CD8 antibodies before 5 weeks of age protected NOD mice from both insulitis and diabetes (Wang et al., 1996). The role of CD8+ T cells in the initiation of IDDM is further supported by the induction of insulitis in β_2m-deficient NOD mice by the expression of MHC class I molecules on β cells (Kay et al., 1996), and by the

inability of T cells from young prediabetic NOD mice to transfer diabetes in NOD/*scid* mice with the β_2m mutation (Serreze et al., 1997). These results argue for the hypothesis that β cell-cytotoxic CD8+ T cells may mediate the initial β cell insult, triggering the shedding of β cell autoantigens and subsequent recruitment and activation of CD4+ T cells.

1.5.2.4 CD4+ T cell subsets

Although additional phenotypes have been suggested, in general, CD4+ T cell subsets are divided into Th1 and Th2 cells based on cytokines they produce and their functional roles in the immune system (Mosmann and Sad, 1996; Abbas et al., 1996; Rabinovitch 1998). Th1 cells secrete IFNγ, IL-2, and TNFβ, and are involved in cell-mediated inflammatory reactions such as delayed-type hypersensitivity reactions. Th2 cells produce IL-4, IL-5, and IL-10, and are involved in antibody, especially IgE, and allergic responses. These Th cell subsets regulate each other through reciprocal downregulatory effects mediated by their respective cytokines. The classification of Th cell subsets was also relevant *in vivo* as proven by their distinct abilities to

control immune responses to infections in animals. Similarly, CD4+ T cell subsets were suggested to be responsible for the induction and regulation of autoimmune diseases.

Th1 cells are believed to be the primary CD4+ T cells mediating IDDM. This is supported by observations from the administration of Th1 cytokines or antibodies specific for them to animals (discussed in detail below). In addition, β cell-specific T cell clones that exhibit a Th1 phenotype efficiently transferred diabetes in syngeneic young NOD recipients (Haskins and McDuffie, 1990; Shimizu et al., 1993; Katz et al., 1995). On the other hand, due to their ability to produce cytokines regulating Th1 cells, Th2 cells are suggested to have a protective role in IDDM. Again, animal studies administering Th2 cytokines support this proposal (discussed in detail below). In addition, Th2-like $CD45RC^{low}$ CD4+ T cells prevented diabetes in rats (Fowell and Mason, 1993).

A functional imbalance between these Th cell subsets is suggested to be a key factor to determine the development of IDDM. A high ratio of IFNγ/IL-4 production was shown to correlate with the destruction of transplanted

islets in NOD mice (Shehadeh et al., 1993). Analysis of the ratio of IFNγ/IL4 mRNA expressed in pancreatic islet-infiltrating leukocytes revealed that female and male NOD mice with high and low incidence of diabetes had a Th1 and Th2 bias, respectively (Fox and Danska, 1997). In addition, a recent study suggested that the roles of $CD45RB^{low}$ subset of CD4+ T cells in NOD mice were correlated with cytokine profiles (Shimada et al., 1996). Their function changed from protective to pathogenic during the development of disease, related to the increased IFNγ/IL-4 ratio. Functional analyses of T cells isolated from CD28-deficient NOD mice showing the promoted development of IDDM also demonstrated that the GAD-specific T cells produced cytokines with high Th1/Th2 ratio (Lenschow et al., 1996).

However, to view the regulation of the disease process strictly in terms of Th1 and Th2 subsets is an oversimplification. For example, CD4+ T cell clones with Th1 phenotypes established from NOD mice secreted an unknown factor, suppressing the adoptive transfer of diabetes (Akhtar et al., 1995), and an insulin-specific CD4+ T cell clone secreting Th1 cytokines inhibited diabetes (Zekzer et al., 1997). In addition, Th2 cells

expressing a diabetogenic TCR failed to mediate protection in NOD neonates (Katz et al., 1995), rather Th2 cells were shown to induce diabetes in immunoincompetent NOD/*scid* mice (Pakala et al., 1997). Moreover, immune deviation therapy inducing a shift from a Th1 to a Th2 phenotype was shown to exacerbate autoimmune disease in some situations (Genain et al., 1996), raising the doubt for use of immune deviation intervention to cure autoimmune diseases.

It is important to recognize that most cytokines are produced by more than one cell type. CD8+ T cells have also been shown to exhibit Th1- and Th2-like phenotypes (Sad et al., 1995), and the contribution of cytokines secreted by non-T cells such as macrophages must be considered. Thus, the development of a Th cell subset and subsequent outcome of the diabetogenic response involve a number of cell types and factors.

1.5.3 Effector molecules

The possible effector mechanisms by which immune cells destroy pancreatic β cells include antigen-non-specific ways such as cytokines and free radicals as well as antigen-specific ways such as Fas/FasL and perforin, requiring the

specific recognition and direct contact between immune cells and β cells.

1.5.3.1 Cytokines

The roles of cytokines in the pathogenesis and regulation of IDDM was investigated by the following approaches: 1) correlation of the expression of cytokines in the pancreatic islets with the development of diabetes, 2) *in vitro* and *in vivo* treatment with a cytokine, or transgenic expression of a cytokine in β cells, and 3) treatment with antibody specific for a cytokine, or cytokine gene knockout experiments.

1.5.3.1.1 Type 1 cytokines

Analysis of cytokine gene expression in the insulitis lesion revealed that the expression of IFNγ was correlated with β cell destruction in the NOD mouse (Rabinovitch et al., 1995). In addition, IL-12, a cytokine known to induce Th1 cell development, was also found to be expressed in the destructive insulitis lesions (Rothe et al., 1996).

It was shown that *in vivo* administration of IL-12 promoted the development of diabetes in NOD mice (Trembleau

et al., 1995), increasing IFNγ/IL-4 production by islet-infiltrating lymphocytes. In contrast, the treatment of IL-12 with different dose and frequency suppressed the development of diabetes (O'Hara et al., 1996). Thus, many factors such as the dose, frequency, and timing of initial administration influence the effect of systemic administration of a given cytokine on the incidence of diabetes. The transgenic expression of IFNγ in pancreatic β cells resulted in the development of insulitis and diabetes in nondiabetes-prone mice (Sarvetnick et al., 1988), and NOD mice expressing IL-2 in pancreatic β cells exhibited the accelerated development of diabetes (Allison et al., 1994).

The roles of type 1 cytokines in the development of diabetes have also been supported by studies using antibody against a cytokine. Administration of antibodies to IFNγ, soluble IFNγ receptor, or IL-2 receptor suppressed the development of diabetes in NOD mice (Campbell et al., 1991; Kelley et al, 1988). The treatment of NOD mice with the IL-12 antagonist was recently shown to prevent the development of cyclophosphamide-induced IDDM (Rothe et al., 1997). In addition, the mutation of the IFNγ receptor gene in NOD mice resulted in the inhibition of insulitis and prevention of

diabetes (Wang et al., 1997). However, the IFNγ gene knockout NOD mouse exhibited the development of diabetes with the delayed onset of IDDM (Hultgren et al., 1996).

1.5.3.1.2 Type 2 cytokines

The gene expression of type 2 cytokines IL-4 and IL-10 was shown to correlate with the protection of NOD mice from autoimmune diabetes (Shehdaeh et al., 1993; Fox and Danska, 1997).

Systemic administration of IL-4 (Rapoport et al., 1993), IL-10 (Pennline et al, 1994) and IL-10/Fc fusion protein (Zheng et al., 1997) resulted in the prevention of diabetes in NOD mice. The transgenic expression of IL-4 in pancreatic β cells led to the prevention of insulitis and diabetes in the NOD mouse (Mueller et al., 1996), inducing tolerance to islet antigens. Unexpectedly, studies using nondiabetes-prone mice expressing IL-10 in pancreatic β cells showed that IL-10 acted as a proinflammatory cytokine (Lee et al, 1994) and, indeed, transgenic expression of IL-10 on β cells promoted β cell destruction in NOD mice (Wongensen et al., 1994).

The evidence stated above generally supports the role of type 1 cytokines in the pathogenesis of IDDM and type 2 cytokine IL-4 in the regulation of diabetes. Some studies, however, have revealed results contradictory to the expectation, as shown by the experiments on the transgenic expression of IL-10. Thus, the role a given cytokine plays in the development of IDDM is likely to depend on the time of expression in β cells with regard to the development of the autoimmune response and the site of expression.

1.5.3.2 Cytotoxic free radicals

There is abundant *in vitro* evidence that islet β cells are sensitive to injury mediated by nitric oxide (NO) and oxygen free radicals, due to their very low free radical scavenging activity. Proinflammatory cytokines such as IL-1β and TNFα inhibited insulin secretion by inducing the production of NO (Southern et al., 1990). Intraislet release of IL-1 by nonendocrine cells such as macrophages was recently reported to inhibit insulin secretion by inducing β cell expression of the inducible form of nitric oxide synthase (iNOS) (Corbett and McDaniel, 1995).

In vivo evidence also suggests the role of NO in the development of IDDM. Treatment of NOD mice with aminoguanidine, a competitive inhibitor of iNOS, delayed the onset of diabetes (Corbett et al., 1993). It was recently shown that the production of NO in pancreatic islets correlated with the development of IDDM (Rabinovitch et al., 1996). Immunohistochemistry revealed the expression of iNOS by β cells as well as islet-infiltrating macrophages in NOD mice, suggesting that both macrophages and β cells contribute to the production of NO in islets of NOD mice developing autoimmune diabetes.

Peroxynitrite is a highly reactive oxidant species produced by the combination of NO and superoxide. Similar to NO, peroxynitrite was found to be cytotoxic to islet cells *in vitro* and produced by both macrophages (Horio et al., 1994) and islet β cells (Surez-Pinzon et al., 1997) of NOD mice developing autoimmune diabetes. This suggests that both nitrogen and oxygen free radicals can contribute to β cell destruction in IDDM via peroxynitrite formation in the islet β cells.

1.5.3.3 Fas/Fas ligand (FasL)

Apoptosis was suggested as the mechanism for the destruction of pancreatic β cells during the progression to the development of diabetes (O'Brien et al., 1997) and the Fas/FasL system is known to be one way to induce apoptosis (Nagata and Suda, 1995). The role of Fas/FasL-mediated β cell destruction in the development of IDDM was recently investigated using transgenic NOD mice expressing FasL on the surface of β cells (Chervonsky et al, 1997). These mice exhibited the accelerated development of diabetes and this was associated with Fas expression on β cells. When a mutation of the Fas gene was introduced into NOD mice, these mice did not develop diabetes and were rather resistant to adoptive transfer of diabetes by an islet-reactive CD8+ T cell clone. These results suggest that Fas expression by pancreatic β cells is necessary for β cell destruction by FasL-expressing activated T cells.

While normal human pancreatic β cells do not express Fas, Fas is expressed on β cells from newly diagnosed IDDM patients (Stassi et al., 1997). Fas expression in normal human pancreatic β cells was induced by IL-1 exposure, rendering β cells then susceptible to Fas-mediated

apoptosis, which also occurs in mice (Yamada et al., 1996). A NOS inhibitor prevented IL-1-induced Fas expression, whereas NO donors induced Fas expression on normal pancreatic β cells. These findings suggest that IL-1-induced Fas expression on β cells is mediated through NO production, contributing to pancreatic β cell damage in IDDM.

1.5.3.4 Perforin

Perforin is one of the contents in the granules of cytotoxic T lymphocytes (CTL) and, upon close contact of CTL with target cells, perforin released from CTL forms pores on the membrane of target cells and subsequent loss of membrane integrity and osmotic lysis.

The role of this perforin-dependent cytotoxicity in the development of IDDM was investigated using perforin knockout mice crossed with transgenic mice expressing glycoprotein of lymphocytic choriomeningitis virus (LMCV) in pancreatic β cells (Kagi et al., 1996). Upon LCMV infection, these mice developed insulitis but not diabetes, whereas transgenic mice expressing perforin developed diabetes. These results suggest that perforin-dependent cytotoxicity is essential for the destruction of β cells in

the final stages of diabetes development but not for the initiation of insulitis. It was more recently shown that perforin knockout mice in the NOD background did not develop diabetes (Kagi et al., 1997).

1.6 Immunoregulation of IDDM

It is a major feature of the immune system that lymphocytes are tolerant to self-antigens. Autoimmunity is believed to result from a breakdown of self-tolerance. T cell tolerance is mainly controlled in the thymus, where self-reactive T cells that survive after contact with self MHC molecules present on the thymic epithelium (positive selection) are eliminated by autoantigen driven apoptosis (negative selection) (Nossal et al., 1992). This mechanism does not, however, delete all autoreactive T cells, particularly specific for subdominant or cryptic epitopes of antigens and autoantigens not present in the thymus. These autoreactive T cells are controlled by either T cell anergy (unresponsiveness after antigen stimulation) or by active immune suppression. Thus, the breakdown of self-tolerance can occur through insufficient intrathymic negative selection, bypass of peripheral anergy, or defective suppression. It was recently demonstrated that

self-tolerance can be broken in NOD mice (Ridgway et al., 1994), as NOD but not other conventional mice mounted an immune response to the self-peptide after immunization with self-peptides.

1.6.1 Deletion

The deletion of T cells reactive with self-antigens expressed in the thymus is a major mechanism of T cell self-tolerance (von Boehmer and Kisielow, 1990). It was recently demonstrated that anatomically distinct sites in the thymus are responsible for the positive and negative selection of thymocytes (Laufer et al., 1996). The positive selection of thymocytes occurs in the thymic cortex via the recognition of the peptide/MHC complex on the thymic epithelial cells. In contrast, the thymic medulla is the place where macrophages and dendritic cells are involved in the negative selection.

It is proposed that the affinity and avidity of the T cell receptor (TCR) on thymocytes with the peptide/MHC complex determine their fate; a high avidity between the TCR on thymocytes and the peptide/MHC complex on the thymic stromal cells results in the negative selection of thymocytes, whereas a weak avidity rescues thymocytes from

death (Sebzda et al., 1994). It was found that, using a fetal thymic organ culture from transgenic mice expressing TCR specific for a viral peptide, the high and low concentration of the peptide led to a decrease and increase in the number of thymocytes bearing transgenic TCR, respectively.

Thus, MHC molecules play an important role in the deletion of autoreactive T cells in the thymus, influencing the autoimmune process in IDDM. A study using NOD mice transgenic for the rearranged TCR of a diabetogenic CD8+ T cell clone recently showed that the introduction of the MHC gene different from the NOD MHC gene resulted in the thymic deletion of T cells bearing transgenic TCR (Schmidt et al., 1997). The role of NOD MHC class II molecules in the pathogenesis of diabetes was further supported by the poor ability of the NOD MHC class II molecule I-A^{g7} to bind peptides (Carrasco-Marin et al., 1996), suggesting that poor binding of MHC molecules to self-antigen allows autoreactive T cells to escape to the periphery.

1.6.2 Anergy

Anergy refers to a functionally defective state of unresponsiveness to an antigen. Upon antigen stimulation,

anergic T cells do not produce IL-2 (Lenschow and Bluestone, 1993), due to defects in the signaling pathways induced by antigen stimulation. It was shown that anergic T cells exhibited defects in the transcriptional activity of activator protein (AP)-1 (Kang et al., 1992) and an early event in the TCR-mediated signaling pathway (Migita et al., 1995).

Full activation of T cells requires two signals through TCR and the costimulatory molecule CD28. Encounter of T cells with antigens without costimulatory signals renders T cells anergic (Harding et al., 1992). CD28 molecules on T cells interact with B7-1 or B7-2 molecules on antigen presenting cells, delivering a costimulatory signal (June et al., 1994). The role of costimulatory signals in the pathogenesis of autoimmune diseases was investigated using antibodies against B-7 molecules. Interestingly, treatment with anti-B7-1 and anti-B7-2 antibodies exerts different effects on distinct autoimmune diseases. Anti-B7-1 antibody slowed the progression of the disease in the EAE model (Kuchroo et al., 1995), by contrast, treatment with anti-B7-1 antibody exacerbated the progress of diabetes in NOD mice (Lenschow et al., 1995).

These apparently contradictory results in different autoimmune disease models have not been explained.

It was recently suggested that the development of Th2 cells is more dependent than that of Th1 cells on costimulatory signals from interactions between CD28 and B7 molecules (Lenschow et al., 1996). Antigen presenting cells from CD28 knockout NOD mice failed to induce the production of IL-4 by NOD T cells, leading to a deficiency in regulatory Th2 cells that compromise effector Th1 cells.

1.6.3 Suppression

Anergic T cell populations have the potential to be activated by their target self-antigens released into the lymphoid system during the course of infections, or by crossreactive epitopes present on infectious agents. Thus, this passive mechanism for self-tolerance may not be sufficient to completely control potentially pathogenic T cells. Evidence for active immune suppression has been accumulating, in which a distinct subset of cells suppresses the activation of autoreactive T cells that have escaped the passive mechanisms of tolerance. A number of experimental data suggest that a defect in these suppressor mechanisms may contribute to the development of diabetes

(Bach et al., 1990). The next chapter will cover these results in detail.

2. OVERALL OBJECTIVE

The existence of suppressor T cells as well as effector T cells has been suggested in the NOD mouse. The onset of diabetes is accelerated by the abrogation of suppressor mechanisms, such as thymectomy at 3 weeks of age (Dardenne et al., 1989) and the administration of cyclophosphamide, a drug known to have selective effects on suppressor T cells (Yasunami et al., 1988). In addition, the transfer of diabetes only occurs in immunodeficient recipients, such as neonates (Bendelac et al., 1987), and adults that were sublethally irradiated (Haskins and McDuffie, 1990) or thymectomized and treated with an anti-CD4 monoclonal antibody (Sempe et al., 1994). Moreover, spleen cells from I-A^d transgenic NOD mice that are protected from diabetes inhibited the ability of splenocytes from diabetic NOD mice to transfer diabetes (Singer et al., 1993).

In spite of the report showing the existence of a CD8+ T cell clone that prevented IDDM (Pankewycz et al., 1991), CD4+ T cells are believed to include a regulatory subset. The treatment with an anti-MHC class II antibody protected young NOD mice from diabetes, and the infusion of CD4+ T cells from these protected mice transferred protection to untreated mice (Boitard et al., 1988). CD4+ T cells from

the spleens of prediabetic female NOD mice delayed and inhibited the transfer of diabetes by spleen cells from diabetic mice into irradiated young NOD mice (Boitard et al, 1989) and into NOD/*scid* recipients (Rohane et al., 1995). Intriguingly, immunostimulation by the administration of staphylococcal superantigens (SEA and SEB) or complete Freund's adjuvant (CFA) prevented the onset of diabetes in NOD mice, and CD4+ T cells from treated mice transferred protection to untreated NOD mice (Kawamura et al., 1993; Qin et al., 1993). It was also reported that, by the administration of anti-CD4+ T cell antibodies, male NOD mice, which are less diabetes-prone than female NOD mice, became susceptible to diabetes transferred by diabetogenic spleen cells (Sempe et al., 1994).

Indeed, CD4+ T cell lines and clones, which exhibit the protective activity in the development of IDDM, have been established from the spleen of male NOD mice (Hutchings et al., 1990). These results suggest that diabetes-resistant animals possess a regulatory CD4+ T-cell subset that provides protection against the development of diabetes. However, the regulatory fraction appears to exist in the diabetes-prone animals as well, as shown by the isolation from islets of acutely diabetic NOD mice of CD4+

T cell lines and clones protecting NOD mice from IDDM (Reich et al., 1989; Utsugi et al., 1994). During the development of diabetes, regulatory T cells may be overwhelmed by the autoreactive T cells. Thus, the role that CD4+ T cells play is now considered important, directing the immune responses toward β cell destruction in the diabetes-prone animals, or toward protection in the diabetes-resistant animals.

As stated in the INTRODUCTION, CD4+ T cells can be generally divided into Th1- and Th2-type cells, dependent on the cytokine gene expression patterns (Mosmann and Sad, 1996; Abbas et al., 1996). It has been proposed that Th1-type autoreactive T cells mediate organ-specific autoimmune diseases involving T cells (Liblau et al., 1995). The immune balance between Th1-type and Th2-type cells is finely regulated in normal conditions. However, once the immune balance breaks down, resulting in a shift to the dominance of Th1 type cells, autoimmune diseases can develop clinically. In the EAE model, T cell clones that induce enchepholomyelitis preferentially exhibited Th1-type T cells, while Th2-type T cells protected against EAE in many cases (Miller and Karpus, 1994). Similarly, in the

inflammatory bowel disease model, Th1-like, $CD45RB^{high}CD4+$ T cells from BALB/c mice transferred colitis to C.B-17 SCID mice, whereas Th2-like, $CD45RB^{low}CD4+$ T cells inhibited the development of colitis (Powrie et al., 1994).

The abnormal shift of islet-reactive CD4+ T cells toward Th1-type cells is also suggested as a mechanism of defective immune suppression in NOD mice. This suggestion is supported by the high IFNγ/IL-4 ratio in invasive insulitis in contrast to the low ratio in noninvasive insulitis (Shehadeh et al., 1993), and by the delaying effect of IL-4 or anti-IFNγ antibody on the onset of diabetes in NOD mice (Rapoport et al., 1993; Campbell et al., 1991). In addition, diabetogenic T cell clones from NOD mice exhibit a Th1 phenotype (Haskins and McDuffie, 1990; Shimizu et al., 1993). Due to the reciprocal downregulating activity of Th1 and Th2 cells, Th2 cells have been proposed as a protective cell subset from the development of IDDM in NOD mice.

Thus, evidence for the existence of suppressor T cells has accumulated and an imbalance of Th1/Th2 cells has been suggested as a mechanism for the defects of immune suppression in NOD mice. However, none of studies have

determined how suppressor T cells can protect NOD mice from IDDM, in part due to the difficulty of *in vitro* cloning of suppressor T cells showing regulatory activities in the immune system *in vivo*. Fortunately, in our lab, such cloned suppressor T cells were established from the pancreatic islet-infiltrating lymphocytes of NOD mice (Nagata and Yoon, 1992; Utsugi et al., 1994). **The overall objective of this study is to elucidate the mechanisms by which CD4+ suppressor T cells prevent the development of IDDM in NOD mice.**

3. MECHANISMS FOR THE PREVENTION OF AUTOIMMUNE DIABETES BY A CD4+ SUPPRESSOR T CELL CLONE, NY4.2, IN NOD MICE

3.1 Introduction

Six CD4+ islet-reactive T cell clones with no cytotoxic activity to NOD islets were previously established from lymphocytes infiltrating the pancreatic islets of acutely diabetic NOD mice in our lab (Nagata and Yoon, 1992). It was found that one of these clones, NY4.2, proliferated in response to both islet cells and splenocytes from NOD mice, but not to spleen cells from other strains, including SJL, C3H, C57BL/6 and DBA-2 mice. Since these cells can proliferate in response to syngeneic antigen-presenting cells (APC) such as irradiated splenocytes in the absence of added foreign antigens, and this response is inhibited by anti-I-A^{g7}-crossreactive monoclonal antibody (10-2.16, data not shown), this clone is I-A^{g7}-restricted autoreactive T cells.

The murine syngeneic mixed lymphocyte reactions (MLR), and its human equivalent, the autologous MLR, which is present in all individuals, provide a model system for studying immunoregulatory mechanisms *in vitro*. It has been shown that autoreactive T lymphocytes, generated during syngeneic or autologous MLR by recognizing self-MHC class II molecules in the absence of foreign antigens, have

immunosuppressive activities *in vitro* (Weksler et al., 1981). The frequent association of impaired autologous MLR with autoimmune diseases including IDDM (Rasanen et al., 1988) further supports the hypothesis that autologous MLR-competent T cells may play a role in maintaining peripheral T cell tolerance to self antigens. Similarly, the NOD mouse exhibited deficient syngeneic MLR (Serreze and Leiter, 1988).

Indeed, autoreactive CD4+ T cells isolated from NOD mice prevented the development of IDDM (Reich et al., 1989; Chosich and Harrison 1993; Akhtar et al., 1995). In line with these reports, when autoreactive NY4.2 T cells were injected into NOD mice five times between the ages of 4 and 20 weeks, none of the animals became diabetic by 35 weeks of age (Utsugi et al., 1994). In contrast, about 75% of PBS-injected NOD mice, as a control, became diabetic by the same age. Similarly, when NY4.2 cells were transfused into acutely diabetic NOD mice 3 days prior to syngeneic islet transplantation, the grafted islets were not destroyed and the animals maintained normoglycaemia for 120 days without insulin or immunosuppressive drug therapy. In contrast, islet-transplanted NOD mice that received PBS, as a control, had islets that were massively infiltrated by

mononuclear cells and the animals developed recurrent diabetes within 20 days of receiving the graft (Utsugi et al., 1994). Thus, the CD4+ T cell clone, NY4.2, clearly prevented the spontaneous occurrence of IDDM in NOD mice and the recurrence of diabetes in islet-transplanted NOD mice. However, the mechanisms by which this T cell clone prevents diabetes were not known. **The first specific objective of this study is to determine how the CD4+ suppressor T cell clone, NY4.2, blocks the autoimmune destruction of pancreatic β cells, resulting in the prevention of diabetes in NOD mice.**

3.2 Materials and methods

3.2.1 The CD4+ suppressor T cell clone, NY4.2

The T cell clone, NY4.2 (1 x 10^6 cells) was maintained by restimulation with 2500 rad-irradiated NOD splenocytes (see 4.2.1) (1 x 10^7 cells) as antigen-presenting cells (APC) (Kruisbeek and Shevach, 1991) every 2 to 3 weeks in 25 cm^2 culture flasks in lymphocyte complete medium (LCM) [RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Life

Technologies), 0.05 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin]. Twenty-four hours after restimulation, recombinant human IL-2 (20 U/10 ng/ml, 0.67 nM, m.w. 15 kD, Boehringer Mannheim, Germany) were added and the cells were expanded. Viable cells were counted using a hematocytometer and trypan blue exclusion test (Strober 1997).

3.2.2 Preparation of the concentrated culture supernatant

NY4.2 T cells were restimulated with APC in serum-free media (LCM without FBS) and the culture supernatant was harvested after 72 h of culture. Since serum itself includes the substantial amounts of TGFβ, and the concentrated serum in media can be toxic to cells, I used serum-free media. The supernatant was concentrated using a Centricon (Amicon, Beverly, MA) with a 10,000 molecular weight cut-off. Concentrated supernatant from cultures of APC alone was used as a control for the concentrated supernatant from NY4.2 T cells.

3.2.3 ConA response assay

NOD splenocytes (2 x 10^5 cells) were incubated in 200 µl of LCM with ConA (5 µg/ml, 185 nM, m.w. 27 kD) (Sigma, St. Louis, MO) for 2 days, with or without restimulated and 2500 rad-irradiated NY4.2 T cells or 100 µl of the concentrated supernatant from restimulated NY4.2 T cells. Similarly, NOD splenocytes were incubated in the presence of ConA and the five-times-concentrated supernatant from NY4.2 cells (100 µl), with or without anti-IL-10, anti-TGFβ1 (R&D Systems, Minneapolis, MN) or anti-IFNγ antibodies (PharMingen, San Diego, CA) (10 µg/ml, 66.7 nM) in triplicate in flat-bottomed 96-well microtiter plates. The concentration of antibodies was determined by the pilot study on the neutralizing activity of antibodies using different concentrations of a given cytokine and antibody against it, and this concentration of antibodies did not affect the ConA response of NOD splenocytes. To measure cell proliferation, cultured cells were added with 1 µCi of ^{3}H-labelled thymidine after 2 days of culture and harvested 16 - 24 h later. ^{3}H-thymidine incorporation was assessed by liquid scintillation counting and expressed as cpm (Shevach 1997).

3.2.4 RT-PCR analysis of cytokine gene expression

NY4.2 T cells restimulated with APC were harvested after 6 days of culture when buoying APC can be removed by centrifugation at 1,000 rpm (200 x *g*) for 5 min (confirmed by microscopic examination) (Kruisbeek and Shevach 1991). Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (Chrom-czynski and Sacchi, 1987). Briefly, cells were homogenized in solution D [4 M guanidium thiocyanate (Sigma), 25 mM sodium citrate (pH 7.0), 0.5% sodium N-laurylsarcosine, and 0.1 M 2-mercaptoethanol]. The homogenized sample was added to an equal volume of water-saturated phenol (Life Technologies), 0.2 volumes of chloroform, and 0.1 volumes of 2 M sodium acetate (pH 4.0) and vortexed vigorously. The mixture was centrifuged at 14,000 rpm for 10 min. The aqueous phase was carefully transferred to a new tube, followed by the addition of 2 volumes of 100% ethanol (Sigma) for precipitation. The total RNA was precipitated after incubation at -20°C for 3 h, dried, and resuspended in diethylpyrocarbonate (DEPC)-treated distilled water.

Two µg of total RNA was subjected to first-strand cDNA synthesis in 20 µl of reaction mixture containing 50 mM

Tris-HCl (pH 8.3), 75 mM KCl, 5 mM $MgCl_2$, 50 pmol of oligo$(dT)_{12-18}$, 10 mM dithiothreitol, 0.2 mM of each dNTP, 15 U of RNase inhibitor (Promega, Wisconsin, WI), and 2.5 U of SuperScriptII RNase H^- reverse transcriptase (Life Technologies) at 37°C for 1 h. After synthesis of first strand cDNA, the tubes were heated to 70°C for 15 min and the sample was diluted with DEPC-treated distilled water. Two µl of sample was used for PCR.

The PCR was run using specific primers for several cytokines (Table 1). Hypoxanthine phosphoribosyl transferase (HPRT) was used as an internal standard. The PCR condition was optimized for each set of primers. PCR was performed using a different number of cycles to ensure that amplification occurred in a linear range. PCR amplification was carried out in 20 µl of the reaction mixture containing 0.2 mM of each dNTP, 50 pmol of specific primers, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 1 U of *Taq* DNA polymerase (Pharmacia) with denaturation at 94°C for 1 min, annealing at 60°C (or 55°C for HPRT) for 1 min, and extension at 72°C for 1 min. After amplification, the PCR products were subjected to electrophoresis on a 1.5% agarose gel and detected by ethidium bromide staining.

Table 1. The upstream and downstream primers for RT-PCR

cytokine (fragment size, cycle number) Primers	amplified sites
IL-2 (307 bp, 40 cycles)	
5'CTTGCCCAAGCAGGCCACAG	285-304 & 572-591 bp
3'GAGCCTTATGTGTTGTAAGC	in the mouse IL-2 mRNA
IL-4 (401 bp, 40 cycles)	
5'TCTTTCTCGAATGTACCAGG	125-144 & 506-525 bp
3'CATGGTGGCTCAGTACTACG	in the mouse IL-4 mRNA
IL-10 (406 bp, 40 cycles)	
5'CAAACAAAGGACCAGCTGGAC	241-261 & 627-646 bp
3'GAGTCCAGCAGACTCAATAC	in the mouse IL-10 mRNA
IFNγ (504 bp, 40 cycles)	
5'AGCTCTGAGACAATGAACGC	58-77 & 542-561 bp
3'GGACAATCTCTTCCCCACCC	in the mouse IFNγ mRNA
TGFβ (303 bp, 30 cycles)	
5'AGGAGACGGAATACAGGGCTTTCG	982-1005 & 1261-1284 bp
3'ATCCACTTCCAACCCAGGTCCTTC	in the mouse TGFβ mRNA
iNOS (222 bp, 32 cycles)	
5'GCATGGACCAGTATAAGGCAAGCA	1949-1972 & 2147-2170 bp
3'GCTTCTGGTCGATGTCATGAGCAA	in the mouse iNOS mRNA
TNFα (374 bp, 27 cycles)	
5'CCTGTAGCCCACGTCGTAGC	434-453 & 788-807 bp
3'TTGACCTCAGCGCTGAGTTG	in the mouse TNFα mRNA
IL-1β (345 bp, 27 cycles)	
5'GAATGACCTGTTCTTTGAAGTT	131-152 & 454-475 bp
3'TTTTGTTGTTCATCTCGGAGCC	in the mouse IL-1β mRNA
IL-12p40 (297 bp, 32 cycles)	
5'AGAGGTGGACTGGACTCCCGA	133-153 & 408-429 bp in
3'TTTGGTGCTTCACACTTCAGC	the mouse IL-12p40 mRNA
HPRT (177 bp, 30 cycles)	
5'GTAATGATCAGTCAACGGGGGAC	404-426 & 557-580 bp
3'CCAGCAAGCTTGCAACCTTAACCA	in the mouse HPRT mRNA

3.2.5 Assay for cytokine production

NY4.2 T cells (1 x 10^5 cells) were cultured in 200 µl of serum-free media with or without APC (1 x 10^6 cells), and the culture supernatant was harvested at 48 h for IL-2, IFNγ, IL-4, and IL-10, or at 72 h for TGFβ (Chen et al., 1994). The concentration of each cytokine was determined by ELISA (R&D Systems) using monoclonal antibodies specific for the corresponding cytokine, following the manufacturer's recommendations and expressed as picogram per millilitre.

3.2.6 Statistical analysis

The statistical significance of differences between two groups was analyzed by the student's *t*-test for unpaired samples, and the one-way ANOVA test was used for statistical analysis of data with more than two groups. Results are given as the mean ± SD.

3.3. Results

3.3.1 The suppressive effect of NY4.2 cells on the proliferative responses of NOD splenocytes

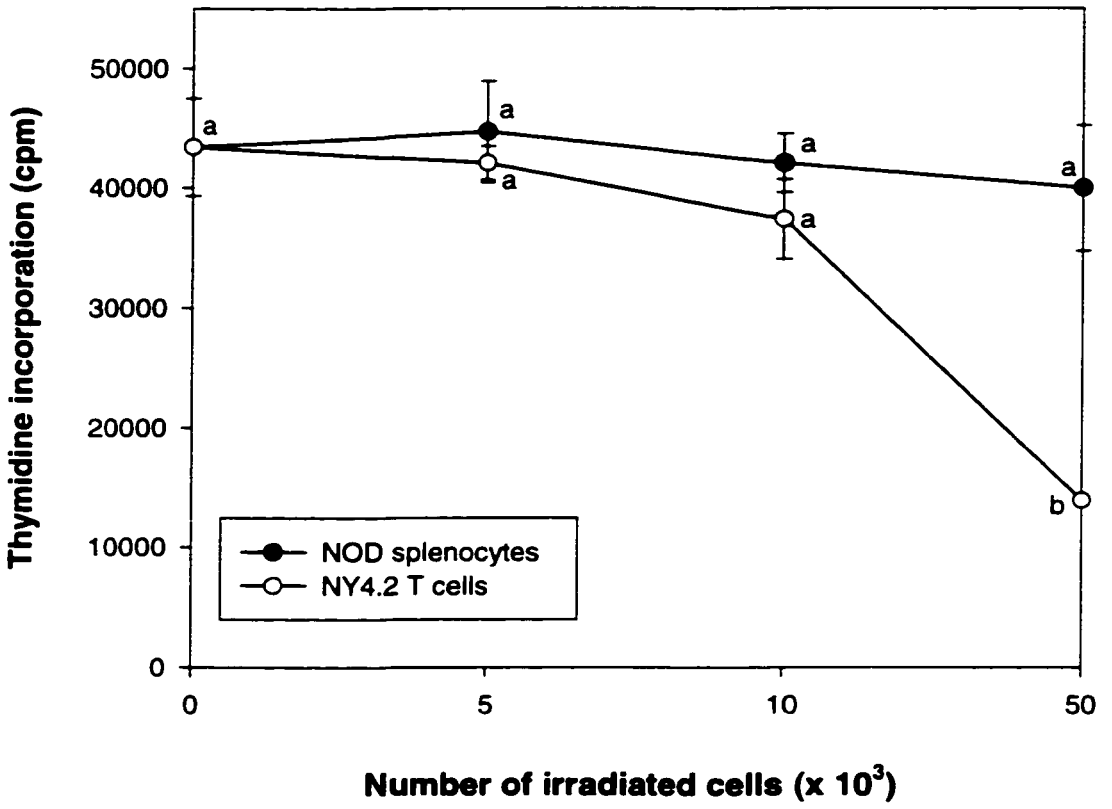
To determine whether NY4.2 T cells, which exhibit the preventive effect on the development of autoimmune diabetes *in vivo*, have also an immunosuppressive activity *in vitro*, I examined the effect of NY4.2 T cells on the ConA response of NOD splenocytes. The NY4.2 T cells or NOD splenocytes, as a control, were 2500 rad-irradiated to prevent their own proliferation (Kruisbeek and Shevach 1991). I found that irradiated NY4.2 T cells had a significant immunosuppressive effect on the proliferative responses of splenic T cells from NOD mice (Figure 1). In contrast, irradiated NOD splenocytes, as a control, did not show any significant suppressive activity.

3.3.2 Secretion of immunosuppressive soluble factors by NY4.2 cells

I found that irradiated NY4.2 T cells had a significant immunosuppressive effect on the proliferative responses of splenic T cells from NOD mice. However, I did not know whether the suppressive activity came from T cells themselves or from soluble factors secreted by the cells. To determine whether the suppressive effect of NY4.2 cells was a result of soluble factors released by the cells, I examined the suppressive activity of culture supernatants

Figure 1. The suppressive effect of NY4.2 cells on the ConA response of NOD splenocytes. NOD splenocytes (2 x 10^5 cells) were incubated with ConA (5 μg/ml, 185 nM) in the presence of different numbers of irradiated NY4.2 T cells or irradiated NOD splenocytes, as control, and ^{3}H-thymidine incorporation was measured. The mean ± SD of representative data of three independent experiments was shown.

* a vs b are significantly different ($p < 0.01$).


Thymidine incorporation (cpm)
50000
40000
30000
20000
10000
0
0
5
10
50
Number of irradiated cells (x 10³)
NOD splenocytes
NY4.2 T cells
a
a
a
a
a
a
b

of NY4.2 cells stimulated with APC in serum-free media. I found that the supernatant alone had a dose-dependent suppressive effect on the proliferative activity of splenic T cells from NOD mice (Figure 2). In contrast, the concentrated supernatant of APC alone, as a control, did not show any significant suppressive activity (Figure 3). This result indicates that the suppressive activity of NY4.2 cells is at least partially a result of soluble factors secreted by the cells, rather than of the cells themselves.

3.3.3 Secretion of cytokines by NY4.2 cells

It is well known that CD4+ T cells function by producing various cytokines. To identify the suppressive soluble factors of NY4.2 T cells, I examined the cytokine gene expression (IFNγ and IL-2 for type 1 cytokines, IL-4 and IL-10 for type 2 cytokines, and transforming growth factor β (TGFβ)) using RT-PCR analysis after stimulation with APC. I found that NY4.2 cells expressed the genes for IL-10, TGFβ, and IFNγ, but I failed to detect gene expression of IL-2 or IL-4 (Figure 4).

Figure 2. The dose-dependent suppressive effect of soluble factors released from NY4.2 cells on the ConA response of NOD splenocytes. Culture supernatant from NY4.2 T cells was collected and concentrated. NOD splenocytes (2 x 10^5 cells) were incubated with ConA (5 µg/ml, 185 nM) in the presence of 100 µl of each concentration of the culture supernatant and ^{3}H-thymidine incorporation was measured. As a control, lymphocyte complete medium (LCM) was used. The mean ± SD of representative data of three independent experiments was shown.

* a vs b are significantly different ($p < 0.01$).

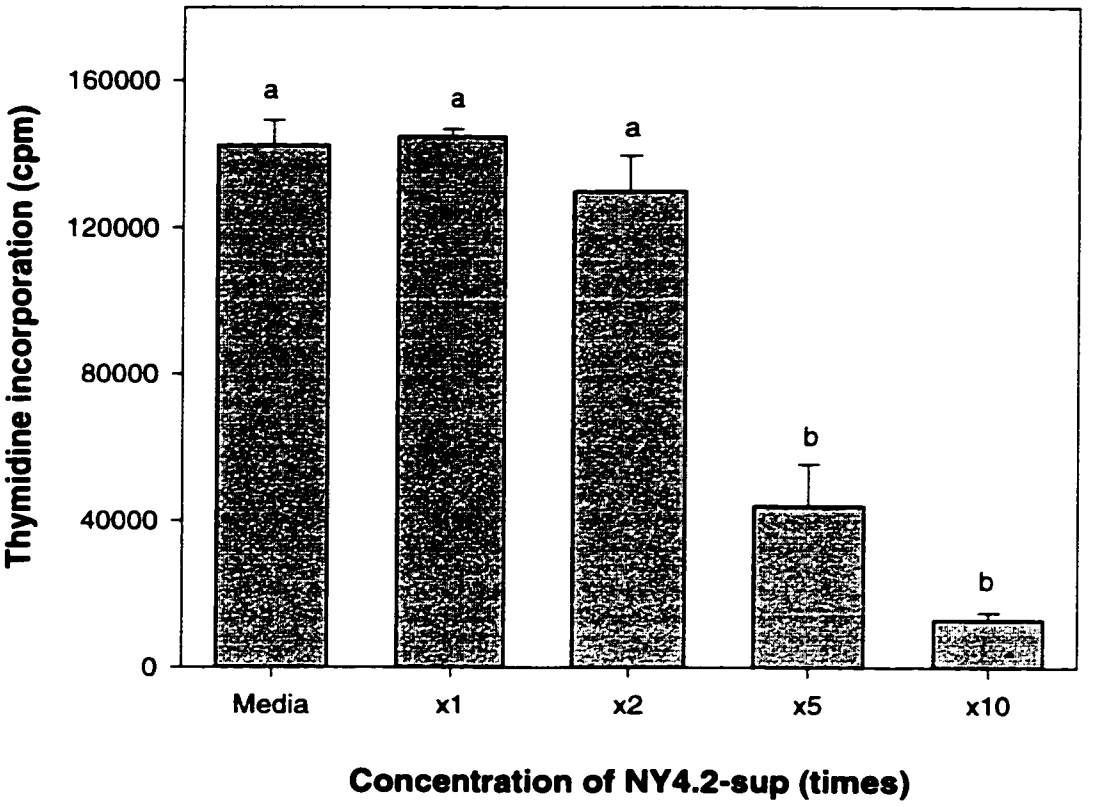

Thymidine incorporation (cpm)
160000
120000
80000
40000
0
a
a
a
b
b
Media
x1
x2
x5
x10
Concentration of NY4.2-sup (times)

Figure 3. The suppressive effect of soluble factors released from NY4.2 cells. NOD splenocytes (2 x 10^5 cells) were incubated with ConA (5 μg/ml, 185 nM) in the presence or absence of 100 μl of the ten-times-concentrated culture supernatant from NY4.2 T cells and ^{3}H-thymidine incorporation was measured. As a control, plain LCM and the ten-times-concentrated culture supernatant from APC alone were used. The mean ± SD of representative data of four independent experiments are shown.

* a vs b are significantly different ($p < 0.01$).

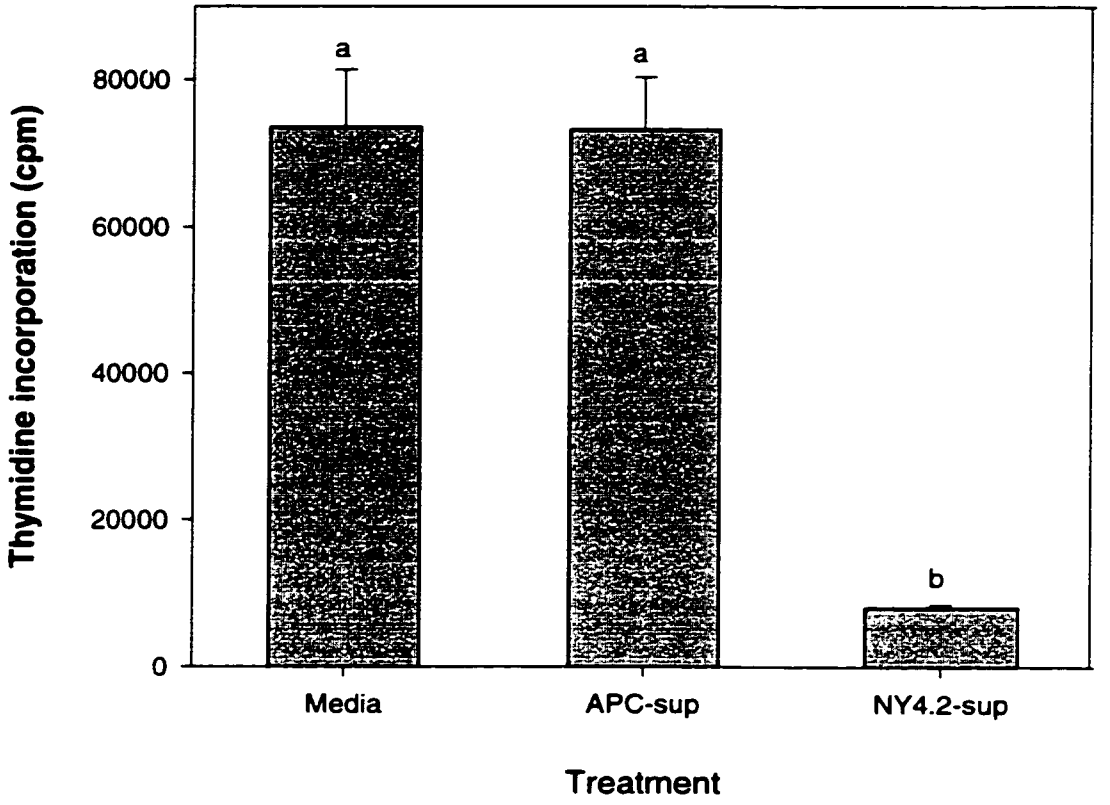
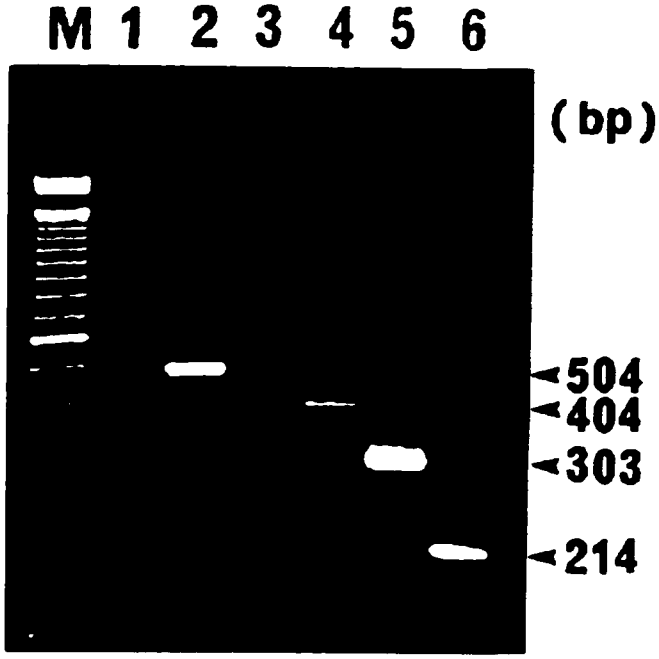

Thymidine incorporation (cpm)
80000
60000
40000
20000
0
a
a
b
Media
APC-sup
NY4.2-sup
Treatment

Figure 4. RT-PCR analysis for cytokine gene expression of NY4.2 cells. RT-PCR was performed using primers specific for IL-2, IFNγ, IL-4, IL-10 and TGFβ. Primers for HPRT were used for a standard. Lane M: Molecular weight standard, 100 bp ladder (Gibco BRL), Lane 1: IL-2, Lane 2: IFNγ, Lane 3: IL-4, Lane 4: IL-10, Lane 5: TGFβ, Lane 6: HPRT. The arrow indicates the amplified product from NY4.2 T cells. The representative data of three independent experiments was shown.


M 1 2 3 4 5 6
(bp)
504
404
303
214

To confirm the secretion of cytokines by NY4.2 cells, I measured the levels of cytokines using ELISA in culture supernatant. I found that, after stimulation with APC, NY4.2 T cells secreted substantial amounts of TGFβ (7 ng/ml), IL-10 (0.6 ng/ml) and IFNγ (5 ng/ml), but not IL-2 or IL-4 (Table 2). In contrast, in the resting state, NY4.2 T cells produce only IFNγ (3 ng/ml). Results of these assays suggest that the NY4.2 cells do not belong to either the classic Th1 subtype of CD4+ T cells, which secretes IFNγ and IL-2, or to the Th2 subtype that secretes IL-4 and IL-10.

3.3.4 Abrogation of the immunosuppressive activity of NY4.2 cells by treatment with anti-TGFβ antibody

To determine which cytokine(s) produced by NY4.2 T cells play a role in the suppression of immune responses, the immunosuppressive activity of supernatants from cultures of NY4.2 cells was measured in the presence of neutralizing antibodies against IL-10, TGFβ, or IFNγ. The supernatant's immunosuppressive effect on the proliferation of NOD splenocytes was abrogated in the presence of anti-TGFβ antibody (Figure 5). In contrast, there was no significant difference between the suppressive activity of

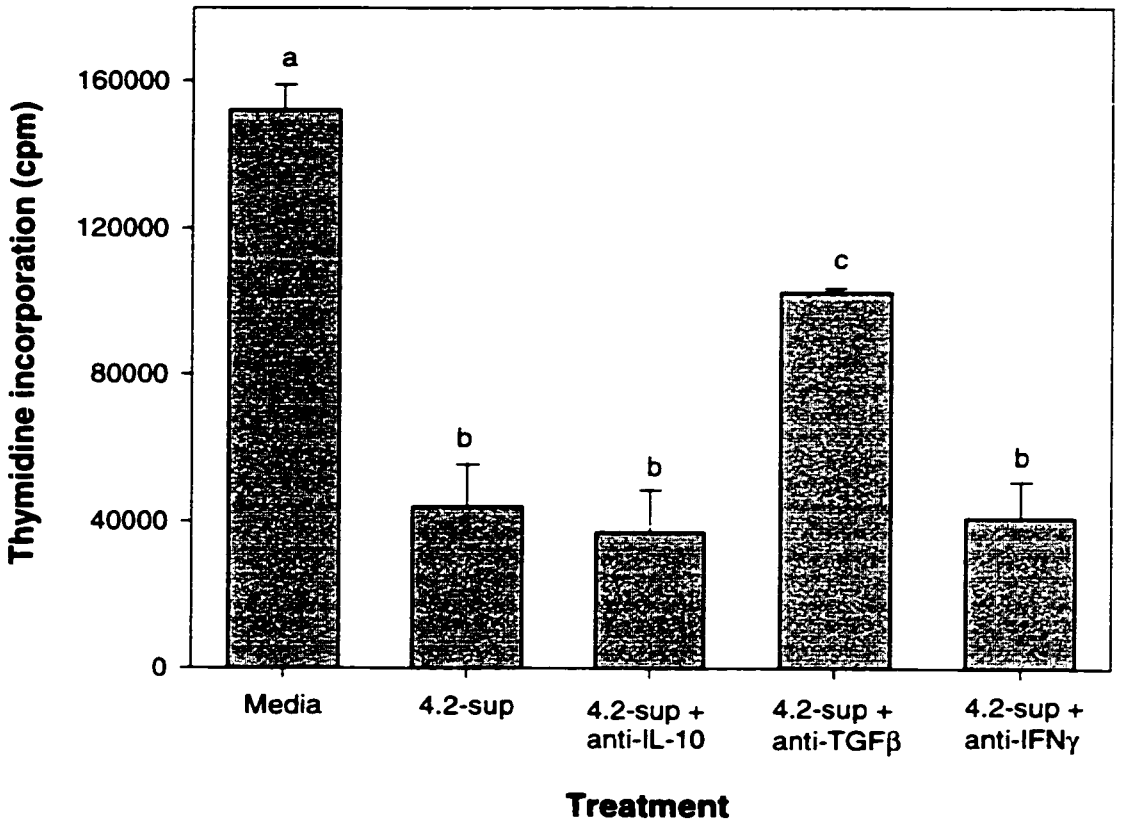
Table 2. Cytokine production by NY4.2 cells

Cytokine	NY4.2 T cells	APC	NY4.2 T cells + APC
IL-2	<2	<2	<2
IL-4	<2	<2	<2
IL-10	36±7	<2	598±35
IFNγ	2,887±90	215±18	5,351±130
TGFβ	30±2	84±9	7,052±130

NY4.2 T cells (1 x 10^5 cells/well), or 2500 rad-irradiated NOD splenocytes (1 x 10^6 cells/well) as APC, were cultured either separately or together in 200 µl of serum-free media. Culture supernatant was collected at 48 h for IL-2, IL-4, IL-10, and IFNγ, and at 72 h for TGFβ. Cytokine concentrations were determined by ELISA and are expressed as picograms per millilitre. The mean ± SD of representative data of three independent experiments was shown.

Figure 5. The effect of anti-cytokine antibodies on the suppressive activity of soluble factors released from NY4.2 cells. NOD splenocytes (2 x 10^5 cells) were incubated with ConA (5 μg/ml, 185 nM) and 100 μl of the five-times-concentrated culture supernatant from NY4.2 T cells in the presence or absence of anti-IL-10, anti-TGFβ, or anti-IFNγ antibodies (10 μg/ml, 66.7 nM). ^{3}H-thymidine incorporation was measured. As a control, plain LCM was used. The mean ± SD of representative data of four independent experiments was shown.

* a vs b, b vs c, and a vs c are significantly different ($p < 0.01$).


Thymidine incorporation (cpm)
160000
120000
80000
40000
0
a
b
b
c
b
Media
4.2-sup
4.2-sup + anti-IL-10
4.2-sup + anti-TGFβ
4.2-sup + anti-IFNγ
Treatment

supernatant with anti-IL-10 or anti-IFNγ antibodies and that of supernatant alone. These results suggest that TGFβ may play a major role in the suppression by NY4.2 T cells of cell-mediated immune responses.

3.4 Discussion

Autoimmune destruction of pancreatic β cells is preceded by the infiltration of macrophages and lymphocytes into pancreatic islets. The development of autoimmune diabetes occurs in two stages. Noninvasive insulitis begins at 3 weeks of age but does not result in destructive insulitis before 3-4 months of age. This long period of clinically silent insulitis and its progression to diabetes are best explained by the involvement of immunoregulatory T cells. Converging data suggest that cytokine imbalance due to the overcome of diabetogenic Th1 cells to regulatory Th2 cells plays a key role in the pathogenesis of diabetes (see INTRODUCTION). In NOD mice, systemic administration of IL-4 or anti-IFNγ antibody prevented diabetes, whereas IL-12, which promotes the development of Th1 cells, accelerated the onset of IDDM. In addition, the high ratio of IFNγ/IL-4

production in the islets of NOD mice correlated with the severity of histological lesions. Furthermore, diabetogenic T cell clones from NOD mice exhibit a Th1 phenotype.

In this study, I found that NY4.2 T cells, which exerted the preventive effect on the development of autoimmune diabetes *in vivo*, have also a significant immunosuppressive effect on the proliferative responses of splenic T cells from NOD mice *in vitro*. The cytokine pattern of NY4.2 T cells suggested that these cells do not belong to typical Th1 or Th2 cell type. I then showed that anti-TGFβ antibody abrogated the immunosuppressive effect of NY4.2 T cells on the proliferation of NOD splenocytes. Although the possibility that proteins coimmunoprecipitated with anti-TGFβ antibody may affect the proliferation of NOD splenocytes cannot be ruled out, these results suggest that TGFβ produced by NY4.2 cells may play a role in the inhibition of ConA-induced immune cell proliferation. Since the ability of anti-TGFβ antibody to recover the proliferation of NOD splenocytes is not complete, it is possible that other soluble mediators from NY4.2 T cells contribute to their immunosuppressive activity or the concentration of antibody was not sufficient. The result

obtained from this study showing that TGFβ contributes to the immunosuppressive activity of CD4+ suppressor T cells, which prevented autoimmune diabetes, was reported for the first time in NOD mice (Han et al., 1996). It was later followed by a study demonstrating that an insulin-specific CD4+ T cell clone, which prevented the development of IDDM in NOD mice, suppressed the proliferative responses of NOD splenocytes to islets *in vitro* by producing TGFβ (Zekzer et al., 1997). These observations add the complexity to the simple proposition on roles Th1/Th2 immune balance plays in the pathogenesis and regulation of autoimmune diabetes. The possible roles of TGFβ in the suppression of the development of autoimmune diabetes will be discussed in detail in the next chapter.

In addition to TGFβ, NY4.2 T cells secreted IL-10. IL-10 has suppressive effects on antigen-stimulated proliferation and IFNγ production by Th1 CD4+ T cells via its inhibition of macrophage function (Fiorentino et al., 1991). Further study showed that IL-10 inhibits the development of a Th1 phenotype by blocking the production of IL-12 by macrophages (Hsieh et al., 1993). In line with the results, IL-10 was shown to prevent the development of

autoimmune diseases, such as autoimmune diabetes (Pennline et al., 1994) and experimental autoimmune thyroiditis (EAT) (Mignon-Godefroy et al., 1995). However, in contrast to the preventive effect of systemic administration of IL-10, transgenic NOD mice expressing IL-10 in pancreatic β cells actually promoted the onset of IDDM (Wogensen et al., 1994). Thus, there is controversy over the effect of IL-10 on the development of diabetes. Regardless of this controversy, my study showed that anti-IL-10 antibody did not abrogate the suppressive effect of the culture supernatant from NY4.2 T cells on the ConA response, allogenic mixed lymphocyte reaction, and cytokine-dependent T cell proliferation (data not shown), whereas anti-TGFβ antibody did. Although I cannot exclude the possibility that the concentration of antibody was not sufficient, these results suggest that IL-10 may not play a role in the suppression by NY4.2 T cells of immune responses. It is difficult, however, to draw any definite conclusions about the role of IL-10 in the pathogenesis of autoimmune IDDM, since the action of a given cytokine is complicated and dependent on interaction with other cytokines present systemically or locally.

NY4.2 T cells secrete IFNγ, as well as TGFβ and IL-10. The evidence supporting that IFNγ is involved in the destruction of pancreatic β cells rather than the prevention of β cell destruction has been accumulated (Rabinovitch et al., 1995). Because substantial amounts of IFNγ are produced by NY4.2 T cells even in the resting state, I speculate that IFNγ may play a role in the infiltration of NY4.2 T cells into the target by, in part, enhancing the expression of adhesion molecules on endothelial cells (Fabry et al., 1992). These T cells can be activated in response to MHC class II-positive cells in pancreatic islets and produce the high amounts of the suppressive cytokine TGFβ. It is proposed that the destruction of β cells may depend on which way the finely tuned balance of cytokines is tipped. If β cell-destructive cytokines dominate cytokines that afford protection from β cell destruction, autoimmune processes leading to β cell destruction may be enhanced. In contrast, an increase in the function of cytokines which protect from β cell destruction may block these destructive processes, resulting in the prevention of β cell destruction. I speculate that the function of TGFβ secreted from NY4.2 T

cells may dominate that of IFNγ, especially upon activation, leading to the prevention of β cell destruction. How these cytokines interact with each other to determine the direction towards the protection rather than the destruction of β cells remains to be determined.

4. MECHANISMS FOR THE PREVENTION OF AUTOIMMUNE DIABETES BY TGFβ, SECRETED FROM A NEW TYPE OF CD4+ SUPPRESSOR T CELLS, IN NOD MICE

4.1 Introduction

The results obtained from the previous chapter indicate that TGFβ, secreted from CD4+ suppressor T cells, NY4.2, could play a role in the prevention of autoimmune IDDM in NOD mice.

The three isoforms of TGFβ, i.e., TGFβ1, 2, and 3, bind to and signal through the same receptors, resulting in almost identical effects on targets. TGFβ is a multifunctional cytokine that has various effects on immune as well as nonimmune cells (McCartney-Francis and Wahl, 1994). TGFβ functions predominantly as a stimulatory cytokine for many nonimmune cells such as fibroblasts (Moses et al., 1990). However, TGFβ has potent inhibitory effects on many cells of the immune system, regulating growth, differentiation, and functions of T cells, B cells, natural killer (NK) cells and macrophages (Ruscetti et al., 1993; Letterio and Roberts, 1998).

TGFβ was shown to suppress cytokine-dependent proliferation of thymocytes and T cells (Wahl et al., 1988; Ruegemer et al., 1990), and the action of cytotoxic T lymphocytes (CTL) and NK cells (Ranges et al., 1987; Rook

et al., 1986). B cells are also affected by TGFβ, resulting in the inhibition of proliferation (Kehrl et al., 1986) and the induction of IgA switching (Coffman et al., 1989). Additionally, it was found that TGFβ inhibited the cytotoxicity mediated by macrophages (Tsunawaki et al., 1988). Moreover, TGFβ has downregulatory effects on the expression of MHC class II molecules on macrophages (Czarniecki et al., 1988; Panek et al., 1995). TGFβ also inhibited the entry of effector immune cells into the target organ such as the brain by interfering with their adhesion to blood vessel endothelia (Fabry et al., 1995). The development of multifocal inflammatory diseases in TGFβ1-deficient mice (Shull et al., 1992) is consistent with the findings from the above *in vitro* studies indicating that TGFβ1 suppresses immune responses.

In organ-specific autoimmune diseases, such as collagen-induced arthritis (CIA) (Thorbecke et al., 1992) and experimental allergic encephalomyelitis (EAE) (Santambrogio et al., 1993), TGFβ affords protection from effector immune cell-mediated destruction of target cells. It was recently demonstrated that regulatory CD4+ T cell clones, isolated from the lymph nodes of mice orally

tolerized with myelin basic protein (MBP), prevented EAE by producing TGFβ (Chen et al., 1994). This result is comparable to the finding obtained from my study in autoimmune diabetes (Han et al., 1996). Although TGFβ appears to play a role in the prevention of autoimmune IDDM in NOD mice, the mechanisms underlying its action remain to be elucidated. Thus, **the second specific objective of this study is to determine the effect of TGFβ on the function of immune cells involved in pancreatic β cell destruction, such as T cells and macrophages in NOD mice**.

4.2 Materials and methods

4.2.1 Cell preparations

Eight to ten week-old female NOD mice were purchased from Taconic Farms (Germantown, NY) and maintained under specific-pathogen-free conditions at the University of Calgary (Calgary, Alberta, Canada). Splenocytes were freshly prepared by mincing the spleen between slide glasses, and red blood cells were depleted by hypotonic lysis using 0.75% NH_4Cl in 0.017 M Tris buffer (pH 7.65).

Macrophages were freshly isolated by peritoneal lavage 4 days after intraperitoneal injection of 2 ml of 4% thioglyco-llate broth (Difco Laboratories, Detroit, Mich.) into 8-10 week-old female NOD mice. Cells were washed with PBS and resuspended in RPMI 1640 medium containing 5% heat-inactivated FBS, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were plated at 1 x 10^6 per well in 24-well plates (Falcon, Becton-Dickinson Co., Lincoln Park, N.J.) for NO release and TNFα secretion studies, and at 1 x 10^7 per 60 mm-diameter petri dish (Falcon) for RT-PCR analysis. The cells were cultured for 2 h at 37 °C with 5% CO_2 and then washed three times with PBS to remove nonadherent cells.

4.2.2 Proliferation assay

For the ConA response, splenocytes (5 x 10^5 cells) were incubated in the presence, or absence, of TGFβ (10 ng/ml, 87 pM, m.w. 115 kD) (R&D Systems) and/or ConA (2.5 μg/ml, 92.5 nM) (Sigma). For IL-2-dependent T cell proliferation, splenocytes (2 x 10^5 cells) were incubated in the presence, or absence, of TGFβ (10 ng/ml, 87 pM) and/or recombinant IL-2 (20 U/10 ng/ml, 0.67 nM) (Boehringer Mannheim). For

proliferative responses to GAD65 peptides, splenocytes (5 x 10^5 cells) were incubated in the presence, or absence, of TGFβ (10 ng/ml, 87 pM), and/or GAD65 peptides (10 μM) (TANA Lab., Houston, TX) and irradiated NOD splenocytes (1 x 10^6 cells) as APC. The sequences of GAD65 peptides are: peptide 247-266 (no. 17), (NMYAMMIARFKMFPEVKEKG); peptide 509-528 (no. 34), (IPPSLRYLEDNEERMSRLSK); and peptide 524-643 (no. 35), (SRLSKVAPVIKARMMEYGTT) (Kaufman et al., 1993). Cells were cultured in 200 μl of LCM for 4 days in triplicate in flat-bottomed 96-well microtiter plates and thymidine incorporation was assessed by liquid scintillation counting and expressed as cpm (see 3.2.3).

4.2.3 RT-PCR analysis of gene expression

Splenocytes (5 x 10^6 cells) were incubated with TGFβ (10 ng/ml, 87 pM) and/or anti-CD3 antibody (1 μg/ml, 6.7 nM, m.w. 150 kD) (Cedarlane, Hornby, ON) in serum-free media for 6 h. Alternatively, splenocytes were treated with cylcloheximide (10 μg/ml, 36 μM, m.w. 281.4) (Sigma) and/or TGFβ (10 ng/ml, 87 pM) for 6 h.

Peritoneal macrophages (1 x 10^7 cells) were incubated with TGFβ (10 ng/ml, 87 pM) and/or IFNγ (100 U/20 ng/ml,

1.3 nM, m.w. 15.6 kD) (PeproTech Inc., Rocky Hill, NJ) in serum-free media for 6 h.

Total cellular RNA was isolated by the TRIzol reagent (Life Technologies) according to the manufacturer's instructions and RT-PCR was performed (see 3.2.4). The density of PCR products was quantified using ImageQuaNT program, and the ratio of density of each sample to that of HPRT as well as sample from untreated cells was shown in Figures.

4.2.4 Assay for cytokine production

Splenocytes were cultured with TGFβ (10 ng/ml, 87 pM) and/or anti-CD3 antibody (1 μg/ml, 6.7 nM) in serum-free media and the culture supernatant was harvested at 48 h and stored at -70°C until use. The concentration of cytokine was determined by ELISA (see 3.2.5).

4.2.5 *In vivo* treatment

Eight to ten week-old female NOD mice were injected intraperitoneally (i.p.) with TGFβ (2 μg in 0.2 ml PBS, 87 nM) (Santambrogio et al., 1993) and, 30 minutes later, injected intravenously (i.v.) with anti-CD3 antibody (1.33

μg in 0.2 ml PBS, 44 nM) (Yoshimoto and Paul, 1994). Different routes were used for injection to decrease the possible, if any, molecular interactions in circulation. Control mice were injected with the same volume of PBS. Ninety minutes after injection of anti-CD3 antibody, RNA was extracted from splenocytes for RT-PCR analysis and the culture supernatant of splenocytes was collected after 2 h or 24 h of culture for ELISA.

4.2.6 NO assay

Peritoneal macrophages were incubated with TGFβ (10 ng/ml, 87 pM) and/or IFNγ (100 U/ml, 1.3 nM) in serum-free media for 24 h. At the end of cultivation, culture supernatants were collected for NO assay and TNF assay. NO formation was measured as the stable end product nitrite (NO_2^-) in culture supernatants with the Griess reagent. Briefly, an aliquot of culture supernatants was added to each well of 96-well plates and mixed with the same volume of Greiss reagent [1:1 (vol/vol); 0.1% N-(1-naphthyl)ethylenediamine dihydro-chloride in H_2O, 1% sulfanilamide in 5% H_3PO_4], and the A_{540} was read with a spectrophotometer (Green et al., 1982).

4.2.7 TNFα assay

TNFα activity was determined by a functional cytotoxic assay using the TNFα-sensitive cell line L929. L929 cells were plated in 96-well culture plates at 1 x 10^5 per well in 100 µl of RPMI 1640 medium containing 5% FBS and incubated for 3 h. The cells were then cultured for an additional 18 h in the presence of serial dilutions of samples (culture supernatants) or TNFα standard (R&D Systems) with 2 µg/ml actinomycin D (Sigma). The viability of the cells was determined by a colorimetric staining assay using crystal violet solution.

4.2.8 Statistical analysis

See 3.2.6.

4.3 Results

4.3.1 Effect of TGFβ on the proliferative responses of splenocytes from NOD mice

Since an important response of lymphocytes occurring upon proper activation is proliferation, I first determined

the effect of TGFβ on the proliferative responses of splenocytes from NOD mice. Consistent with the findings that NY4.2 suppressor T cells inhibited the ConA response of NOD splenocytes (Figure 1) and that the responsible soluble factor is TGFβ (Figure 5), TGFβ suppressed the proliferative responses of NOD splenocytes in response to ConA (Figure 6A). Also, TGFβ inhibited the proliferative responses of NOD splenocytes to IL-2, a cytokine known to be a T cell growth factor (Figure 6A).

GAD is an important islet antigen involved in the pathogenesis of IDDM, and T cell responsiveness to GAD coincides with the onset of insulitis in NOD mice. Since TGFβ suppressed antigen-non-specific proliferation of NOD splenocytes in response to ConA or IL-2, I determined whether TGFβ influenced T cell reactivity to this islet antigen. As shown in Figure 6B, TGFβ also inhibited the antigen-specific response of NOD splenocytes to GAD65 peptides.

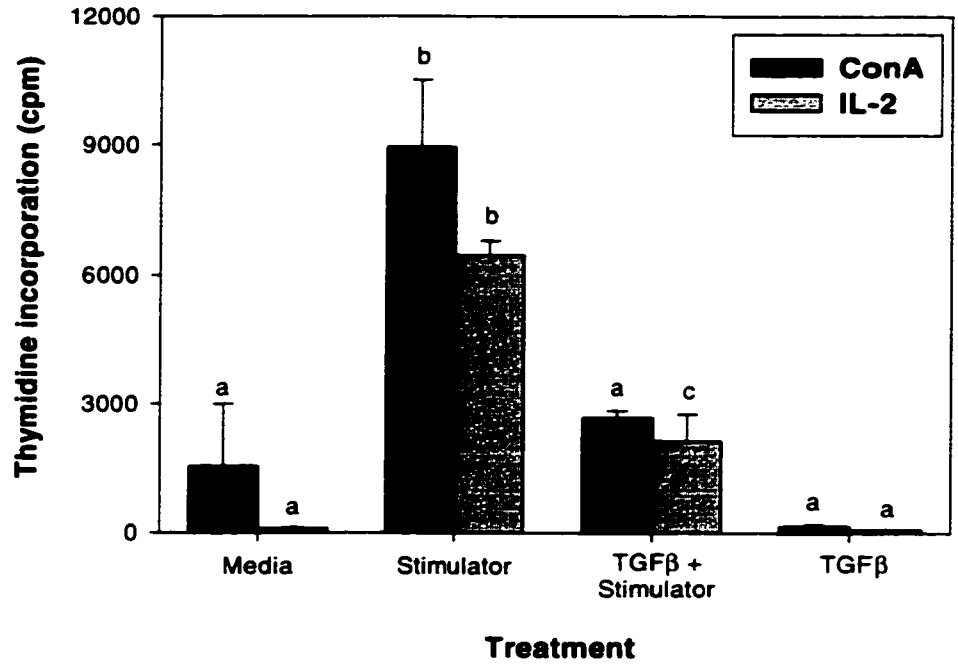
4.3.2 Effect of TGFβ on the gene expression of T cell-derived cytokines in splenocytes from NOD mice

I next determined the effect of TGFβ on the function of

Figure 6. The effect of TGFβ on the proliferative responses of NOD splenocytes. (A) NOD splenocytes were cultured in the presence, or absence, of ConA (2.5 μg/ml, 92.5 nM) or IL-2 (20 U/ml, 0.67 nM) and/or TGFβ (10 ng/ml, 87 pM). (B) NOD splenocytes were cultured in the presence, or absence, of GAD65 peptides (10 μM) and APC, and/or TGFβ (10 ng/ml, 87 pM). Cells were cultured for 4 days and ^{3}H-thymidine incorporation was measured. As a control, plain LCM was used. The mean ± SD of the representative data of three independent experiments was shown.

* a vs b, b vs c, and a vs c are significantly different ($p < 0.01$).

(A)


12000
9000
6000
3000
0
Thymidine incorporation (cpm)
ConA
IL-2
a
a
b
b
a
c
a
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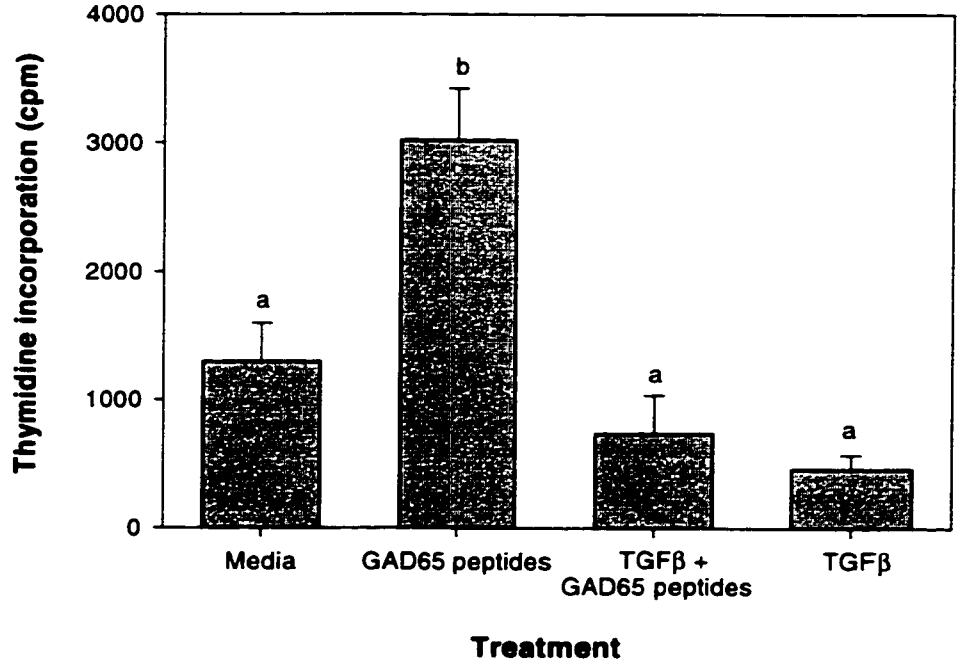
Media
Stimulator
TGFβ + Stimulator
TGFβ
Treatment

(B)

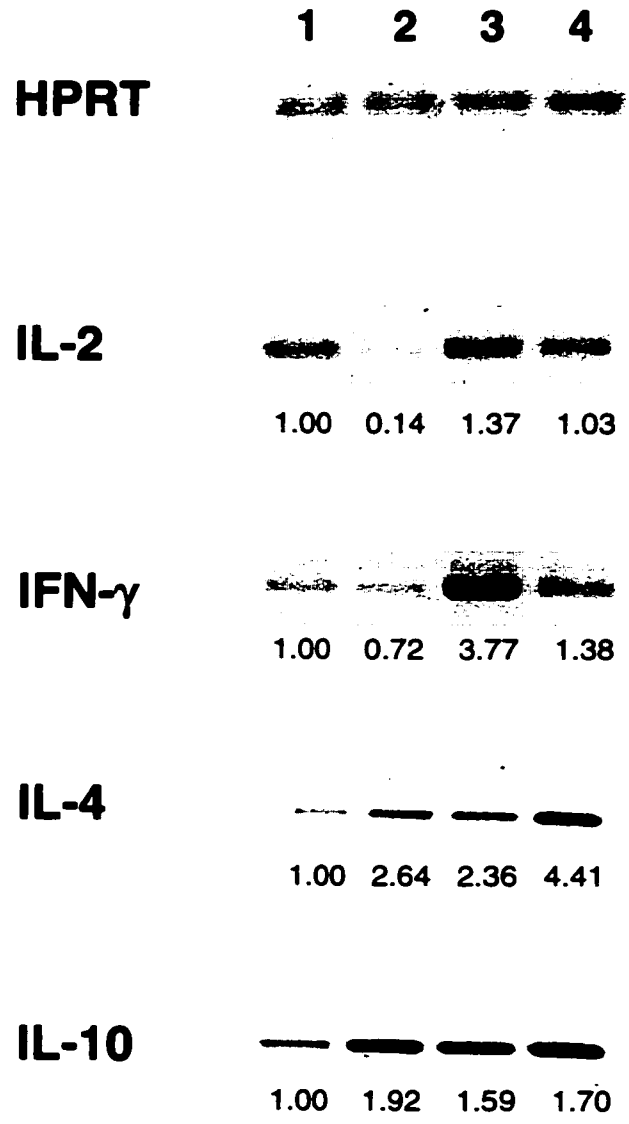

4000
3000
2000
1000
0
Thymidine incorporation (cpm)
a
b
a
a
Media
GAD65 peptides
TGFβ + GAD65 peptides
TGFβ
Treatment

T cells in NOD mice by examining the production of cytokines by them. The gene expression using RT-PCR analysis of T cell-derived cytokines such as type 1 cytokines (IFNγ and IL-2) and type 2 cytokines (IL-4 and IL-10) was examined after treatment of NOD splenocytes with TGFβ and/or anti-CD3 antibody. With or without T cell activation by anti-CD3 antibody, the gene expression of type 2 cytokines such as IL-4 and IL-10 was increased by TGFβ treatment, whereas TGFβ inhibited the expression of genes for type 1 cytokines such as IFNγ and IL-2 (Figure 7A).

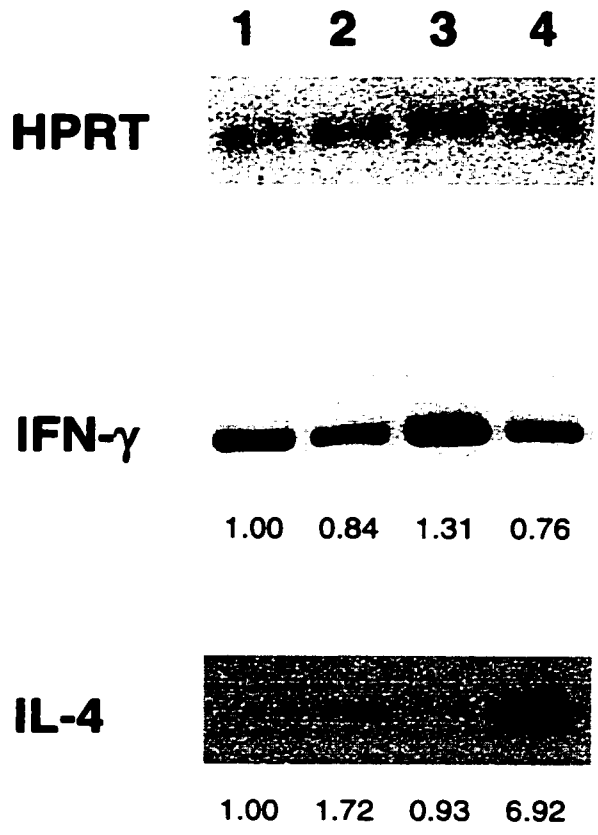
Since whole NOD splenocytes including various cell types were used for experiments, the above results can be due to the paracrine reaction of other cytokines such as IL-12, a macrophage-derived strong stimulator for type 1 cytokines and of which the gene expression was also inhibited by TGFβ treatment (Figure 9). To address this question, splenocytes were treated with TGFβ and/or cycloheximide, a protein synthesis inhibitor. Cycloheximide did not affect the decrease of IFNγ gene expression and an increase in IL-4 gene expression after TGFβ treatment (Figure 7B), suggesting that the protein synthesis may not

Figure 7. The *in vitro* effect of TGFβ on the gene expression of T cell-derived cytokines in NOD splenocytes. (A) NOD splenocytes (5 x 10^6 cells) were incubated in the presence, or absence, of TGFβ (10 ng/ml, 87 pM) and/or anti-CD3 antibody (1 μg/ml, 6.7 nM) for 6 h. (B) NOD splenocytes (5 x 10^6 cells) were incubated in the presence, or absence, of cycloheximide (CHX) (10 μg/ml, 36 μM) and/or TGFβ (10 ng/ml, 87 pM) for 6 h. RT-PCR analysis was performed using primers specific for each cytokine. Primers for HPRT were used for an internal standard. (A) Lane 1: untreated, Lane 2: TGFβ-treated, Lane 3: anti-CD3-treated, Lane 4: TGFβ- and anti-CD3-treated. (B) Lane 1: untreated, Lane 2: TGFβ-treated, Lane 3: CHX-treated, Lane 4: CHX- and TGFβ-treated. Representative data of four independent experiments was shown.

(A)


1 2 3 4
HPRT
IL-2
1.00 0.14 1.37 1.03
IFN-γ
1.00 0.72 3.77 1.38
IL-4
1.00 2.64 2.36 4.41
IL-10
1.00 1.92 1.59 1.70

(B)



be required for the effect of TGFβ on cytokine gene expression. Rather cycloheximide appeared to potentiate the effect of TGFβ on cytokine gene expression, probably due to the ability of cycloheximide to stabilize unstable cytokine mRNAs by blocking the binding of nuclease responsible for degrading mRNA to the ribosomes (Alberts et al., 1994; Shaw et al., 1987). These findings suggest that TGFβ induces the polarization of T cells towards a type 2 phenotype and these effects result from its direct influence to T cells. This suggestion was confirmed by experiments using T cell populations enriched by T cell columns from NOD splenocytes (data not shown).

I next attempted to determine whether TGFβ can induce the polarization of T cells to type 2 responses *in vivo*. I injected TGFβ (2 μg, i.p.) with or without anti-CD3 antibody (1.33 μg, i.v.) into NOD mice and splenocytes were isolated for experiments. Similar to the results from *in vitro* experiments, with or without T cell activation by injection of anti-CD3 antibody, the gene expression of type 2 cytokines in splenocytes from TGFβ-injected NOD mice was increased compared to splenocytes from control mice, whereas TGFβ injection resulted in the decrease of type 1

cytokines in NOD splenocytes (Figure 8). These observations suggest that TGFβ affects the function of T cells *in vivo* as well as *in vitro*, resulting in their polarization towards a type 2 phenotype.

4.3.3 Effect of TGFβ on the secretion of T cell-derived cytokines by splenocytes from NOD mice

I attempted to confirm quantitatively using ELISA the polarization by TGFβ of T cells to a type 2 phenotype. Without stimulation by anti-CD3 antibody, I could barely detect the secretion of any cytokine in culture supernatants (Table 3). When splenocytes were stimulated with anti-CD3 antibody, they clearly produced all T cell-derived cytokines with different concentrations. Thus, the effect of stimulation of T cells with anti-CD3 antibody on gene expression and secretion of cytokines seems to be different. One possible explanation is that T cell stimulation with anti-CD3 antibody may affect cytokine secretion at the posttranscriptional as well as transcriptional levels. I confirmed that TGFβ inhibited the secretion of type 1 cytokines such as IFNγ and IL-2. Compared to TGFβ-mediated significant inhibition of type 1

Figure 8. The *in vivo* effect of TGFβ on the gene expression of T cell-derived cytokines in NOD splenocytes. Eight to ten week-old female NOD mice were injected with TGFβ (2 μg in 0.2 ml PBS, 87 nM, i.p.) and/or anti-CD3 antibody (1.33 μg in 0.2 ml PBS, 44 nM, i.v.). As a control, PBS was injected. Ninety minutes later, splenocytes (5 x 10^6 cells) were isolated from mice and RT-PCR analysis was performed using primers specific for each cytokine. Primers for HPRT were used for an internal standard. Lane 1: PBS-injected mice, Lane 2: TGFβ-injected mice, Lane 3: anti-CD3-injected mice, Lane 4: TGFβ- and anti-CD3-injected mice. Representative data of four independent experiments was shown.

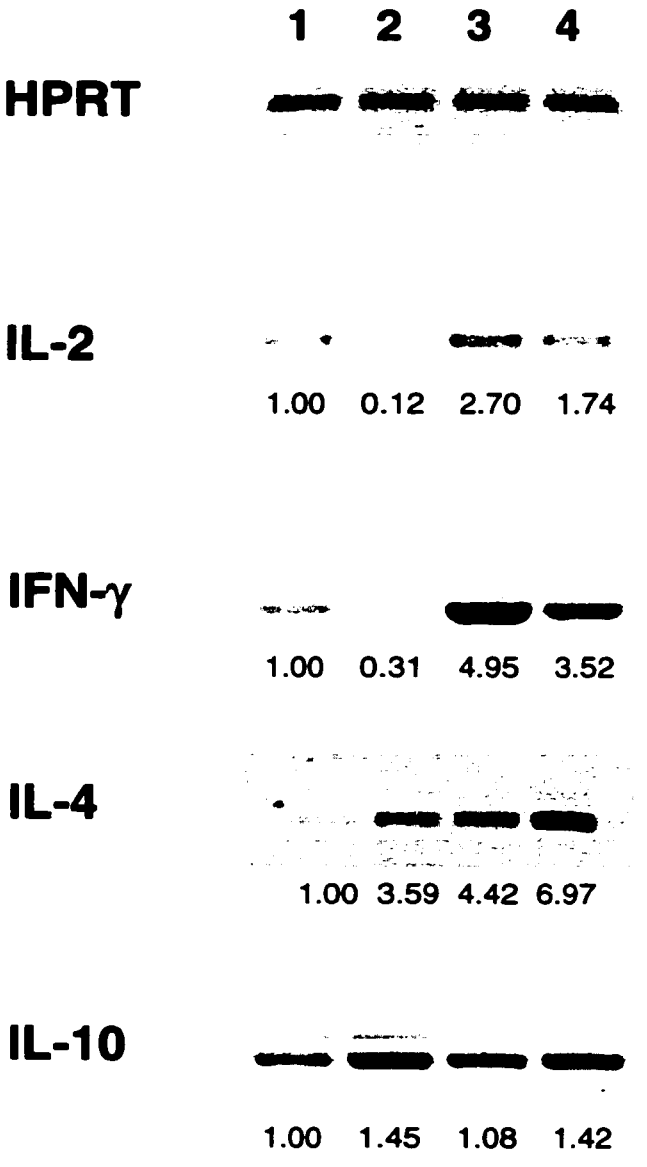

1 2 3 4
HPRT
IL-2
1.00 0.12 2.70 1.74
IFN-γ
1.00 0.31 4.95 3.52
IL-4
1.00 3.59 4.42 6.97
IL-10
1.00 1.45 1.08 1.42

Table 3. The *in vitro* effect of TGFβ on the cytokine production of NOD splenocytes

Treatment					
Anti-CD3	TGFβ	IL-2	IFNγ	IL-4	IL-10
-	-	<3	<2	<2	<4
-	+	<3	<2	<2	<4
+	-	128±9	3,709±180	2±1	43±2
+	+	88±5*	2,135±90*	4±1	45±1

Splenocytes were cultured in the presence, or absence, of TGFβ (10 ng/ml) and/or anti-CD3 antibody (1 μg/ml) and culture supernatant was collected at 48 h. Cytokine concentrations were determined by ELISA and are expressed as picograms per millilitre. Results represent the mean ± SD of the representative of four independent experiments.
* $P<0.05$ as compared to the culture supernatant in the absence of TGFβ.

cytokine production, TGFβ did not affect the production of IL-4 and IL-10. TGFβ-mediated inhibition of the production of type 1 cytokines by T cells was also confirmed using enriched T cell populations from NOD splenocytes (data not shown). Similar results were obtained when I determined the *in vivo* effect of TGFβ on cytokine secretion by measuring the cytokine concentrations in the supernatant after splenocytes from TGFβ- and/or anti-CD3 antibody-injected NOD mice were cultured for 2 h (Table 4). When the culture supernatant was examined after 24 h, IFNγ production was increased, which is inhibited by TGFβ, whereas IL-4 secretion remained the similar level (data not shown).

4.3.4 Effect of TGFβ on the function of macrophages from NOD mice

I also determined the effect of TGFβ on the function of macrophages, another cell type involved in pancreatic β cell destruction in NOD mice. Peritoneal macrophages from NOD mice were treated with TGFβ and/or IFNγ and the gene expression was examined using RT-PCR analysis of macrophage-derived cytokines such as TNFα, IL-1β, the p40

Table 4. The *in vivo* effect of TGFβ on the cytokine production of NOD splenocytes

Treatment					
Anti-CD3	TGFβ	IL-2	IFNγ	IL-4	IL-10
-	-	<3	<2	<2	<4
-	+	<3	<2	<2	<4
+	-	232±7	103±4	12±1	32±4
+	+	176±5*	60±2*	18±2	40±1

NOD mice were injected with TGFβ (2 μg, i.p.) and/or anti-CD3 antibody (1.33 μg, i.v.). As controls, PBS was injected. Ninety minutes later, splenocytes were isolated from mice and culture supernatant was collected at 2 h. Cytokine concentrations were determined by ELISA and are expressed as picograms per millilitre. Results represent the mean ± SD of the representative of four independent experiments.
* $P<0.05$ as compared to splenocytes from NOD mice not treated with TGFβ.

subunit of IL-12, and iNOS responsible for nitric oxide (NO) production. With or without macrophage activation with IFNγ, the gene expression for all cytokines and iNOS was decreased by TGFβ treatment (Figure 9). Moreover, TGFβ-mediated inhibition of NO and TNF secretion induced by IFNγ in macrophages was confirmed quantitatively (Figure 10). However, in macrophages without activation by IFNγ, I failed to see the inhibition by TGFβ of NO production in contrast to the inhibition of iNOS gene expression. This may be due to the different sensitivities of assays, so that the effect of TGFβ on gene expression, which was determined by sensitive RT-PCR analysis, may not be found by NO assay. These results suggest that TGFβ may suppress the cytotoxic activity of macrophages to pancreatic β cells by inhibiting the production of cytotoxic molecules.

4.4 Discussion

Although the effect of TGFβ on the function of immune cells is reported, NOD mice have defects in the immune system such as deficiency of cytokine production (Serreze

Figure 9. The effect of TGFβ on the gene expression of cytokines and iNOS in IFNγ-stimulated peritoneal macrophages from NOD mice. Peritoneal macrophages (5 x 10^6 cells) were incubated in the presence, or absence, of TGFβ (10 ng/ml, 87 pM) and/or IFNγ (100 U/ml, 1.3 nM) for 6 h and RT-PCR analysis was performed using primers specific for each cytokine and iNOS. Primers for HPRT were used for an internal standard. Lane 1: untreated, Lane 2: TGFβ-treated, Lane 3: IFNγ-treated, Lane 4: TGFβ- and IFNγ-treated. Representative data of three independent experiments was shown.

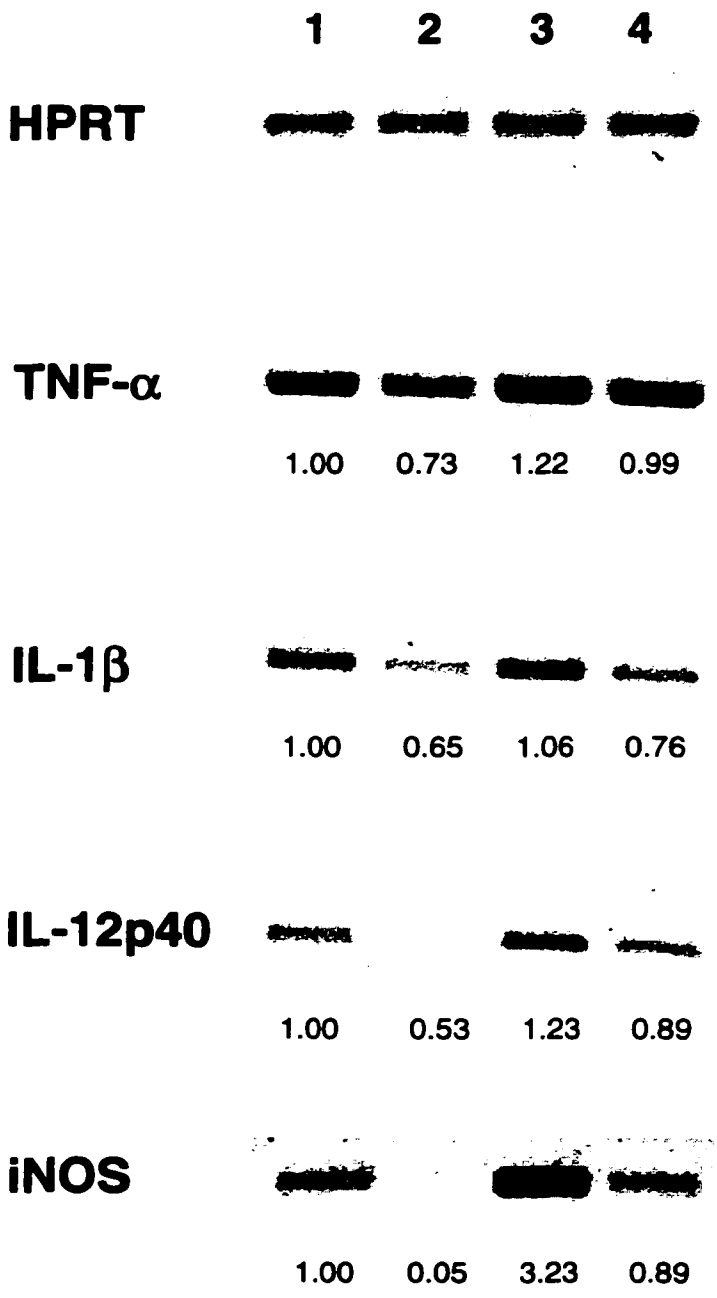
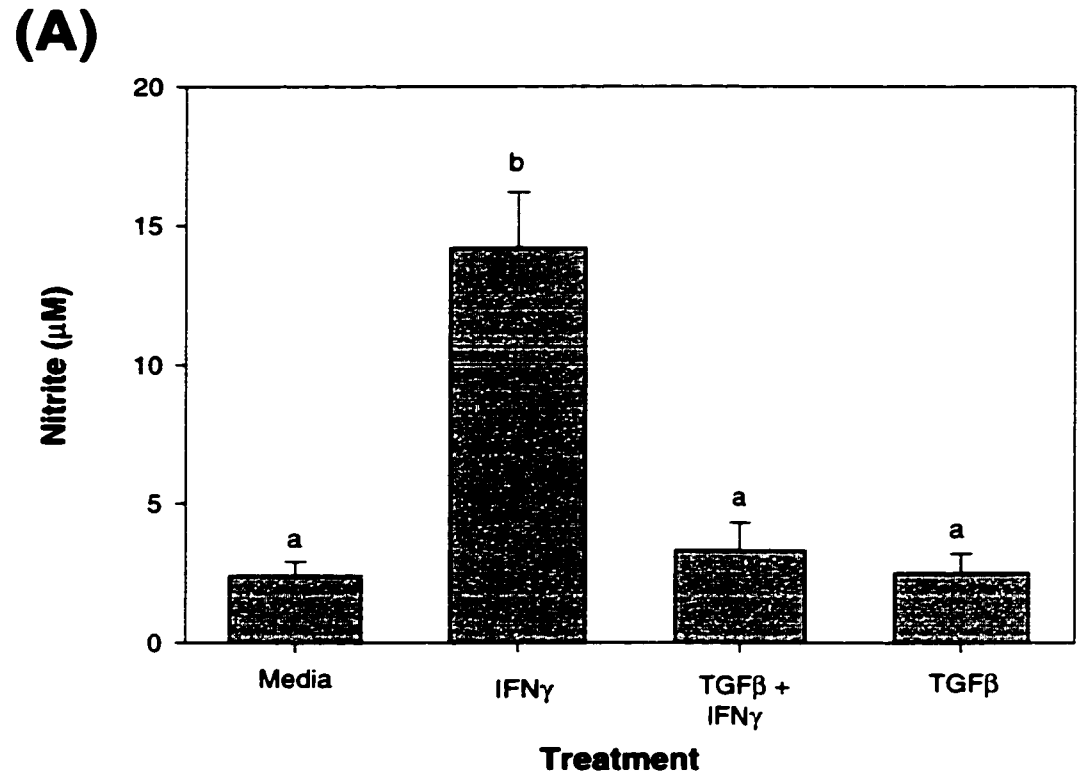
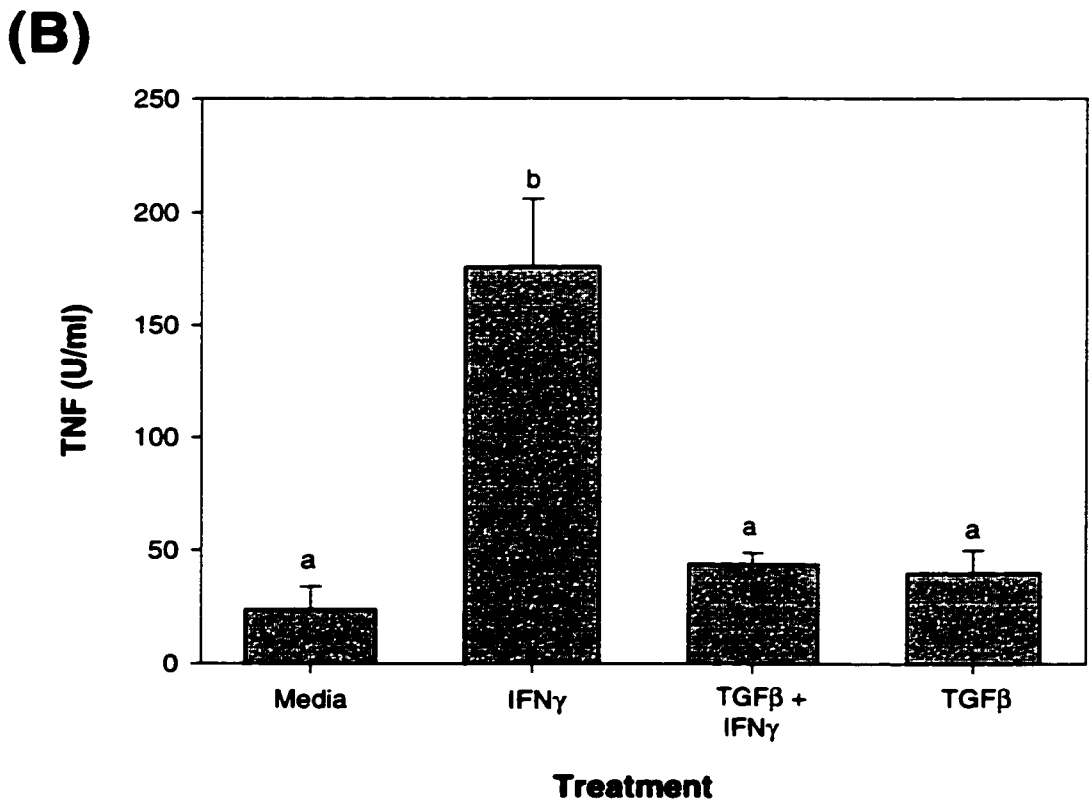

1 2 3 4
HPRT
TNF-α
1.00 0.73 1.22 0.99
IL-1β
1.00 0.65 1.06 0.76
IL-12p40
1.00 0.53 1.23 0.89
iNOS
1.00 0.05 3.23 0.89

Figure 10. The effect of TGFβ on the secretion of NO (A) and TNF (B) by IFNγ-stimulated peritoneal macrophages from NOD mice. Peritoneal macrophages (1 x 10^6 cells) were cultured in the presence, or absence, of TGFβ (10 ng/ml, 87 pM) and/or IFNγ (100 U/ml, 1.3 nM) and culture supernatant was collected at 24 h for NO assay and TNF assay. The mean ± SD of the representative data of four independent experiments was shown.

* a vs b are significantly different ($p < 0.01$).


(A)
20
15
10
5
0
Nitrite (μM)
b
a
a
a
Media
IFNγ
TGFβ + IFNγ
TGFβ
Treatment


(B)
250
200
150
100
50
0
TNF (U/ml)
b
a
a
a
Media
IFNγ
TGFβ + IFNγ
TGFβ
Treatment

and Leiter, 1988; Rapoport et al., 1993), therefore, it is necessary to examine systematically the various activities of TGFβ in the regulation of immune cells in NOD mice.

Upon appropriate antigen stimulation, the responses of mature T cells include proliferation, differentiation to effector T cells, and cytokine production. TGFβ has a large number of pleiotropic effects on T cells, acting differentially dependent upon their differentiation state. TGFβ has been reported to have both growth-inhibitory and growth-promoting effects. Most studies have stressed its growth-inhibitory effects (Moses et al., 1992), but, when TGFβ was added to naive CD4+ T cells, long-term growth-promoting effects was shown (Swain et al., 1991). In addition, TGFβ, alone and in synergy with IL-2, was shown to block apoptosis and promote the expansion of antigen-specific, Th2-polarized effector cells (Zhang et al., 1995). In my study, I found that TGFβ inhibited the proliferative responses of NOD splenocytes in response to antigen-non-specific stimulators such as ConA and IL-2 as well as GAD autoantigen. Thus, the inhibition by TGFβ of the proliferation of effector T cells upon stimulation with antigen and/or cytokine may be one mechanism for the

protective effect of TGFβ-producing CD4+ suppressor T cells on IDDM in NOD mice.

Results from previous studies on the regulation by TGFβ of Th1/Th2 responses have been contradictory. Several reports showed that TGFβ enhanced the generation of Th1 cells *in vitro* (Swain et al., 1991). However, a more recent study showed that TGFβ can promote or inhibit the development of Th1 cells, depending on the mouse strain and the amount of IL-2 present in priming culture (Hoehn et al., 1995). *In vivo* studies on the role TGFβ plays in the regulation of Th1/Th2 responses are also inconsistent. In experimental models of leishmanial infection, the administration of TGFβ clearly inhibited the generation of a Th1 immune response (Barral-Netto et al., 1992). In contrast, the administration of TGFβ delayed the progression of disease in mice infected with *Candida albicans*, concomitant with lower levels of IL-4 (Spaccapelo et al., 1995).

In my study, I found that TGFβ inhibited the secretion as well as gene expression of type 1 cytokines such as IFNγ and IL-2 in NOD mice. In contrast, TGFβ did not affect the

production of type 2 cytokines such as IL-4 and IL-10. While I found that the gene expression of type 2 cytokines was enhanced by TGFβ, I failed to see the promotion of their secretion by T cells. In the case of IL-4, this difference may result from too low production of IL-4 in NOD mice, as reported previously (Rapoport et al., 1993), not allowing visualization of a clear effect of TGFβ on IL-4 production by ELISA. In the case of IL-10, the enhancing effect of TGFβ on its gene expression and secretion in T cells is unlikely to be significant. This TGFβ-mediated influence on the production of T cell-derived cytokines was shown *in vivo* as well as *in vitro*. Considering the pathogenic role Th1-type T cells play in β cell destruction, the protective activity of TGFβ-producing CD4+ suppressor T cells in the development of IDDM in NOD mice may be attributed to the inhibition by TGFβ of the production of type 1 cytokines by T cells rather than the enhancement of type 2 cytokine secretion.

In addition to the effect of TGFβ on the function of T cells, a role TGFβ plays in the regulation of macrophage function was investigated in NOD mice. I found that TGFβ inhibited the gene expression of macrophage-derived

cytokines such as IL-1β, TNFα, and IL-12, and iNOS as well as the secretion of TNF and NO in NOD mice. The IL-12 expressed in macrophages is known to play an important role in the development of Th1-type CD4+ T cells and its systemic administration promoted the development of IDDM in NOD mice (Trembleau et al., 1995). A recent study showed that the involvement of macrophages in the pathogenesis of IDDM is, in part, due to their production of IL-12, inducing a shift toward a Th1 phenotype (Jun et al., 1999). Thus, TGFβ-mediated inhibition of cytokine production by Th1 cells can be attributed to an indirect influence on macrophages as well as a direct effect on T cells. The cytokines such as IL-1β and TNFα, and free radicals such as NO, released from activated macrophages, are believed to mediate toxic effects exerted by activated macrophages on β cells. The cytokines produced by islet-infiltrating macrophages may contribute to β cell damage by inducing the production of free radicals in the islets. Thus, TGFβ-mediated suppression of the production of β-cell toxic mediators by macrophages may account for the inhibition by

TGFβ-producing CD4+ suppressor T cells of macrophage-mediated pancreatic β cell destruction in NOD mice.

Recently, to evaluate the role of TGFβ in the regulation of IDDM, TGFβ was transgenically expressed in pancreatic β cells (King et al., 1998) or α cells (Moritani et al., 1998), resulting in decreased incidence of IDDM in NOD mice. However, the mechanisms for the preventive effect of transgenic expression of TGFβ on pancreatic β cell destruction were not fully elucidated. One mechanism shown by King et al. is the alteration in APC preferences toward a Th2 phenotype. They showed that NOD T cells use B cells as APC to present GAD autoantigen, whereas T cells from NOD mice expressing TGFβ on β cells utilize macrophages, inducing the deviation of GAD-specific T cells toward a Th2 phenotype. These results are consistent with my observation on TGFβ-mediated inhibition of Th1 cytokines. However, the underlying mechanisms inducing a shift toward Th2 cells that they suggested are still questionable. In line with a known effect of IL-12 on the development of Th1-type CD4+ T cells, the involvement of macrophages in the pathogenesis of IDDM was recently reported to be, in part, due to their

production of IL-12, inducing a shift toward a Th1 phenotype (Jun et al., 1999).

5. MOLECULAR MECHANISMS FOR TGFβ-MEDIATED SUPPRESSION OF EFFECTOR IMMUNE CELLS INVOLVED IN THE PATHOGENESIS OF AUTOIMMUNE DIABETES

5.1 Effect of TGFβ on IL-2-induced signal transduction pathways in T cells

5.1.1 Introduction

The results gained from the previous chapter suggest that TGFβ-mediated suppression of the function of immune cells in NOD mice may play a role in the prevention of immune cell-mediated pancreatic β cell destruction. **The third specific objective of this study is to determine the molecular role TGFβ plays in the suppression of function of immune cells involved in the pathogenesis of autoimmune IDDM, such as T cells and macrophages.**

As mentioned in the INTRODUCTION, a substantial amount of evidence supports a critical role for T cells in the destruction of β cells. Anti-T cell reagents prevent the development of autoimmune diabetes, and T cells from diabetic mice can transfer IDDM to immunodeficient or young NOD mice. Although TGFβ may act on multiple targets to prevent and reverse autoimmune diseases, the inhibition of T cell proliferation is an important means by which TGFβ mediates immunosuppression. However, the molecular mechanisms by which TGFβ inhibits T cell proliferation has remained elusive.

Priming of resting T cells occurs through T cell receptor and the expansion of primed T cells is induced by IL-2, a T cell growth factor. Thus, IL-2 has long been known to play an important role in the activation of T lymphocytes (Theze et al., 1996) and the role IL-2 plays in the development of autoimmune diabetes has been demonstrated (Allison et al., 1994; Kelly et al., 1988). The high-affinity IL-2 receptor (IL-2R) is composed of α-, β- and γ-chains, whereas the IL-2R β- and γ-chains form an intermediate-affinity IL-2R (Theze et al., 1996). The α-chain only expressed after T cell activation is dispensable for the signaling pathways, while the β- and γ-chains are essential for signal transduction. IL-2-induced signal transduction requires the heterodimerization of the IL-2R β- and γ-chains (Nakamura et al., 1994), leading to the aggregation of IL-2R-associated signaling molecules. IL-2R itself has no tyrosine kinase activity, but stimulation with IL-2 results in tyrosine phosphorylation of cellular proteins, including IL-2R β- and γ-chains. It was shown that the IL-2R β-chain and γ-chain are associated with the JAK protein tyrosine kinases JAK1 and JAK3, respectively (Miyazaki et al., 1994) and that JAK1 and JAK3 are

activated by tyrosine phosphorylation after stimulation with IL-2 (Johnston et al., 1995).

The best known event following the activation of JAK kinases is the activation of STAT proteins, which are present as latent transcription factors in the cytoplasm (Ihle, 1995; Ihle, 1996). STAT proteins are tyrosine-phosphorylated by JAK kinases and form homodimers or heterodimers in order to become active transcription factors. These proteins translocate to the nucleus where they induce the activation of gene expression. It was shown that IL-2 activates STAT proteins, such as STAT3 and STAT5 (Johnston et al., 1995). Ahuja et al. demonstrated that TGFβ inhibits T cell proliferation by downregulating IL-2-mediated proliferative signals (Ahuja et al., 1993). In order to investigate the molecular mechanisms for the suppression by TGFβ of T cell proliferation, I therefore determined the effect of TGFβ on the activation of signal transduction pathways in T cells stimulated with IL-2.

5.1.2 Materials and methods

5.1.2.1 Antibodies and cytokines

Anti-phosphotyrosine monoclonal antibody (4G10) and anti-JAK3 antibody were purchased from Upstate Biotechnology (Lake Placid, NY) for immunoprecipitation and immunoblotting. Anti-JAK1, anti-STAT3, anti-STAT5b, and anti-ERK2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human IL-2 was purchased from Boehringer Mannheim. Recombinant human TGFβ1 and anti-TGFβ1 antibody were purchased from R&D Systems (Minneapolis, MN).

5.1.2.2 Cell preparations

Activated T cells were developed by culturing splenocytes from 8-10 week-old NOD mice in the presence of ConA (5 μg/ml, 185 nM) (Sigma) in LCM for 2 d. These ConA blasts, which respond to IL-2, were used for experiments.

HT-2, an IL-2-dependent murine T cell line, was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells (1 x 10^6 cells/ml) were maintained in LCM containing 20 U/ml recombinant IL-2 (0.67 nM).

5.1.2.3 *In vitro* proliferative responses to IL-2

ConA blasts from NOD splenocytes (2 x 10^4 cells) or HT-2 cells were incubated in 200 μl of LCM in the presence of

recombinant IL-2 (20 U/ml, 0.67 nM) and NY4.2 T cell culture supernatant (concentrated five times, 100 µl, see 3.2.2), with or without anti-TGFβ antibody (10 µg/ml, 66.7 nM) (R&D Systems) in flat-bottomed 96-well microtiter plates, in triplicate. As a control, the supernatant (concentrated five times) from cultures of APC alone was used. After 24 h, cells were pulsed with 1 µCi of ^{3}H-labelled thymidine and harvested 4 h later. ^{3}H-thymidine incorporation was assessed by liquid scintillation counting and expressed as cpm.

5.1.2.4 Flow cytometric analysis

ConA blasts from NOD splenocytes (1 x 10^{6} cells) or HT-2 cells were cultured in serum-free LCM in the presence, or absence, of TGFβ (10 ng/ml, 87 nM) for 12 h. After washing with staining buffer (PBS containing 1% FBS and 0.1% sodium azide), cells were incubated with FITC-conjugated anti-CD25 (IL-2R α-chain) antibody (Cedarlane, Hornby, ON) for 30 min at 4°C. Cells were then washed with staining buffer three times and analyzed using FACScan (Becton Dickinson, Sunnyvale, CA).

5.1.2.5 Immunoprecipitation and immunoblot analysis

ConA blasts from NOD splenocytes were deprived of serum by culturing in 1% FBS-containing LCM for 12 h in order to synchronize cells to G_1 phase, and HT-2 cells were cultured in IL-2-free LCM for 4-6 h. ConA blasts or HT-2 cells (5 x 10^7 cells) were then cultured in serum-free LCM in the presence, or absence, of TGFβ (10 ng/ml, 87 nM) for 12 h. After stimulation with IL-2 (20 U/ml, 0.67 nM) for 5 min, cells were lysed in ice-cold lysis buffer (50 mM Tris (pH7.6), 1% NP-40, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin). Lysates were cleared of debris by centrifugation at 12,000 x *g* for 15 min. The supernatant was precleared with protein A-Sepharose (Pharmacia, Uppsala, Sweden) to prevent non-specific binding and were incubated with 1 μg of the designated antibodies for 1 h at 4°C. Immune complexes were precipitated with protein A-Sepharose (Pharmacia) overnight at 4°C and washed extensively in lysis buffer. Proteins were eluted with sample buffer, separated by SDS-PAGE, and transferred to nitrocellulose membranes (Amersham Life Science Inc., Canada). Membranes were probed with the designated

antibodies, visualized with the enhanced chemiluminescence (ECL) detection system (Amersham Life Science Inc.), stripped and reprobed as directed by the manufacturer. The density of immunoblots was quantified using ImageQuaNT program and the ratio of density of tyrosine-phosphorylated proteins to that of total proteins was shown in figure legends.

5.1.2.6 Statistical analysis

See 3.2.6.

5.1.3. Results

5.1.3.1 Inhibition of IL-2-dependent T cell proliferation by TGFβ secreted from NY4.2 cells

To determine whether TGFβ, secreted from NY4.2 suppressor T cells, inhibits IL-2-dependent T cell proliferation, ConA blasts from NOD splenocytes were incubated with IL-2 and the culture supernatant of NY4.2 cells in the presence, or absence, of anti-TGFβ antibody, and a thymidine incorporation assay was performed. The culture supernatant from NY4.2 T cells inhibits IL-2-

dependent proliferation of ConA blasts from NOD splenocytes (Figure 11). This suppressive activity of NY4.2 cell culture supernatant was abolished by treatment with anti-TGFβ antibody (Figure 11). These results indicate that TGFβ, secreted from NY4.2 T cells, inhibits IL-2-dependent T cell proliferation, which is consistent with the inhibitory effect of TGFβ on the proliferative responses to IL-2 of NOD splenocytes (Figure 6A).

5.1.3.2 TGFβ-mediated inhibition of IL-2-dependent T cell proliferation is not the result of downregulation of IL-2R on T cells.

To determine whether TGFβ-mediated inhibition of T cell proliferation induced by IL-2 results from the downregulation of IL-2R on the surface of T cells, I examined the surface expression of the IL-2R α-chain by flow cytometric analysis of CD25 (IL-2R α-chain). I found that the expression of the IL-2R α-chain on the surface of T cells did not change in ConA blasts from NOD splenocytes (Figure 12) treated with TGFβ for 12 h. These observations suggest that the inhibition by TGFβ of IL-2-dependent T cell

Figure 11. The effect of anti-TGFβ antibody on the suppressive activity of soluble factor(s) released from NY 4.2 cells in the IL-2-dependent proliferation of ConA blasts from NOD splenocytes. ConA blasts from NOD splenocytes (2 x 10^4 cells) were cultured with 20 U/ml IL-2 (0.67 nM) and NY4.2 T cell culture supernatant (concentrated five times) in the presence, or absence, of anti-TGFβ antibody (10 μg/ml, 66.7 nM). ^{3}H-thymidine incorporation was measured. As a control, plain media and the concentrated culture supernatant from APC alone were used. The mean ± SD of the representative data of three independent experiments was shown.

* a vs b are significantly different ($p < 0.01$).

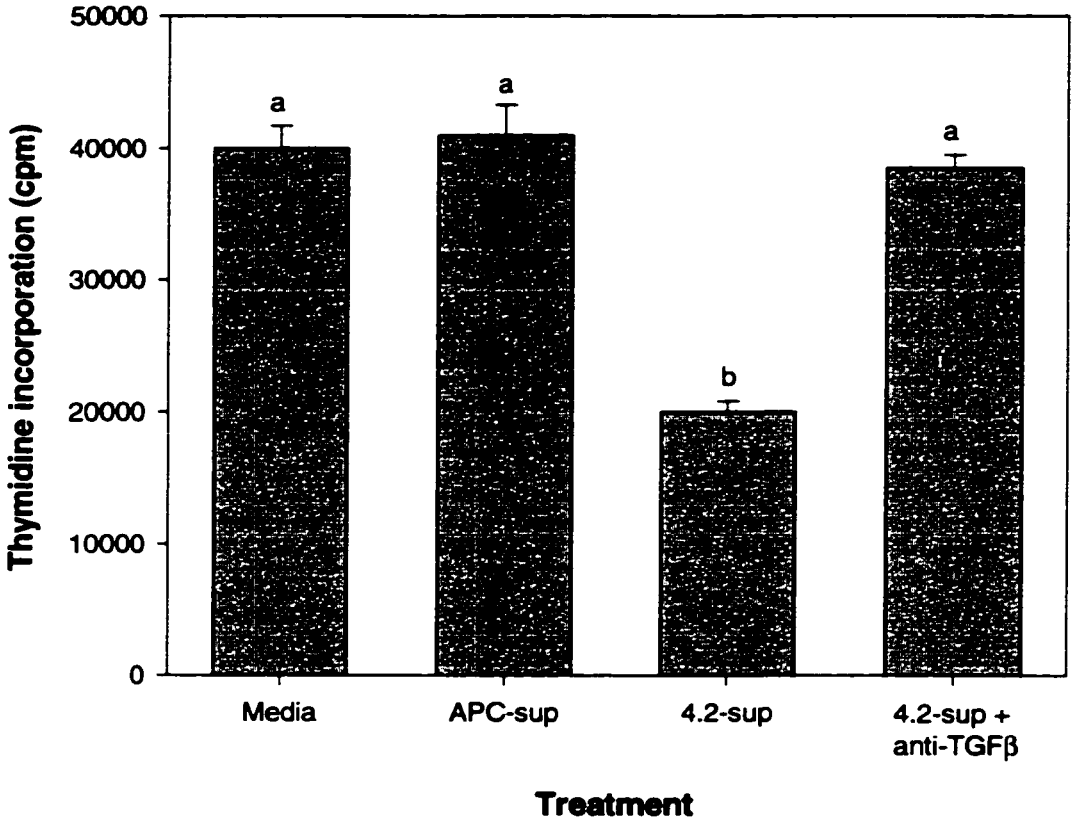
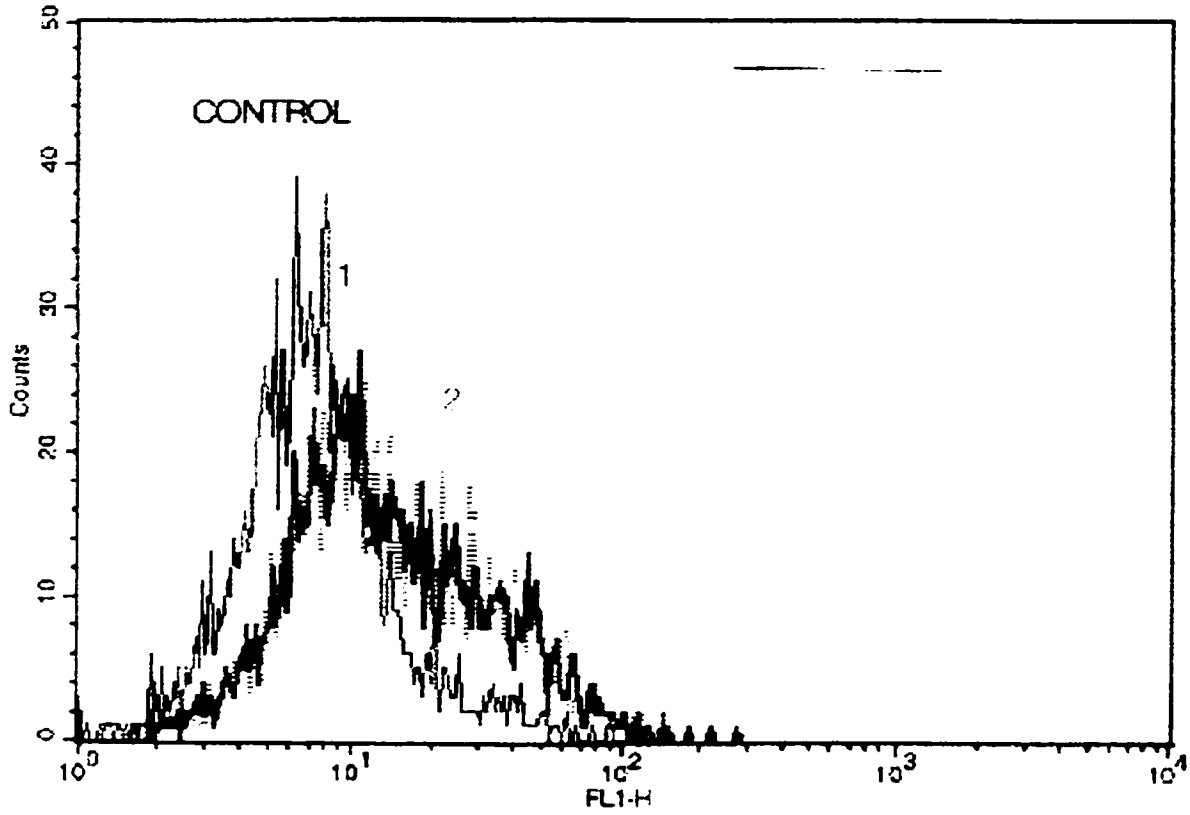

50000
40000
30000
20000
10000
0
Thymidine incorporation (cpm)
a
a
b
a
Media
APC-sup
4.2-sup
4.2-sup +
anti-TGFβ
Treatment

Figure 12. The effect of TGFβ on the expression of IL-2R α-chain on the surface of ConA blasts from NOD splenocytes. ConA blasts from NOD splenocytes (1 x 10^6 cells) were incubated in the presence, or absence, of TGFβ (10 ng/ml, 87 pM) for 12 h. Flow cytometric analysis was performed using antibodies against IL-2R α-chain (CD25). Data is the representative of four independent experiments. Control: without antibody (violet line), 1: untreated cells with antibody (green line), 2: TGFβ-treated cells with antibody (pink line).


CONTROL
1
2
Counts
0
10
20
30
40
50
10^0
10^1
10^2
10^3
10^4
FL1-H

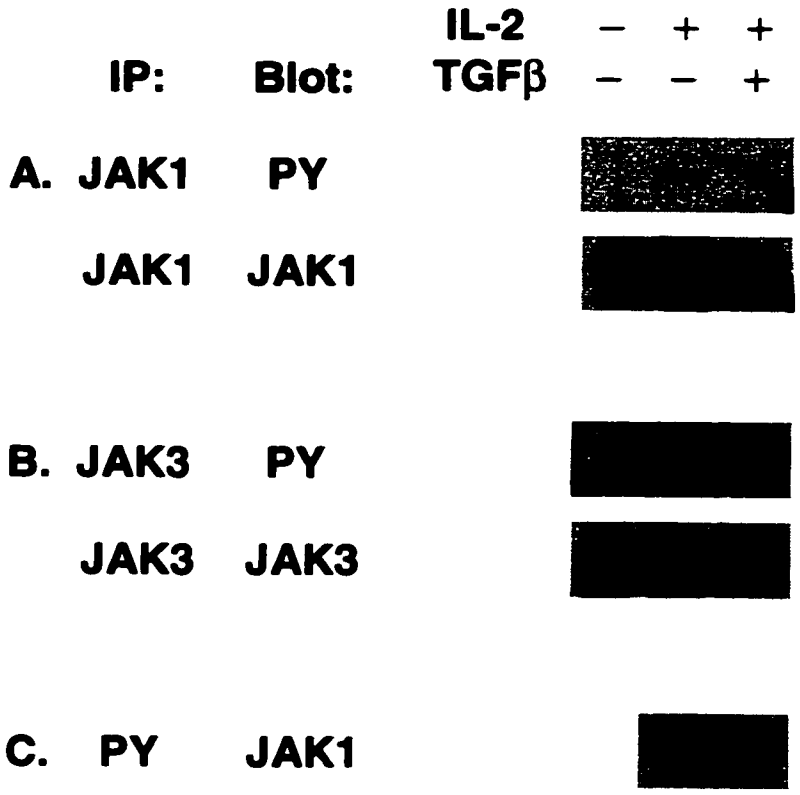
proliferation is not a result of the downregulation of IL-2R on the surface of T cells. Rather it may be a result of the inhibition of a signaling pathway inside the cells.

5.1.3.3 Inhibition by TGFβ of IL-2-induced tyrosine phosphorylation of JAK kinases

To determine whether TGFβ inhibits the activation of the IL-2-induced signaling pathways, I measured the level of tyrosine phosphorylation of JAK1 and JAK3 in ConA blasts from NOD splenocytes stimulated with IL-2 after treatment with TGFβ. I found that TGFβ treatment resulted in an inhibition of tyrosine phosphorylation of JAK1 (Figure 13A) and JAK3 (Figure 13B), while IL-2 stimulation induced tyrosine phosphorylation of JAK1 and JAK3. The inhibition of tyrosine phosphorylation of JAK1 by TGFβ was confirmed by immunoblot analysis using anti-JAK1 antibody and the phosphotyrosine immunoprecipitates (Figure 13C). These results suggest that the suppression by TGFβ of IL-2-dependent T cell proliferation may be a result of the inhibition of IL-2-induced activation of JAK kinases.

5.1.3.4 Inhibition by TGFβ of IL-2-induced tyrosine phospho-

Figure 13. The effect of TGFβ on tyrosine phosphorylation of IL-2-induced JAK1 and JAK3 in ConA blasts from NOD splenocytes. (A, B) *Upper panels:* ConA blasts from NOD splenocytes (5 x 10^7 cells) were incubated in the presence, or absence, of TGFβ (10 ng/ml, 87 pM) for 12 h. After stimulation with IL-2 (20 U/ml, 0.67 nM) for 5 min, cells were lyzed, immunoprecipitated with anti-JAK1 or anti-JAK3 antibody, and immunoblotted with anti-phosphotyrosine monoclonal antibody (4G10). *Low panels*: the nitrocellulose membranes were stripped and reprobed with anti-JAK1 or anti-JAK3 antibody. (C) Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and immunoblotted with anti-JAK1 antibody. Data is the representative of four independent experiments. The ratios of density of tyrosine-phosphorylated proteins to that of total proteins are 1.00, 15.86, 5.84 for Lane 1, 2, 3 of JAK1, and 1.00, 2.07, 1.32 for Lane 1, 2, 3 of JAK3.


IL-2 − + +
IP: Blot: TGFβ − − +
A. JAK1 PY
JAK1 JAK1
B. JAK3 PY
JAK3 JAK3
C. PY JAK1

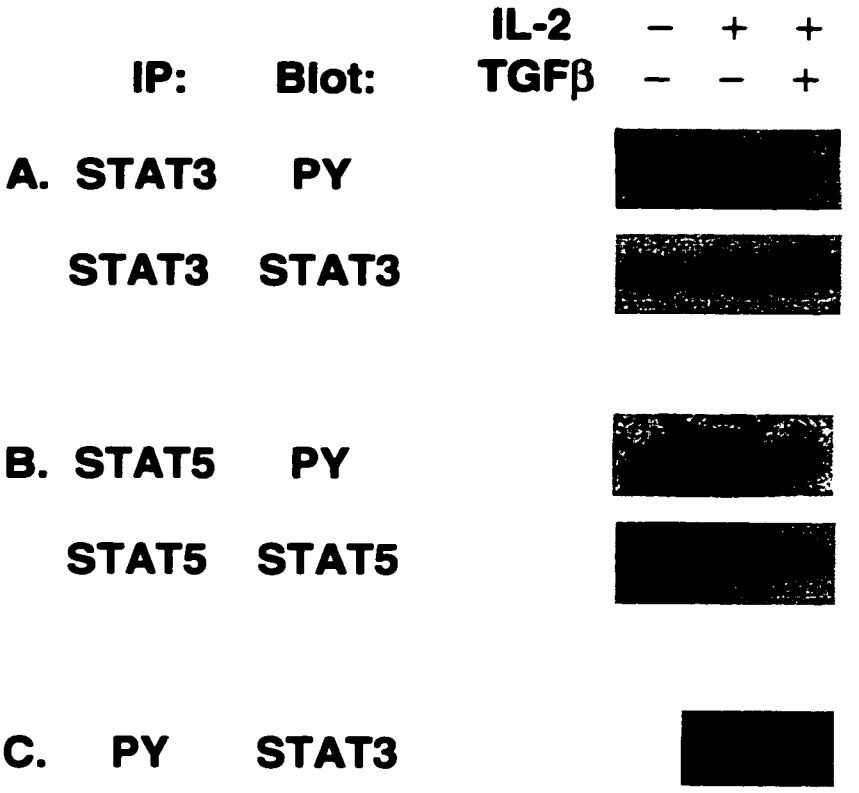
rylation of STAT proteins

Since JAK kinases were shown to induce tyrosine phosphorylation of STAT proteins (Ihle 1996), I then determined whether TGFβ inhibits tyrosine phosphorylation of STAT3 and STAT5 induced by IL-2 in T cells. I found that IL-2 stimulation induced tyrosine phosphorylation of STAT3 and STAT5. In contrast, TGFβ inhibited tyrosine phosphorylation of STAT3 (Figure 14A) and STAT5 (Figure 14B) in ConA blasts from NOD splenocytes. TGFβ-mediated inhibition of tyrosine phosphorylation of STAT3 was confirmed by immunoblot analysis using anti-STAT3 antibody and the phosphotyrosine immunoprecipitates (Figure 14C). These results suggest that TGFβ inhibits JAK kinase activation, leading to the inhibition of tyrosine phosphorylation of STAT proteins.

5.1.3.5 Inhibition by TGFβ of IL-2-induced tyrosine phosphorylation of ERK2

From the above studies, which determined the effect TGFβ has on tyrosine phosphorylation of IL-2-induced JAK kinases and STAT proteins, I learned that a 42 kD tyrosine-phosphorylated protein co-immunoprecipitated with JAK kina-

Figure 14. The effect of TGFβ on tyrosine phosphorylation of IL-2-induced STAT3 and STAT5 in ConA blasts from NOD splenocytes. (A, B) *Upper panels:* ConA blasts from NOD splenocytes (5 x 10^7 cells) were incubated in the presence, or absence, of TGFβ (10 ng/ml, 87 pM) for 12 h. After stimulation with IL-2 (20 U/ml, 0.67 nM) for 5 min, cells were lyzed, immunoprecipitated with anti-STAT3 or anti-STAT5 antibody and immunoblotted with anti-phosphotyrosine monoclonal antibody (4G10). *Lower panels:* the nitrocellulose membranes were stripped and reprobed with anti-STAT3 or anti-STAT5 antibody. (C) Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and immunoblotted with anti-STAT3 antibody. Data is the representative of four independent experiments. The ratios of density of tyrosine-phosphorylated proteins to that of total proteins are 1.00, 1.82, 1.06 for Lane 1, 2, 3 of STAT3, and 1.00, 5.12, 1.35 for Lane 1, 2, 3 of STAT5.


IL-2 − + +
IP: Blot: TGFβ − − +
A. STAT3 PY
STAT3 STAT3
B. STAT5 PY
STAT5 STAT5
C. PY STAT3

ses and STAT proteins, and it was found to be ERK2, as suggested by immunoblot analysis of the same membranes with anti-ERK2 antibody (data not shown).

It was recently suggested that mitogen-activated protein kinase (MAPK), such as extracellular signal-regulated kinase 2 (ERK2) (a 42 kD protein also activated by IL-2), is required for full activation of the JAK/STAT pathway (Zhang et al., 1995; Wen et al., 1995). Furthermore, ERK2 was shown to be directly associates with cytokine receptors such as IFNα/β receptors and STAT proteins such as STAT1 (David et al., 1995). In addition, it was suggested that JAK kinases are required for activation of the ERK/MAPK pathway as well as STAT proteins (Winston and Hunter, 1996). Thus, new proposed model for cytokine signaling pathway is that MAPK directly associates with the receptor and phosphorylates STAT proteins in the receptor complexes (Ihle, 1996). It is feasible that TGFβ may affect the activation of the ERK/MAPK pathway, resulting in a decrease of T cell responsiveness to IL-2. I therefore determined whether TGFβ inhibits IL-2-induced ERK activation in T cells. I found that IL-2 stimulation induced tyrosine phosphorylation of ERK2, while TGFβ

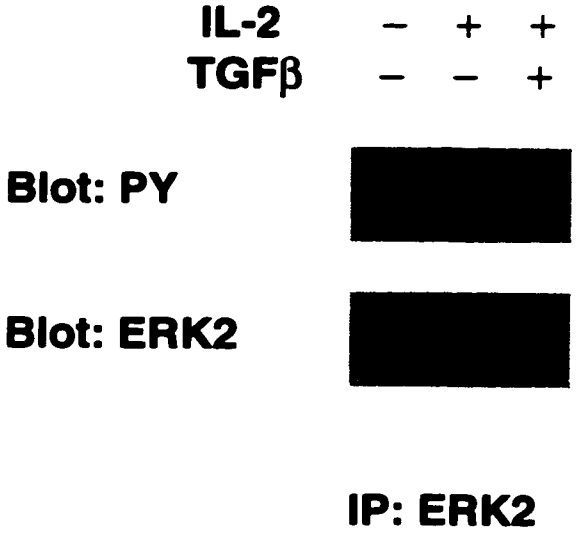
inhibited IL-2-induced tyrosine phosphorylation of ERK2 in T cells (Figure 15). These results suggest that TGFβ-mediated inhibition of ERK activation induced by IL-2 may be another mechanism by which TGFβ suppresses IL-2-dependent T cell proliferation.

5.1.4 Discussion

In this study, I first found that TGFβ secreted from NY4.2 CD4+ suppressor T cells, inhibited IL-2-dependent T cell proliferation, which is consistent with TGFβ-mediated inhibition of the proliferative responses to IL-2 of NOD splenocytes (Figure 6A). However, the molecular mechanisms by which TGFβ inhibits IL-2-dependent T cell proliferation are not known. It is reasonable to postulate that TGFβ-mediated inhibition of IL-2-dependent T cell proliferation is a result of the modulation of the IL-2R, such as downregulation of IL-2R on the surface of T cells. When I examined whether there is any change of IL-2R expression on the surface of T cells after TGFβ treatment, I found that it is not the case.

Since no change of IL-2R expression is evident on the

Figure 15. The effect of TGFβ on tyrosine phosphorylation of ERK2 in ConA blasts from NOD splenocytes. *Upper panels:* ConA blasts from NOD splenocytes (5 x 10^7 cells) were incubated in the presence, or absence, of TGFβ (10 ng/ml, 87 pM) for 12 h. After stimulation with IL-2 (20 U/ml, 0.67 nM) for 5 min, cells were lyzed, immunoprecipitated with anti-ERK2 antibody and immunoblotted with anti-phosphotyrosine monoclonal antibody (4G10). *Lower panels:* the nitrocellulose membranes were stripped and reprobed with anti-ERK2 antibody. Data is the representative of three independent experiments. The ratios of density of tyrosine-phosphorylated proteins to that of total proteins are 1.00, 18.40, 5.42 for Lane 1, 2, 3.


IL-2 − + +
TGFβ − − +
Blot: PY
Blot: ERK2
IP: ERK2

T cell surface, TGFβ may affect an IL-2-induced signal transduction pathway within T cells. I, therefore, examined whether TGFβ inhibits the activation of molecules involved in the IL-2-induced signal transduction pathways. I found that treatment with TGFβ resulted in the inhibition of tyrosine phosphorylation of JAK1 and JAK3, followed by the suppression of activation of STAT3 and STAT5 in T cells. Experiments using HT-2 cells, the murine IL-2-dependent T cell line, were also performed, providing similar results to those in this study using ConA blasts from NOD splenocytes (data not shown). These results suggest that TGFβ-mediated inhibition of the activation of the JAK/STAT pathway may be one mechanism for the suppression by TGFβ of IL-2-dependent T cell proliferation (Han et al., 1997).

My studies substantiate the previous findings on the significant effect of TGFβ on T cell proliferation dependent on IL-2 (Ruegemer et al., 1990), and the association of this effect with the inhibition of IL-2R signal transduction (Ahuja et al., 1993). Similar to my findings, a study showing that the mechanism of TGFβ-mediated inhibition of T cell proliferation is linked to the IL-2R signal transduction pathway, involving JAK-1 and STAT5 but

not JAK3 and STAT3, was later reported (Bright et al., 1997). Difference between their results and mine may be due to the different incubation time with TGFβ. They treated TGFβ for 30 min before IL-2 stimulation, while I treated TGFβ for 12 h. Therefore, their results might be due to the direct interference of proximal events of TGFβ signaling with the IL-2 signaling pathway, whereas my results are from the overall effect of TGFβ on T cells, including the effect of proteins induced by TGFβ. However, while they showed that TGFβ did not affect the activation of JAK3, the importance of JAK3 in the IL-2 signaling pathway was recently reported (Oakes et al., 1996). JAK3 mutant cells lost the ability to induce tyrosine phosphorylation, in response to IL-2, of IL-2R β-chain, JAK1, and STAT5. Thus, TGFβ-mediated inhibition of JAK3 activation induced by IL-2 may be important for the effect of TGFβ on T cell proliferation.

I found that TGFβ inhibited the activation of JAK kinases in T cells. One possible mechanism for the inhibition by TGFβ of JAK kinases is an increase of protein tyrosine phosphatase (PTP) activity by TGFβ, resulting in

the inactivation of JAK kinases and termination of IL-2-induced signaling in T cells. This proposition is supported by the ability of the tyrosine phosphatase inhibitor vanadate to restore IL-2-induced tyrosine phosphorylation of JAK1 kinase inhibited by TGFβ (Bright et al., 1997). It has been suggested that, among PTPs identified to date, SHPTP1 plays a negative role in the activation of T cells via a T cell receptor (TCR) signaling pathway (Pani et al., 1996). Moreover, it was reported that receptors for cytokines, such as erythropoietin (EPO) (Klingmuller et al., 1995) or IL-3 (Yi et al, 1993), interacts with SHPTP1, resulting in the inactivation of JAK kinases and termination of the proliferative signals induced by the cytokines. However, I failed to find any changes in tyrosine phosphorylation of SHPTP1 and its phosphatase activity in T cells treated with TGFβ (data not shown). These results suggest that the inhibitory effects of TGFβ on IL-2-induced activation of JAK kinases may not result from the activation of the PTP activity of SHPTP1, although the possible involvement of other PTPs in TGFβ action cannot be ruled out.

Cell growth arrest induced by TGFβ is known to be accompanied by the inhibition of cyclin-dependent kinases (CDKs) important for cell cycle progression, resulting in the accumulation of hypophosphorylated retinoblastoma (Rb) proteins. This effect is due to the upregulation of CDK inhibitors and/or the downregulation of CDK-activating phosphatase (Hu et al., 1998). However, the integration of positive and negative signals through IL-2R and TGFβR, respectively, in terms of cell growth is not well understood in T cells. In a recent study, the STAT5 protein was shown to be an essential mediator for IL-2-induced cell cycle progression of T cells (Moriggl et al., 1999). Genes implicated in cell cycle progression, including cyclin D2, cyclin D3, cyclin E, cyclin A, and CDK6, but not CDK2 or CDK4, were shown to be regulated by the STAT5 protein in response to IL-2. Thus, the suppression of STAT5 activation, leading to the regulation of the expression of genes involved in cell cycle progression, may contribute to the inhibition by TGFβ of cell cycle progression induced by IL-2 in T cells.

I showed that the JAK/STAT pathway is one candidate target for TGFβ in mediating growth-inhibitory effect on IL-

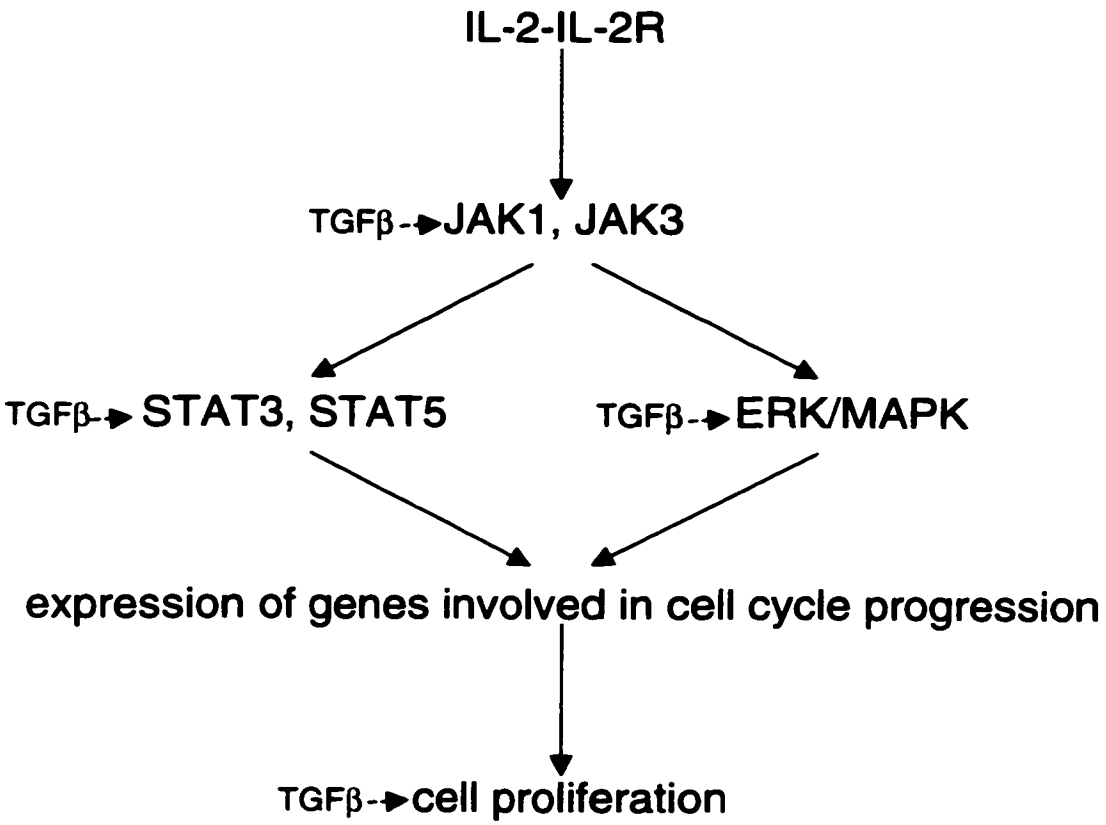
2-stimulated T cells. Similarly, IL-5-induced tyrosine phosphorylation of JAK2 and STAT1 in eosinophils (Pazdrak et al., 1995) and IL-12-induced activation of the JAK/STAT pathway in T cells (Pardoux et al., 1999) were inhibited by TGFβ. However, the inhibitory effect of TGFβ on the cytokine-associated JAK/STAT pathway is not likely to be universal since IFNγ-induced JAK/STAT activation was not affected by TGFβ in glial cells (Panek et al., 1995).

The other signaling pathway induced by IL-2 in T cells is the ERK/MAPK pathway (Theze et al., 1996). Intriguingly, MAPK, such as ERK2, was recently shown to play an important role in the full activation of STAT proteins and associate with STAT proteins and cytokine receptors (Ihle, 1996; Zhang et al., 1995; Wen et al., 1995; David et al., 1995). In addition, JAK kinases, which associate with cytokine receptors and induce tyrosine phosphorylation of STAT proteins, are suggested to be required for MAPK activation induced by cytokine receptors such as growth hormone receptors (Winston and Hunter, 1996). Consistent with TGFβ-mediated inhibition of the activation of JAK kinases induced by IL-2, I found that TGFβ inhibited IL-2-induced activation of ERK2, coimmunoprecipitated with STAT

proteins, in T cells. These results suggest that TGFβ-mediated inhibition of ERK activation in T cells may be another mechanism responsible for the suppression by TGFβ of IL-2-dependent T cell proliferation (Figure 16).

Taken together, my results indicate that, although how TGFβ inhibits IL-2-induced activation of JAK kinases is not known, TGFβ interferes with the activation of JAK/STAT pathway and ERK/MAPK pathway without modulation of the surface expression of IL-2R on T cells, resulting in the inhibition of IL-2-dependent T cell proliferation. This effect of TGFβ is a potential molecular mechanism by which TGFβ-producing CD4+ suppressor T cells prevent the development of effector T cell-mediated autoimmune diabetes in NOD mice.

Figure 16. Model for IL-2-induced signal transduction pathways interfered with by TGFβ in T cells. Binding of IL-2 to the IL-2R results in the aggregation of α-, β-, and γ-chains and the subsequent activation of JAK1 and JAK3 (by tyrosine phosphorylation), which are associated with IL-2R β- and γ-chains, respectively. STAT proteins are then recruited to IL-2R tyrosine phosphorylated by JAK kinases. The recruited STAT proteins are phosphorylated on the tyrosine residues by receptor-associated JAK kinases. The tyrosine-phosphorylated STAT proteins dimerize and then translocate to the nucleus where STAT proteins bind to the promoters of IL-2-responsive genes. Activation of JAK kinases may also result in ERK/MAPK activation. Maximal activation of STAT protein requires serine phosphorylation, probably by ERK, as well as tyrosine phosphorylation. TGFβ may interfere with the tyrosine phosphorylation of JAKs, resulting in the inhibition of tyrosine phosphorylation of STAT proteins. In addition, TGFβ may block the activation of ERK, leading to a decrease of the transcriptional activity of STAT proteins.


IL-2-IL-2R
TGFβ-►JAK1, JAK3
TGFβ-►STAT3, STAT5
TGFβ-►ERK/MAPK
expression of genes involved in cell cycle progression
TGFβ-►cell proliferation

5.2 Effect of TGFβ on signal transduction pathways involved in macrophage activation

5.2.1 Introduction

As described in the INTRODUCTION, macrophages have been implicated in the development of IDDM, based on their early presence in the infiltrated islet and the ability of macrophage depletion to prevent diabetes. Attention has been focused on the cytokine TGFβ as a potent inhibitor of macrophage activation. Treatment of macrophages *in vitro* with TGFβ1 has been reported: 1) to inhibit their ability to express MHC class II genes (Czarniecki et al., 1988), 2) to initiate a respiratory burst (Tsunawaki et al., 1988), 3) to produce cytokines (Bogdan et al, 1992) and reactive nitrogen intermediates (Vodovotz et al., 1993), and 4) to kill intracellular microbes (Silva etl al., 1991) or extracellular tumor cells (Pinson et al., 1992). Moreover, TGFβ1-deficient mice exhibited the constitutive expression of cytokine and iNOS genes that are expressed by macrophages only upon activation (Shull et al., 1992; Vodovotz et al., 1996).

Although the mechanisms by which TGFβ inhibits macrophage responses to cytokines such as IFNγ have become better defined (Nandan and Reiner 1997; Ulloa et al.,

1999), less attention has been paid to a role for TGFβ in host responses to bacterial endotoxin and the mechanisms for the inhibition by TGFβ of macrophage responses induced by bacterial lipopolysaccharide (LPS). LPS, a conserved component of the Gram-negative bacterium's outer membrane, is one of the best-studied and most important triggers of macrophage activation. Macrophage responses to LPS include the production of a number of potent proinflammatory mediators responsible for septic shock (Ulevitch and Tobias, 1995; Sweet and Hume, 1996). The major cell surface receptor for LPS on macrophages is CD14. LPS binding to CD14 leads to activation of protein tyrosine kinases (PTKs) such as members of *src* tyrosine kinase family in macrophages (Stefanova et al., 1993), although the precise mechanism is unknown. Upon LPS stimulation, numerous proteins become tyrosine phosphorylated, in particular MAPKs (Reimann et al., 1994; Han et al., 1994; Hambleton et al., 1996). Studies utilizing PTK inhibitors indicate that PTKs play critical roles in LPS signaling (Shapira et al., 1994) and that MAPK activation occurs downstream of tyrosine phosphorylation (Reimann et al., 1994). Activation of the transcription factor NFκB is also well described

signaling pathway initiated by LPS treatment of macrophages (Ulevitch and Tobias, 1995). Tyrosine phosphorylation events may be important for NFκB activation since PTK inhibitor blocked NFκB activation (Ishikawa et al., 1995). In addition, the other signaling pathways induced by LPS include heterotrimeric GTP-binding proteins (Zhang and Morrison, 1993), protein kinase C (Shapira et al., 1994) and ceramide-activated protein kinase (CAK) (Joseph et al., 1994). Thus, how LPS induces the production of inflammatory soluble mediators is only partly understood.

While extensive studies have demonstrated that many cytokines including IFNγ can enhance LPS-initiated macrophage activation (Hausmann et al., 1994), much less is known about negative regulation of these responses. From my study, I learned that TGFβ-mediated suppression of macrophage activation in NOD mice contributes to the prevention of immune cell-mediated pancreatic β cell destruction. In order to elucidate the molecular mechanisms for the suppression by TGFβ of macrophage activation, I determined the effect of TGFβ on the signal transduction pathways involved in macrophage activation induced by LPS.

5.2.2 Materials and methods

5.2.2.1 Reagents

Recombinant human TGFβ1 was purchased from R&D Systems and LPS (from *E. coli* 026:B6) was from Sigma. For immunoprecipitation and immunoblot analysis, anti-phosphotyrosine monoclonal antibody (4G10) was purchased from Upstate Biotechnology, and anti-NFκB p50, anti-c-Rel, anti-c-Fos, anti-c-Jun, anti-IκBα, anti-IKKα, anti-ERK2, anti-JNK1, and anti-$p90^{rsk1}$ antibodies were purchased from Santa Cruz Biotechnology.

5.2.2.2 Cell preparations and stimulation

Peritoneal macrophages were isolated by protocol described in 4.2.1. RAW 264.7, the murine macrophage-like cell line, was obtained from the American Type Culture Collection and cells were maintained in RPMI 1640 medium containing 5% FBS, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were plated at 1 x 10^6 per well in 24-well plates (Falcon) for NO release and TNF secretion studies, at 1 x 10^7 per 60 mm-diameter petri dish (Falcon) for RT-PCR analysis, the preparation of nuclear extracts

and immunoblot analysis, and at 2 x 10^7 per 100 mm-diameter petri dish (Falcon) for immunoprecipitation.

Cells were incubated in the presence, or absence of TGFβ (10 ng/ml, 87 pM) in serum-free medium and stimulated with LPS (1 ng/ml, 0.25 nM, m.w. 4 kD). Stimulation times were 24 h for NO assay and TNF assay, 6 h for RT-PCR analysis, 1 h for preparation of nuclear extracts, and 30 min for immunoprecipitation and immunoblot analysis. For NO and TNF assays, at the end of cultivation, culture supernatants were collected and stored at -70°C until use.

5.2.2.3 NO assay and TNF assay

See 4.2.6 and 4.2.7.

5.2.2.4 RT-PCR analysis of gene expression

See 4.2.3.

5.2.2.5 Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Cells were suspended in 400 µl of cold buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 2 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml

aprotinin) and incubated on ice for 15 min after which 25 µl of 10% NP-40 was added and the tube was vortexed for 15 sec. The homogenate was centrifuged for 30 sec in a microfuge and the nuclear pellet was resuspended in 50 µl of cold buffer (50 mM HEPES pH 7.9, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 µg/ml aprotinin and 10% glycerol). After rocking for 15 min at 4°C, the nuclear extract was centrifuged for 5 min in a microfuge and the supernatant was kept at -70°C until used for EMSA. The protein concentration was measured (BioRad).

The specific double-stranded oligonucleotides (Santa Cruz Biotechnology) were P^{32}-labelled with [γ-^{32}P] ATP, using polynucleotide kinase (Pharmacia Biotech., Uppsala, Sweden) (50,000 cpm/sample). Binding reaction mixtures (20 µl), containing 0.5 ng DNA probes and 3 - 10 µg nuclear extract in 10 mM Tris (pH 7.5) buffer with 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol and 1 µg of poly(dI-dC) (Pharmacia Biotech.) to inhibit non-specific binding of the labeled probe to nuclear extract proteins, were incubated at room temperature for 20 min. DNA-protein complexes were resolved by electrophoresis through 6% polyacrylamide gels. The gels

were subsequently dried and autoradiographed at -70ºC. The density of autoradiograms was quantified using ImageQuaNT program and the ratios to the density of untreated controls were shown in Figures.

5.2.2.6 Immunoprecipitation and immunoblot analysis

See 5.1.2.5.

5.2.2.6 *In vitro* kinase assay

Cells were lyzed in ice-cold lysis buffer and the lysates were immunoprecipitated with the designated antibodies and protein A-Sepharose as described in 5.1.2.5. The immunoprecipitates were washed twice with lysis buffer and suspended in 30 µl of kinase assay buffer (25 mM HEPES [pH 7.6], 20 mM $MgCl_2$, 100 µM Na_3VO_4, 20 mM β-glycerolphosphate, 20 mM p-nitrophenyl phosphate, 2 mM DTT, 50 µM ATP, 5 µCi of [γ-^{32}P]ATP, and 0.5 µg of IκBα or c-Jun GST fusion protein [Santa Cruz] as a substrate). After incubation for 20 min at 30°C, the reaction was stopped by adding 5x sample buffer and the products were resolved by SDS-PAGE. The gels were subsequently dried and autoradiographed at -70°C. The density of autoradiograms was

quantified using ImageQuaNT program and the ratios to the density of untreated controls were shown in Figures.

5.2.2.8 Statistical analysis

See 3.2.6.

5.2.3 Results

5.2.3.1 Inhibition by TGFβ of the secretion of nitric oxide (NO) and TNF

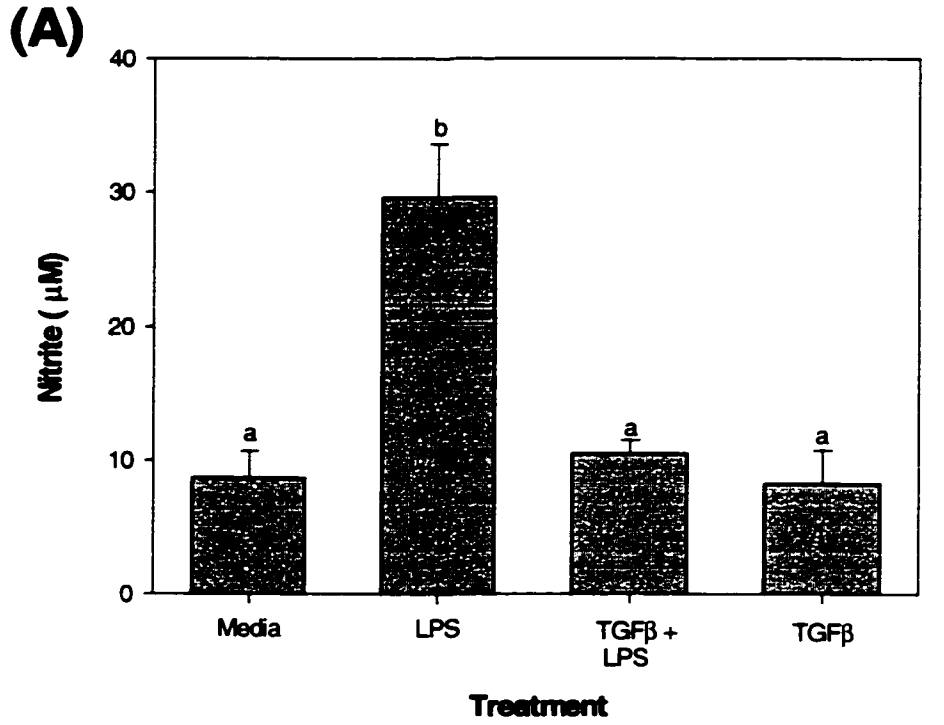
I first determined whether TGFβ inhibits the activation of macrophages by examining the effect of TGFβ on the secretion of NO and TNF, important proinflammatory mediators produced by LPS-activated mouse peritoneal macrophages. In agreement with reported studies using RAW 264.7 cells (Hausmann et al., 1994), I found that TGFβ inhibited the secretion of NO and TNF by macrophages stimulated with LPS (Figure 17).

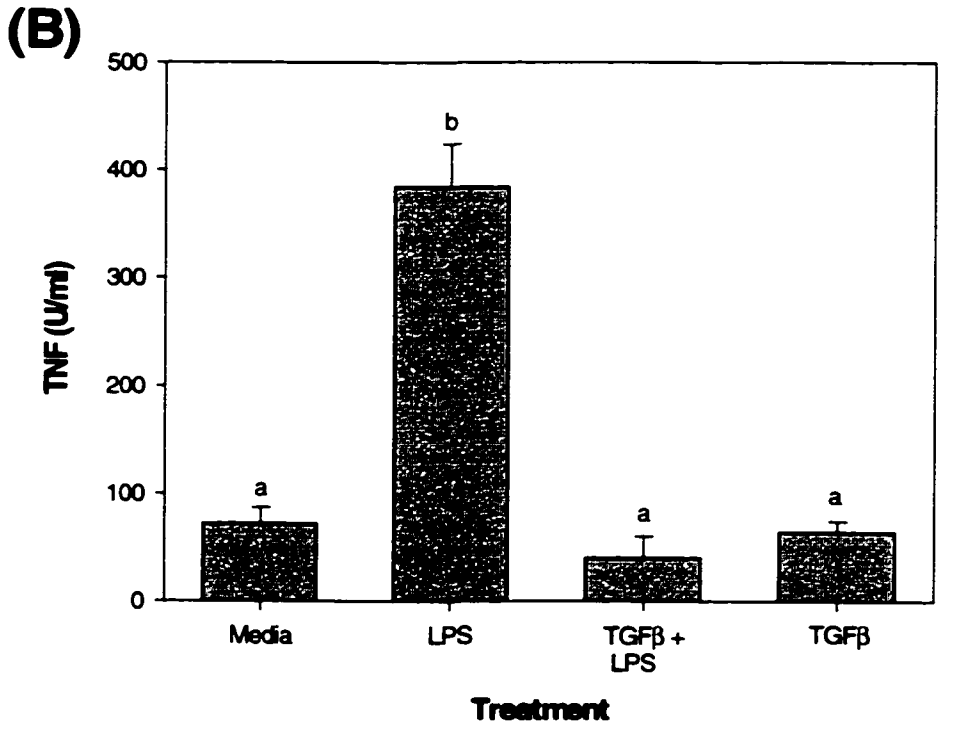
5.2.3.2 Inhibition by TGFβ of the gene expression of iNOS

To see whether TGFβ-mediated inhibition of the secretion of NO and TNF results from the suppression of

Figure 17. The effect of TGFβ on the secretion of nitric oxide (NO) (A) and TNFα (B) by macrophages stimulated with LPS. Thioglycollate-elicited peritoneal macrophages from NOD mice were treated with TGFβ (10 ng/ml, 87 pM) for 4 h and stimulated with LPS (1 ng/ml, 0.25 nM). Twenty-four hours later, culture supernatants were collected to perform NO assay and TNFα assay. The mean ± SD of representative data of four independent experiments was shown.

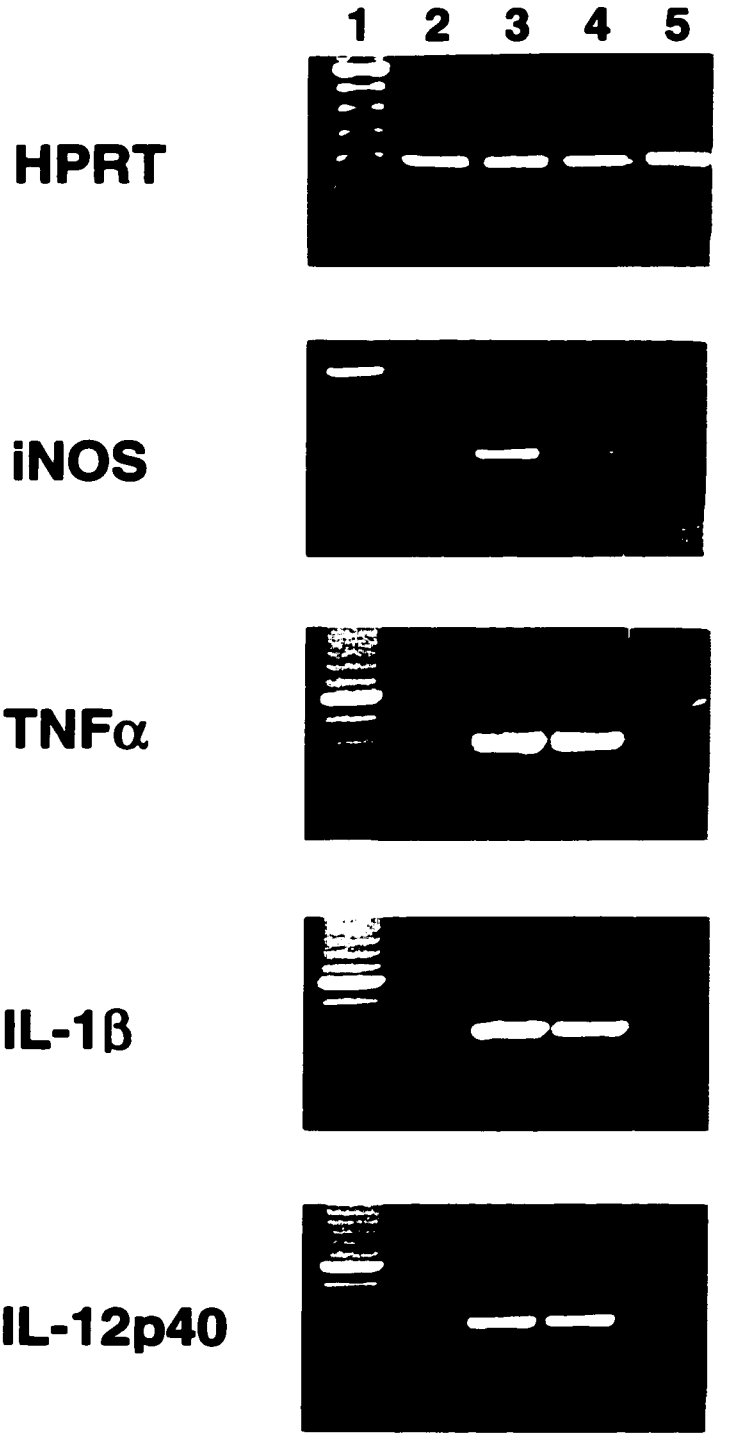
* a vs b are significantly different ($p < 0.01$).


(A)
Nitrite (μM)
40
30
20
10
0
a
b
a
a
Media
LPS
TGFβ + LPS
TGFβ
Treatment


(B)
TNF (U/ml)
500
400
300
200
100
0
a
b
a
a
Media
LPS
TGFβ + LPS
TGFβ
Treatment

gene expression, and whether TGFβ also affects the gene expression of other cytokines such as IL-1β and IL-12 produced by activated macrophages, I determined the effect of TGFβ on gene expression by RT-PCR analysis of iNOS, TNFα, IL-1β and the p40 subunit of IL-12 (IL-12p40) in macrophages stimulated with LPS. I found that the expression of genes for all of iNOS, TNFα, IL-1β, and IL-12p40 was induced by LPS stimulation (Figure 18). In contrast to the inhibition by TGFβ of IFNγ-induced gene expression of all macrophage-derived cytokines and iNOS (Figure 9), only iNOS gene expression was inhibited by TGFβ in LPS-stimulated macrophages (Figure 18). These results suggest that the molecular mechanisms for the inhibition by TGFβ of macrophage responses to different stimuli are distinct. Since TGFβ did not affect the gene expression of TNFα in LPS-stimulated macrophages, TGFβ-mediated inhibition of TNF production may be due to the regulation at the posttranscriptional levels rather than the suppressed expression of the TNFα gene, while TGFβ-mediated inhibition of NO production is, at least partially, due to the suppressed expression of the iNOS gene. Thus, the

Figure 18. The effect of TGFβ on the gene expression of iNOS, TNFα, IL-1β, and p40 subunit of IL-12 in macrophages stimulated with LPS. Thioglycollate-elicited peritoneal macrophages from NOD mice were treated with TGFβ (10 ng/ml, 87 pM) for 4 h and stimulated with LPS (1 ng/ml, 0.25 nM) for 6 h. RNA was isolated and RT-PCR analysis was performed using specific primers. Lane 1: 100 bp DNA ladder, Lane 2: untreated, Lane 3: LPS-stimulated, Lane 4: TGFβ-treated and LPS-stimulated, Lane 5: TGFβ-treated. Representative data of four independent experiments was shown.


1 2 3 4 5
HPRT
iNOS
TNFα
IL-1β
IL-12p40

mechanisms for the inhibitory effect of TGFβ on LPS-induced NO and TNF are distinct, suggesting that the induction of their synthesis is regulated independently in macrophages.

5.2.3.3 Inhibition by TGFβ of the activation of transcription factors NFκB and AP-1 involved in iNOS gene expression

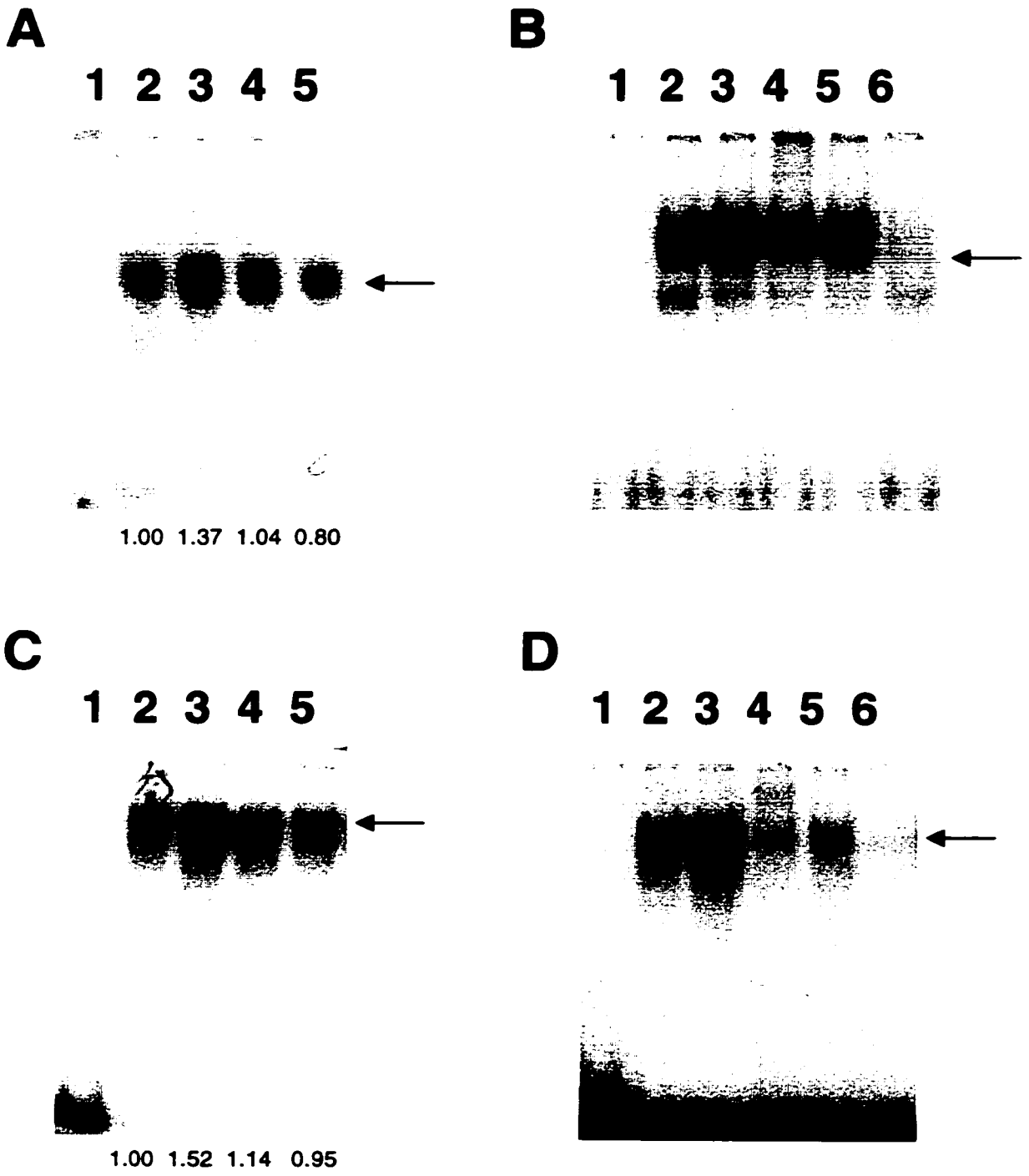
Since I found that TGFβ inhibited the expression of the iNOS gene in response to LPS, I determined whether TGFβ affects the activity of transcription factors involved in the expression of the iNOS gene by EMSA using P^{32}-labelled specific oligonucleotides. From this point, I used RAW 264.7 cells rather than peritoneal macrophages from mice to decrease the possible variations of primarily isolated macrophages in terms of responsiveness to LPS (Sweet and Hume, 1996). It was reported that the promoter region of murine iNOS gene includes the binding sites for transcription factors such as nuclear factor for κB (NFκB) and activator protein (AP)-1 (Xie et al, 1993; Lowensrein et al, 1993; Xie et al., 1994). I found that LPS stimulation induced the activation of NFκB and AP-1, while TGFβ inhibited the DNA-binding activity of NFκB and AP-1

(Figure 19). In contrast, the DNA-binding activity of a transcription factor interferon regulatory factor (IRF)-1, which is also shown to be important for iNOS gene expression in macrophages (Kamijo et al., 1994), was not affected by TGFβ treatment (data not shown). These results suggest that TGFβ-mediated inhibition of iNOS gene expression in LPS-activated macrophages is, at least in part, due to the inhibition of the activation of transcription factors such as NFκB and AP-1 involved in the expression of the iNOS gene.

5.2.3.4 Inhibition by TGFβ of the degradation of IκBα

Since the DNA-binding activity of NFκB was affected by TGFβ treatment, in order to elucidate the molecular mechanisms for the inhibitory effects of TGFβ on macrophage activation, I then examined the effect of TGFβ on the signaling pathways involved in the activation of NFκB. NFκB is sequestered in the cytoplasm by tightly bound inhibitory proteins called IκBs (Baldwin Jr., 1996; Baeuerle and Baltimore, 1996). NFκB activation can be induced by serine phosphorylation and subsequent degradation of the IκBs,

Figure 19. The effect of TGFβ on the DNA-binding activity of NFκB and AP-1 in macrophages stimulated with LPS. RAW 264.7 cells were treated with TGFβ (10 ng/ml, 87 pM) for 4 h and stimulated with LPS (1 ng/ml, 0.25 nM) for 1 h. Cells were then lysed and nuclear extracts were prepared to be subject to EMSA with P^{32}-labelled specific oligonucleotides. (A, C) Lane 1: no nuclear extracts, Lane 2: untreated, Lane 3: LPS-stimulated, Lane 4: TGFβ-treated and LPS-stimulated, Lane 5: TGFβ-treated. (B, D) Investigation of the identity of nuclear complex bound to the oligonucleotide. Lane 1: no nuclear extracts, Lane 2: untreated, Lane3: LPS-stimulated, Lane 4: LPS-stimulated, and pretreated with anti-NFκB p50 for B or anti-c-Fos for D, Lane 5: LPS-stimulated, and pretreated with anti-c-Rel for B or anti-c-Jun for D, Lane 6: LPS-stimulated, and pretreated with unlabeled oligonucleotide. Representative data of four independent experiments was shown.

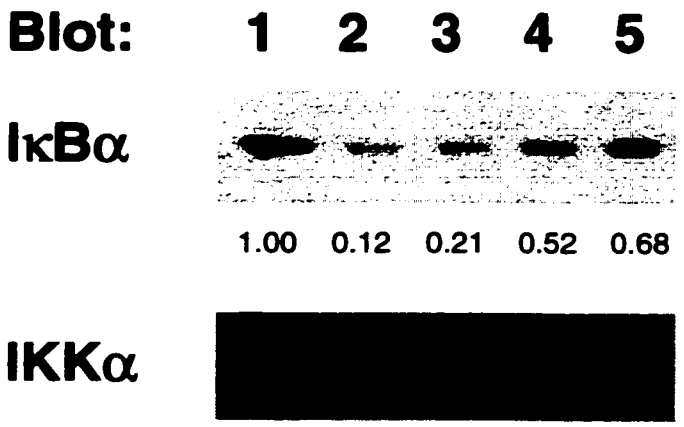

A
1 2 3 4 5
1.00 1.37 1.04 0.80
B
1 2 3 4 5 6
C
1 2 3 4 5
1.00 1.52 1.14 0.95
D
1 2 3 4 5 6

allowing NFκB to translocate into the nucleus and activate target genes. When the amounts of IκBα protein was examined by immunoblot analysis, I found that IκBα was degraded after LPS stimulation and TGFβ inhibited LPS-induced degradation of IκBα (Figure 20). The amount of loaded proteins was normalized by immunoblot analysis of the same membrane with antibody to another protein such as IκB kinase α. These results suggest that TGFβ-mediated inhibition of the activation of NFκB induced by LPS may be due to the suppression by TGFβ of LPS-induced IκBα degradation in macrophages.

5.2.3.5 Inhibition by TGFβ of the activation of IKKα

IκB kinases (IKKs) were recently identified, which specifically phosphorylates IκBα on the sites that trigger its degradation, serines 32 and 36 (Regnier et al., 1997; DiDonato et al., 1997; Mercurio et al., 1997; Woronicz et al, 1997). Therefore, I determined whether LPS stimulation induces the activation of IKK and whether TGFβ-mediated inhibition of IκBα degradation results from the suppression of IKK activity by examining the kinase activity of IKKα

Figure 20. The effect of TGFβ on the degradation of IκBα in macrophages stimulated with LPS. RAW 264.7 cells were treated with TGFβ (10 ng/ml, 87 pM) for different periods and stimulated with LPS (1 ng/ml, 0.25 nM) for 30 min. Cells not treated with TGFβ were left in the same media for 16 h and stimulated with LPS. *Upper panels:* cells were then lysed and immunoblotted with anti-IκBα antibody. *Lower panels:* the nitrocellulose membranes were stripped and reprobed with anti-ERK2 antibody. Lane 1: untreated, Lane 2: LPS-stimulated, Lane 3: TGFβ-treated for 30 min and LPS-stimulated, Lane 4: TGFβ-treated for 4 h and LPS-stimulated, Lane 5: TGFβ-treated for 16 h and LPS-stimulated. Representative data of five independent experiments was shown. Numerical values represent the ratios of density of IκB to that of IKKα.

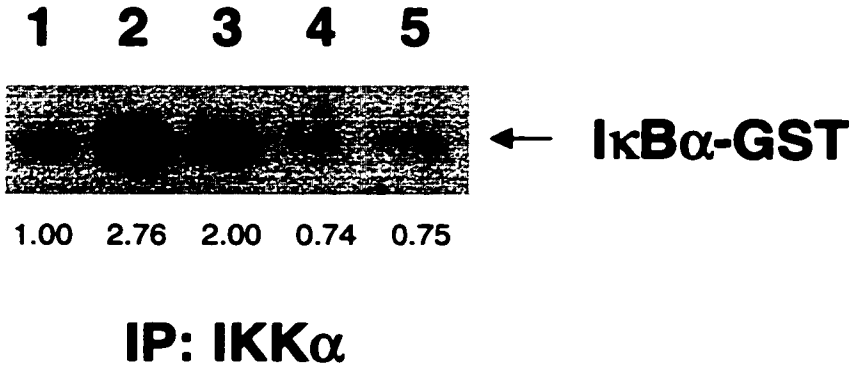

Blot:
1 2 3 4 5
IκBα
1.00 0.12 0.21 0.52 0.68
IKKα

using IκBα GST fusion protein as a substrate. I found that the kinase activity of IKKα was increased by LPS stimulation and TGFβ treatment resulted in an inhibition of the IKKα activity (Figure 21). These results suggest that one signaling pathway interfered with by TGFβ in LPS-stimulated macrophages may include the activation of IKKα, the degradation of IκBα and the activation of NFκB, resulting in iNOS gene expression and NO production.

5.2.3.6 Inhibition by TGFβ of tyrosine phosphorylation of ERK2

The early signaling event of LPS stimulation is tyrosine phosphorylation of multiple proteins (Ulevitch and Tobias, 1995; Sweet and Hume, 1996). It was shown that protein tyrosine kinase (PTK) inhibitors cause a severe impairment in LPS-induced production of cytokines, emphasizing an important role of tyrosine phosphorylation of cellular proteins in mediating the macrophage response to LPS. To determine whether TGFβ inhibits tyrosine phosphorylation of proteins, proximal signaling events induced by LPS, I measured the level of tyrosine phosphory-

Figure 21. The effect of TGFβ on the kinase activity of IKKα in macrophages stimulated with LPS. RAW 264.7 cells were treated with TGFβ (10 ng/ml, 87 pM) for different periods and stimulated with LPS (1 ng/ml, 0.25 nM) for 30 min. Cells not treated with TGFβ were left in the same media for 16 h and stimulated with LPS. Cells were then lysed and immunoprecipitated with anti-IKKα antibody. *In vitro* kinase assay was performed using P^{32}-labelled IκBα-GST fusion protein. Lane 1: untreated, Lane 2: LPS-stimulated, Lane 3: TGFβ-treated for 30 min and LPS-stimulated, Lane 4: TGFβ-treated for 4 h and LPS-stimulated, Lane 5: TGFβ-treated for 16 h and LPS-stimulated. Representative data of three independent experiments was shown.


1 2 3 4 5
IκBα-GST
1.00 2.76 2.00 0.74 0.75
IP: IKKα

lation after treatment with TGFβ. I found that LPS stimulation induced tyrosine phosphorylation of cellular proteins and TGFβ inhibited tyrosine phosphorylation of these proteins, especially 42 and 44 kD proteins (Figure 22). These prominently tyrosine-phosphorylated proteins were found to be extracellular signal-regulated kinase 1 (ERK1) (44 kD) and ERK2 (42 kD), as demonstrated by immunoblot analysis of the same membrane with anti-ERK2 antibody (Figure 22). TGFβ-mediated inhibition of tyrosine phosphorylation of ERK2 was confirmed by the immunoblot analysis of ERK2 immunoprecipitates with anti-phosphotyrosine antibody (Figure 23). This TGFβ-mediated inhibition of the activation of ERK/MAPK may contribute to the suppression by TGFβ of the activation of AP-1 shown in Figure 19 in LPS-stimulated macrophages (Figure 26), since it was known that ERK induces the activation of transcription factor Elk-1, leading to gene expression of *c-fos*, of which the protein is a component of AP-1 (Su and Karin, 1996; Karin et al., 1997).

5.2.3.7 Inhibition by TGFβ of the activation of JNK1

Figure 22. The effect of TGFβ on tyrosine phosphorylation of cellular proteins in macrophages stimulated with LPS. RAW 264.7 cells were treated with TGFβ (10 ng/ml, 87 pM) for 16 h and stimulated with LPS for 30 min. *Upper panels:* cells were then lysed and immunoblotted with anti-phosphotyrosine antibody (4G10). *Lower panels:* the nitrocellulose membranes were stripped and reprobed with anti-ERK2 antibody. Lane 1: untreated, Lane 2: LPS (1 ng/ml, 0.25 nM)-stimulated, Lane 3: TGFβ-treated and LPS (1 ng/ml, 0.25 nM)-stimulated, Lane 4: LPS (10 ng/ml, 2.5 nM)-stimulated, Lane 5: TGFβ-treated and LPS (10 ng/ml, 2.5 nM)-stimulated. Representative data of three independent experiments was shown.

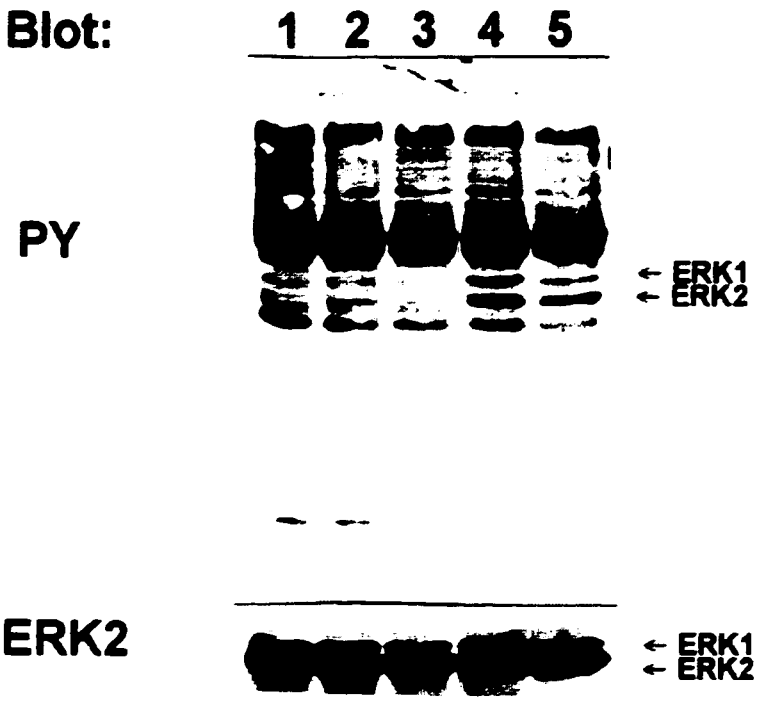
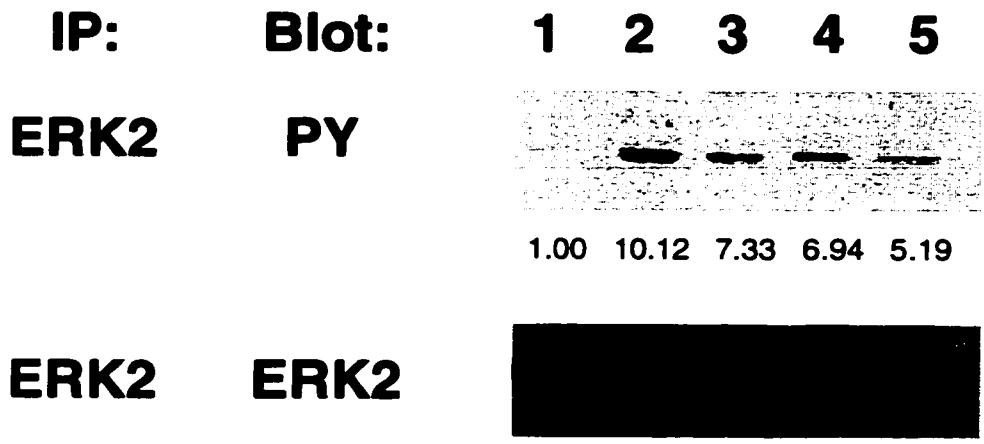

Blot:
1 2 3 4 5
PY
ERK1
ERK2
ERK2
ERK1
ERK2

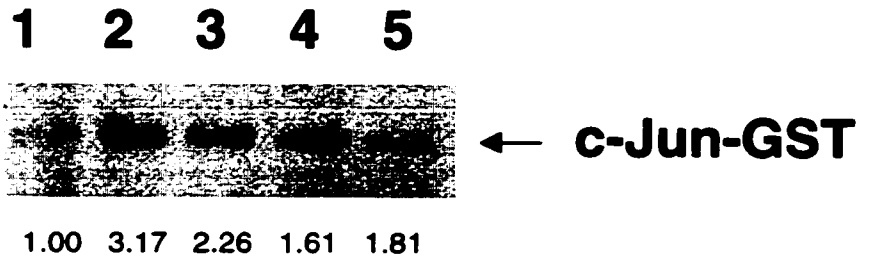
Figure 23. The effect of TGFβ on tyrosine phosphorylation of ERK2 in macrophages stimulated with LPS. RAW 264.7 cells were treated with TGFβ (10 ng/ml, 87 pM) for different periods and stimulated with LPS (1 ng/ml, 0.25 nM) for 30 min. Cells not treated with TGFβ were left in the same media for 16 h and stimulated with LPS. *Upper panels:* cells were then lysed, immunoprecipitated with anti-ERK2 antibody, and immunoblotted with anti-phosphotyrosine (PY) monoclonal antibody (4G10). *Lower panels:* the nitrocellulose membranes were stripped and reprobed with anti-ERK2 antibody. Lane 1: untreated, Lane 2: LPS-stimulated, Lane 3: TGFβ-treated for 30 min and LPS-stimulated, Lane 4: TGFβ-treated for 4 h and LPS-stimulated, Lane 5: TGFβ-treated for 16 h and LPS-stimulated. Representative data of three independent experiments was shown. Numerical values represent the ratios of density of tyrosine-phosphorylated proteins to that of total proteins.


IP:
Blot:
1 2 3 4 5
ERK2
PY
1.00 10.12 7.33 6.94 5.19
ERK2
ERK2

In addition to the inhibition by TGFβ of ERK activation, TGFβ-mediated suppression of AP-1 activation in macrophages stimulated with LPS could result from, in part, the inhibitory effect on c-Jun N-terminal kinase (JNK) activation, the other kinase involved in the regulation of AP-1. JNK, another subfamily of MAP kinase family, mediate the activation of c-Jun, constitutively present in cells in an inactive form, by serine phosphorylation (Su and Karin, 1996; Karin et al., 1997). Recently, JNK has been shown to become activated after LPS treatment of macrophages (Hambleton et al., 1996). To see whether TGFβ-mediated inhibition of the activation of AP-1 results from, in part, the suppression of JNK activity, I examined the kinase activity of JNK1 using c-Jun GST fusion protein as a substrate. I found that LPS stimulation induced the activation of JNK1 and the kinase activity of JNK1 was inhibited by TGFβ (Figure 24). These results suggest that the inhibition of AP-1 activation by TGFβ may result from the suppression of JNK as well as ERK activation in macrophages stimulated with LPS (Figure 26).

5.2.3.8 Inhibition by TGFβ of the activation of $p90^{rsk1}$

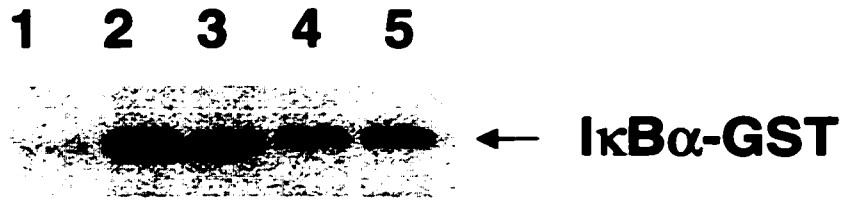
Figure 24. The effect of TGFβ on the kinase activity of JNK1 in macrophages stimulated with LPS. RAW 264.7 cells were treated with TGFβ (10 ng/ml, 87 pM) for different periods and stimulated with LPS (1 ng/ml, 0.25 nM) for 30 min. Cells not treated with TGFβ were left in the same media for 16 h and stimulated with LPS. Cells were then lysed and immunoprecipitated with anti-JNK1 antibody. *In vitro* kinase assay was performed using P^{32}-labelled c-Jun-GST fusion protein. Lane 1: untreated, Lane 2: LPS-stimulated, Lane 3: TGFβ-treated for 30 min and LPS-stimulated, Lane 4: TGFβ-treated for 4 h and LPS-stimulated, Lane 5: TGFβ-treated for 16 h and LPS-stimulated. Representative data of three independent experiments was shown.



IP: JNK1

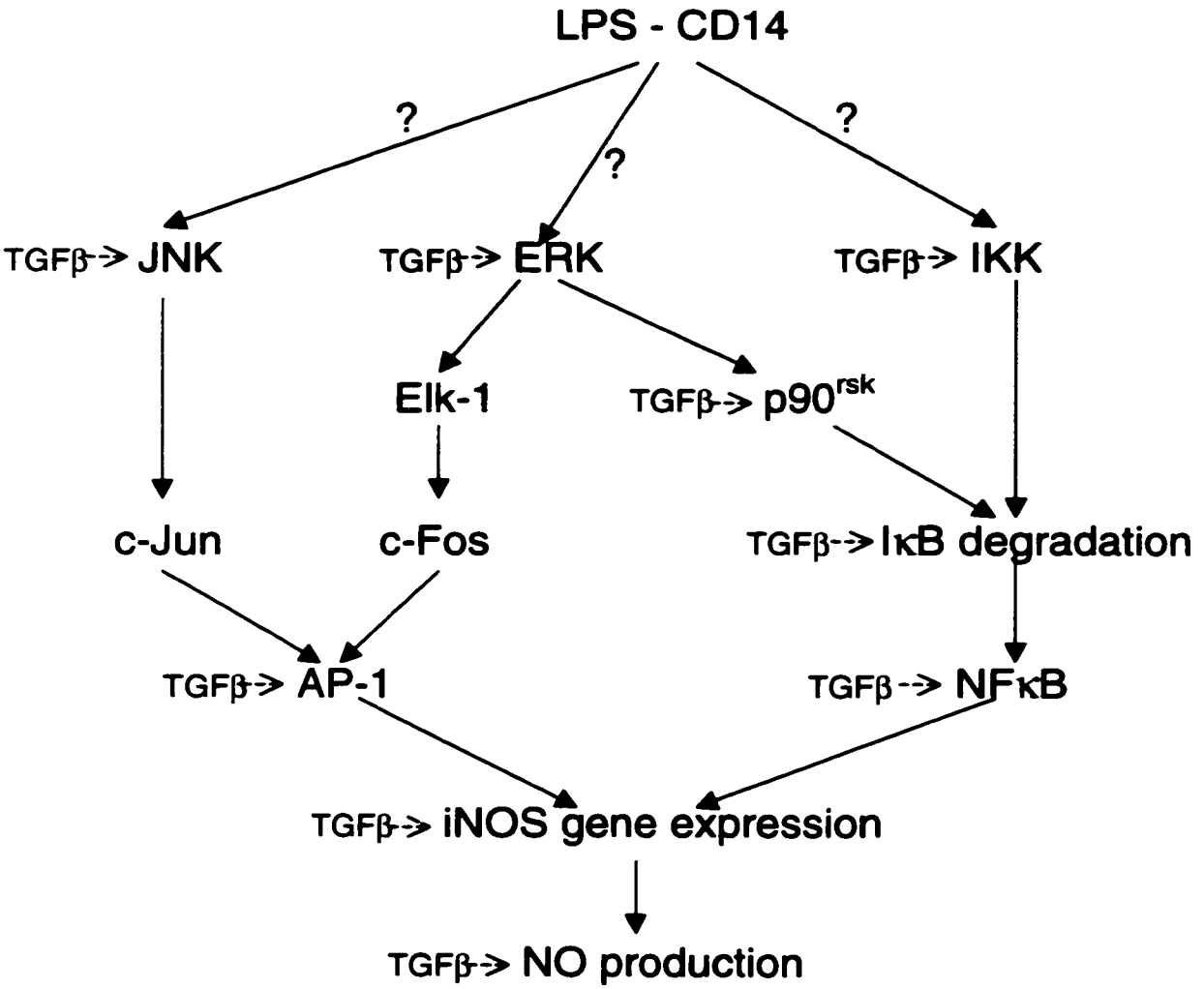
One of the best known downstream players of the ERK/MAPK pathway is the 90 kDa ribosomal S6 kinase ($p90^{rsk}$) (Blenis, 1993). Intriguingly, it was recently reported that $p90^{rsk1}$, known to be phosphorylated and activated by ERK kinases, can directly phosphorylate the N-terminus of IκBα, principally on serine 32, resulting in the degradation of IκBα and activation of NFκB (Ghoda et al, 1997; Schouten et al., 1997). I therefore determined the effect of TGFβ on the activity of $p90^{rsk1}$ in LPS-stimulated macrophages to see whether TGFβ-mediated inhibition of IκBα degradation results from, in part, the suppression of $p90^{rsk1}$ activity, another kinase involved in IκBα degradation. I found that LPS stimulation induced the activation of $p90^{rsk1}$ and TGFβ treatment resulted in an inhibition of the kinase activity to IκBα of $p90^{rsk1}$ (Figure 25). These results suggest that TGFβ-mediated inhibition of ERK activation may contribute to the suppression by TGFβ of NFκB activation via $p90^{rsk1}$ as well as AP-1 activation in LPS-stimulated macrophages and that $p90^{rsk1}$ may play a role as a connector of the NFκB pathway and the ERK/MAPK pathway (Figure 26).

Figure 25. The effect of TGFβ on the kinase activity of $p90^{rsk1}$ in macrophages stimulated with LPS. RAW 264.7 cells were treated with TGFβ (10 ng/ml, 87 pM) for different periods and stimulated with LPS (1 ng/ml, 0.25 nM) for 30 min. Cells not treated with TGFβ were left in the same media for 16 h and stimulated with LPS. Cells were then lysed and immunoprecipitated with anti-$p90^{rsk1}$ antibody. *In vitro* kinase assay was performed using P^{32}-labelled IκBα-GST fusion protein. Lane 1: untreated, Lane 2: LPS-stimulated, Lane 3: TGFβ-treated for 30 min and LPS-stimulated, Lane 4: TGFβ-treated for 4 h and LPS-stimulated, Lane 5: TGFβ-treated for 16 h and LPS-stimulated. Representative data of five independent experiments was shown.



IP: $p90^{rsk1}$

Figure 26. Model for LPS-induced signal transduction pathways interfered with by TGFβ in macrophages. LPS binding to CD14 receptors on the surface of macrophages activates MAPKs such as ERK and JNK by as-yet-to-be-identified mechanisms. ERK, in turn, phosphorylates and activates Elk-1, leading to the transcriptional activation of *c-fos* gene. JNK phosphorylates and increases the transcriptional activity of c-Jun. On the other hand, LPS stimulation also induce IKK activation and IκB degradation, resulting in the translocation of NFκB into the nucleus, which, in turn, with AP-1 coordinately activate iNOS gene expression. In addition, $p90^{rsk}$ phosphorylated and activated by ERK can contribute to IκB degradation. TGFβ may block the activation of MAPKs and IKK, leading to the decreased expression of the iNOS gene and subsequent NO production.


LPS - CD14
?
?
?
TGFβ-> JNK
TGFβ-> ERK
TGFβ-> IKK
Elk-1
TGFβ-> p90rsk
c-Jun
c-Fos
TGFβ-> IκB degradation
TGFβ-> AP-1
TGFβ -> NFκB
TGFβ-> iNOS gene expression
TGFβ-> NO production

5.2.4 Discussion

In the present study, I attempted to clarify the molecular mechanism of the inhibition by TGFβ of LPS-induced macrophage activation. I first confirmed that TGFβ inhibited the secretion of NO and TNF, which are important proinflammatory mediators produced by LPS-activated macrophages. And then I found that the inhibition by TGFβ of the secretion of NO resulted from the suppression of gene expression of iNOS, while TGFβ did not affect the expression of the TNFα gene in LPS-stimulated macrophages. In order to elucidate the molecular mechanisms for the inhibitory effects of TGFβ on iNOS gene expression, I determined whether TGFβ affects the activity of transcription factors involved in the expression of the iNOS gene. Candidate transcription factors involved in the murine iNOS gene include NFκB and AP-1 (Xie et al, 1993; Lowensrein et al, 1993; Xie et al., 1994). The DNA-binding activity of NFκB and AP-1 was inhibited by TGFβ in LPS-stimulated macrophages. These results suggest that TGFβ-mediated inhibition of iNOS gene expression in LPS-activated

macrophages may result from the suppression of the activation of NFκB and AP-1.

The transcription factor NFκB was shown to mediate key immune and inflammatory responses (Baldwin Jr, 1996; Baeuerle and Baltimore, 1996). NFκB is the prototype of a family of dimeric transcription factors made from monomers that have the Rel homology domain which bind to DNA, interact with each other, and bind a class of the inhibitory proteins called IκBs. In LPS-stimulated macrophages, the important role NFκB plays in the production of NO was demonstrated with a specific inhibitor of NFκB and a nuclear protein complex that binds specifically to a NFκB binding site contained the heterodimer p50/p65 and p50/c-Rel (Xie et al., 1994). Tightly bound IκBs regulate NFκB via its sequestration in the cytoplasm as inactive complexes. The activation of NFκB by a variety of stimuli involves the phosphorylation of IκBα on serines 32 and 36. Quite recently, IκB kinases (IKKs) IKKα and IKKβ were identified (Regnier et al., 1997; DiDonato et al., 1997; Mercurio et al., 1997; Woronicz et al, 1997). Phosphorylation of IκBs is followed by their ubiquitination and proteosome-mediated

degradation. This allows NFκB to translocate into the nucleus where they activate target genes. The effect of TGFβ on the signaling pathway inducing the activation of NFκB was next determined. I found that TGFβ inhibited the degradation of IκBα, the most studied IκB protein and the kinase activity of IKKα induced by LPS, which is consistent with the finding that TGFβ inhibited LPS-mediated activation of NFκB. Thus, one possible signaling pathway interfered with by TGFβ in LPS-stimulated macrophages include the activation of IKKα, the degradation of IκBα and the activation of NFκB, resulting in iNOS gene expression and NO production (Figure 26).

Intriguing question about the regulation of IKKs in LPS-stimulated macrophages remains to be addressed. The IKK activity appears sensitive to treatment with the phosphatase (DiDonato et al., 1997), suggesting that phosphorylation may control its activity. A MAP kinase kinase kinase (MAP3K) known as the NFκB-inducing kinase (NIK) was shown to associate with IKK, and coexpression of IKKα with NIK leads to the phosphorylation of IKKα and to an increase in its kinase activity (Regnier et al., 1997).

It was recently proposed that LPS may employ the analogous signaling pathways to those induced by IL-1 receptors, leading to NF•B activation. Since CD14, the LPS receptor, is glycosylphosphatidylinositol (GPI)-linked cell surface protein, it lacks the ability to transduce signals (Ulevitch and Tobias 1995). Quite recently, Toll-like receptors (TLRs), human homologues of the *Drosophila* Toll protein, especially TLR4, were identified to serve as a cell-surface coreceptor for CD14 and mediate LPS-induced signal transduction, leading to NFκB activation (Chow et al., 1999). Intriguingly, cytoplasmic domains of TLRs have homology to the intracellular portion of the IL-1 receptor (Rock et al., 1998) and constitutively active TLR4 activated proximal components of the IL-1 signaling pathway (Muzio et al., 1998). The intracellular domains of IL-1 receptors interact with adaptor protein TNF receptor-associated factor 6 (TRAF6) and NIK, resulting in the activation of NFκB (Regnier et al., 1997; Malinin et al., 1997). Thus, it is likely that LPS-induced IKK activation involves the signal transduction pathways similar to those through IL-1 receptors.

NFκB can be activated by an extraordinarily large number of different signals, ranging from ultraviolet irradiation to T cell activation, therefore, the mechanism by which these various signals converge on IκBα degradation is an important question. Intriguingly, although both IKKα and IKKβ appear to be essential contributors to the IKK activity, it was found that they differ in their phosphorylation efficiency between IκBα and IκBβ, and possibly even between phosphorylation sites on the same molecule (Stancovski and Baltimore, 1997). This observation raises the possibility that IκB degradation may be tightly regulated by a network of kinases, with different regulation and preferences for IκB family members.

Whether all of the diverse stimuli known to activate NFκB lead to the activation of IKK is also still unclear. Several lines of evidence indicate that the Ras/MAPK cascade induced by various growth factors and phorbol ester mediates ligand-induced activation of NFκB (Diaz-Meco et al., 1993; Devary et al., 1993; Finco and Baldwin Jr., 1993). Two recent reports suggest that the involvement of the Ras/MAPK cascade is through the transduction of

mitogen-induced signals to the $p90^{rsk}$, which also functions as an IκB kinase (Ghoda et al., 1997; Schouten et al., 1997). $p90^{rsk}$ is activated by phorbol ester, a known inducer of NFκB, but not by other NFκB inducers including TNFα. $p90^{rsk}$ is associated with IκBα, and phosphorylates it mainly at serine 32. Moreover, dominant-negative $p90^{rsk}$ inhibited IκBα degradation in response to mitogenic stimuli (Schouten et al., 1997). These results suggest that IKKα and IKKβ may not be the only integrators of NFκB response, and that certain stimuli may follow other pathways to IκB phosphorylation and NFκB activation. I therefore determined the effect of TGFβ on the activity of $p90^{rsk1}$ in LPS-stimulated macrophages to see whether TGFβ-mediated inhibition of IκBα degradation results, in part, from the suppression of $p90^{rsk1}$ activity, another kinase involved in IκBα degradation. I found that the kinase activity of $p90^{rsk1}$ was also inhibited by TGFβ. Thus, TGFβ-mediated inhibition of IκBα degradation and NFκB activation may result from the suppression by TGFβ of $p90^{rsk}$ as well as IKKα activation in LPS-stimulated macrophages. At present, the extent to which

IKK and $p90^{rsk}$ contributes to the phosphorylation of IκBα in LPS-stimulated macrophages is not known.

The AP-1 refers to the transcription factors that bind to TPA-response element (TRE) sites, which consists of both homodimers and heterodimers of members of the Jun family and Fos family (Su and Karin, 1996; Karin et al., 1997). The AP-1 activity is regulated by transcription of the *c-fos* and *c-jun* gene, or by posttranscriptional modification of their products by kinases. The ternary complex factors (TCFs) such as Elk-1 are important mediators of *c-fos* gene induction by a large variety of extracellular stimuli. ERK rapidly activated by most mitogens induces phosphorylation of Elk-1, leading to mitogen-induced *c-fos* transcription. It was found that treatment with LPS induced the phosphorylation of Elk-1 by the activated ERK kinase (Reimann et al., 1994) and experiments using a specific MEK1 inhibitor suggested the important role of ERK/MAPK in the production of NO in LPS-stimulated macrophages (data not shown). When I determined the effect of TGFβ on the signaling pathway involved in the activation of AP-1 activity, I found that TGFβ treatment resulted in an inhibition of tyrosine phosphorylation of ERK1 and ERK2 in

LPS-stimulated macrophages. This TGFβ-mediated inhibition of the activation of ERK kinases may account for the suppression by TGFβ of the activation of AP-1. Intriguingly, one of the best known downstream players of ERK/MAPK pathway is $p90^{rsk}$ (Blenis, 1993), which was shown to induce the degradation of IκBα. Thus, $p90^{rsk}$ can be a connector of ERK/MAPK pathway and NFκB pathway, and TGFβ-mediated inhibition of ERK activation may contribute to the suppression by TGFβ of NFκB via $p90^{rsk}$ as well as AP-1 activation in LPS-stimulated macrophages (Figure 26).

On the other hand, the AP-1 activity could be, in part, regulated by the phosphorylation of c-Jun by JNK, which increases its transcriptional activity. Recently, the JNK was shown to be activated after LPS stimulation of macrophages (Hambleton et al., 1996) and experiments using inhibitors of MAPKs showed the important role of JNK in the production of NO in LPS-stimulated macrophages (data not shown). I therefore determined whether, in addition to the inhibition by TGFβ of ERK activation, TGFβ-mediated suppression of AP-1 activation could result from, in part, the inhibitory effect of TGFβ on JNK activation in LPS-stimulated macrophages. I found that the kinase activity of

JNK was also inhibited by TGFβ, suggesting that the inhibition by TGFβ of AP-1 activation may result from the suppression of JNK as well as ERK activation.

The mechanism by which LPS activates MAPKs is not yet completely established. It was shown that Raf-1, MEK and MAPK are activated upon treatment of macrophages with LPS, resulting in phosphorylation of the transcription factor Elk-1 (Reimann et al., 1994). MAPKs are the most prominently tyrosine-phosphorylated proteins (Ulevitch and Tobias 1995; Sweet and Hume 1996), and tyrosine kinase inhibitors caused a severe impairment in LPS-induced cytokine production and inhibited Raf-1/MAPK activation (Reimann et al., 1994), suggesting that the activation of Raf-1/MAPK pathway occurs as a downstream event of tyrosine kinases. The evidence suggests that tyrosine kinases, especially the Src-family kinases Hck, Fgr, and Lyn, play important roles in LPS signaling (Stefanova et al., 1993). A recent study using *hck*$^{-/-}$*fgr*$^{-/-}$*lyn*$^{-/-}$ mice, however, showed that those Src-family kinases are not obligatory for LPS-initiated signal transduction (Meng and Lowell 1997). In addition, activated Raf-1 alone was not sufficient to ensure MEK/MAPK activation, and Raf-1 activation is p21^{ras}-

independent in LPS signaling in macrophages (Buscher et al., 1995).

Taken together, although the upstream signaling pathway interfered with by TGFβ has not been identified, my results suggest that, in LPS-stimulated macrophages, TGFβ inhibits the activation of IKK and $p90^{rsk}$, leading to the suppression of NFκB activity, and inhibits the activation of ERK and JNK MAP kinases, resulting in the suppression of AP-1 activity (Figure 26). This TGFβ-mediated suppression of NFκB and AP-1 contributes to the decreased iNOS gene expression and NO production in macrophages.

As described in the INTRODUCTION, islet β cells are sensitive to injury mediated by NO, and intra-islet release of IL-1 by macrophages induced β cell expression of iNOS (Corbett and McDaniel, 1995). The production of NO by β cells as well as islet-infiltrating macrophages in pancreatic islets correlated with the development of IDDM in NOD mice (Rabinovitch et al., 1996). Even though LPS is not a physiological activator inducing NO production by β cells, the transmembrane receptor for LPS, TLR-4, may employ similar signaling pathways to those of the receptor

for IL-1, a physiological activator for NO production by β cells as well as macrophages. Thus, it would be of interest to examine the effect of TGFβ on IL-1-induced NO production in pancreatic β cells as well as islet-infiltrating macrophages during the development of IDDM.

6. OVERALL DISCUSSION AND FUTURE PERSPECTIVES

One of the primary features of the immune system is the discrimination of self from non-self. Self-tolerance occurs mainly through clonal deletion in the thymus of self-reactive T cells at the early stage of development. However, the immune system is also exposed to extrathymic self-antigens and to repetitive stimulation by non-pathogenic antigens. Therefore, the peripheral immune compartment needs to have some mechanisms to suppress responsiveness to such stimuli. Mechanisms of peripheral immune tolerance includes T cell anergy (Schwartz, 1990), T cell deletion by apoptosis (Rocha and von Boehmer, 1991), and active immune suppression. Analysis of active immune suppression mediated by T cells has been hampered by the inability to generate and clone these cells *in vitro* and to demonstrate their regulatory activities in the immune system *in vivo*. Understanding these mechanisms of tolerance induction is clinically relevant for the treatment of autoimmune diseases and in transplantation, where the graft must ultimately be recognized as self.

The functional heterogeneity of CD4+ T cells as a result of their different cytokine profiles has explained the ability of some T cells to produce autoimmunity and other T cells to regulate these autoreactive T cells

(O'Garra et al., 1997). Thus, it has been proposed that type 1 immune responses are elicited inappropriately against self antigens, leading to tissue destruction and pathology, whereas type 2 immune responses against the same antigens could explain the immune deviation from cell-mediated autoimmunity to tolerance. It would appear that the studies performed in the NOD mouse confirm a role of type 1 T cells in the pathogenesis of IDDM. A deficiency of a type 2 cytokine IL-4 has been implicated in the pathogenesis of IDDM in the NOD mouse (Gombert et al., 1996) but this view is not universally accepted, as demonstrated by the induction of diabetes in immunoincompetent NOD mice by Th2 cells (Pakala et al., 1997). In addition, IL-4 plays no important role in the prevention of Th1-mediated inflammatory bowel disease in mice (Powrie et al., 1996), and regulatory T cells that prevent this pathology appear not to produce IL-4 *in vitro* (Groux et al., 1997). Thus, more recent studies suggest that the strict definition of type 1 T cells as pathogenic and type 2 T cells as regulatory in autoimmune diseases is not as simple as originally proposed.

There is now a large body of evidence that TGFβ, which does not belong to either type 1 or type 2 cytokines, plays a major role in controlling several autoimmune or inflammatory diseases. Many studies suggest that alternative regulatory populations rather than type 2 T cells exist and that the T cell subsets that was shown to transfer protection from autoimmune diseases are functionally and phenotypically diverse.

Oral tolerance refers to a state of unresponsiveness that follows feeding a soluble antigen. The role of TGFβ as an immunoregulatory molecule in the induction of oral tolerance has been demonstrated by the ability of anti-TGFβ antibodies to reverse the suppression of EAE after oral tolerization to MBP (Miller et al., 1992). CD4+ T cell clones, which were isolated from mice fed with MBP and suppressed EAE, produced TGFβ but little or no IL-4 and IL-10 (Chen et al., 1994).

The expression of CD45 was shown to explain the functional heterogeneity of CD4+ T cells in the rat as well as mouse models. A role of the rat $CD45RC^{low}$ subset in regulating immune responses to self has been demonstrated by its ability to inhibit diabetes and insulitis (Fowell

and Mason, 1993). Quite recently, it was shown that autoimmune thyroditis, humoral autoimmunity induced by a similar protocol to that used for inducing autoimmune diabetes, was prevented by the same subset of T cells, due to a specific suppression of the autoimmune responses involving both TGFβ and IL-4 (Seddon and Mason, 1999). Similarly, in SCID mice, inflammatory bowel disease was induced by reconstitution with $CD45RB^{high}$ CD4+ T cells, while the cotransfer of $CD45RB^{low}$ CD4+ T cells inhibited this pathology in a TGFβ-dependent manner (Powrie et al., 1996). Alternatively, the coadministration of IL-10 also was effective for inhibiting this disease (Powrie et al., 1994), suggesting that the $CD45RB^{low}$ CD4+ population is heterogeneous.

It was recently shown that *in vitro* chronic activation of CD4+ T cells in the presence of IL-10 induced regulatory CD4+ T cell clones (Groux et al., 1997). These antigen-specific clones produced high levels of IL-10, low levels of IL-2 and no IL-4, and suppressed the proliferation of naïve CD4+ T cells in response to the same antigen, mediated by IL-10 and TGFβ. Another type of regulatory CD4+ T cell lines was described to inhibit Th2-type autoimmunity

in rats (Bridoux et al., 1997). The protective T cell lines against Th2-mediated autoimmunity produced IL-2, IFNγ and TGFβ, and the protective effect was dependent upon TGFβ.

Even though these regulatory CD4+ T cell populations, which were produced under different conditions, have distinct cytokine profiles, they have common ability to regulate proliferative or pathogenic responses, dependent on TGFβ. In some cases, IL-10 as well as TGFβ can explain their inhibitory effects. In addition to the development of multiple inflammation in TGFβ1-deficient mice, IL-10 gene knockout mice exhibited chronic enterocolitis, implying that both cytokines may be important regulators of immunopathology of inflammatory reactions.

The regulation of the differentiation of TGFβ-producing cells was recently investigated (Seder et al., 1998). Similar conditions required for the induction of Th2 cells, low amounts of IL-12 and IFNγ and high amounts of IL-4, induced the generation of cells producing high levels of TGFβ. While IL-4 and IFNγ have a direct positive and negative effect, respectively, the main factor for regulating TGFβ production from naive CD4+ T cells is TGFβ itself. Thus, it was suggested that the presence of high

amounts of IL-4 and low amounts of IFNγ may set up the conditions for initial TGFβ production and, then, TGFβ exerts a positive feedback effect that can further enhance its production. Considering the role of IL-4 in the prevention of autoimmunity, the secretion of high levels of TGFβ by T cells activated in the presence of IL-4 raises the possibility that IL-4 is a growth factor for regulatory T cells, while TGFβ acts as an effector suppressive cytokine, so that a deficiency of either cytokine results in the breakdown of regulation. Indeed, both TGFβ and IL-4 were shown to play an essential role in the prevention by regulatory T cells of autoimmune thyroditis (Seddon and Mason, 1999).

The nature of regulatory T cells in autoimmune diabetes is poorly understood, in part due to the limited availability of cloned T cells exerting such effects. Some T cell clones protecting IDDM were isolated but their mechanism of action is not well known. None of the cloned T cells with protective effects have exhibited the Th2 phenotype. In this thesis, I showed that a CD4+ T cell clone isolated from the islet-infiltrating lymphocytes of NOD mice, which prevented diabetes in NOD mice, produces

TGFβ and IL-10, and that their immunosuppressive effect on the proliferative responses of NOD splenocytes is abrogated by treatment with antibodies directed against TGFβ (Han et al., 1996). The report was followed by similar observation showing that an insulin-specific CD4+ T cell clone, which prevented the development of IDDM in NOD mice, suppressed the proliferative responses of NOD splenocytes to islets *in vitro* by producing TGFβ (Zekzer et al., 1997). In order to investigate the role of TGFβ in the development of IDDM, quite recently, TGFβ1 was transgenically expressed in the pancreatic β cells (King et al., 1998) or in the pancreatic α cells (Moritani et al., 1998). These mice exhibited the clearly decreased incidence of diabetes. Based on these results, it is suggested that TGFβ plays an important role in the regulation of autoimmune diabetes in NOD mice. Since TGFβ prevents T cell activation but does not kill activated cells, it appears that regulatory T cells producing TGFβ suppress but do not eliminate autoreactive T cells. This suggestion is compatible with the observation that the normal T cell repertoire includes such autoreactive T cells.

Although the immunosuppressive effect of TGFβ has been reported in a number of systems, the mechanism of action of TGFβ is not yet fully understood in NOD mice, which have some intrinsic defects in the immune system. I, therefore, determined the effect of TGFβ on the function of immune cells involved in β cell destruction in NOD mice, such as T cells and macrophages. I found that TGFβ inhibited the proliferative responses of NOD splenic T cells. I, next, attempted to investigate the molecular mechanisms for the suppression by TGFβ of T cell proliferation in NOD mice. When I determined the effect of TGFβ on signal transduction pathways induced by IL-2, a T cell growth factor, in T cells, I found that TGFβ interfered with IL-2-induced signal transduction pathways, such as the JAK/STAT pathway and the ERK/MAPK pathway, resulting in the inhibition of IL-2-dependent T cell proliferation (Han et al., 1997). These results suggest that one mechanism by which TGFβ-producing CD4+ suppressor T cells inhibit autoimmune diabetes in NOD mice is through the interference by TGFβ of a growth factor-induced expansion of autoaggressive CD4+ and CD8+ T cells.

When I determined the effect of TGFβ on the gene expression and secretion of T cell-derived cytokines in NOD splenocytes, I found that TGFβ inhibited the secretion as well as gene expression of type 1 cytokines such as IFNγ and IL-2. In contrast, TGFβ did not affect the production of type 2 cytokines such as IL-4 and IL-10. These results suggest that another mechanism for the inhibition of autoimmune diabetes by TGFβ-producing CD4+ suppressor T cells in NOD mice is the specific inhibition by TGFβ of type 1 cytokine production by autoaggressive CD4+ and CD8+ T cells.

An understanding of the mechanisms involved in the differentiation and/or activation of Th1 and Th2 cells might provide potential targets for therapeutic intervention of a particular disease, to which susceptibility or resistance is determined by a specific Th cell subset. A bidirectional communication between the CD4+ T cells and APC might influence this process. Thus, it is of importance to elucidate mechanisms controlling the expression of IFNγ and IL-4 during the differentiation and/or activation of Th1 and Th2 cells, and the expression of specific cytokines such as IL-12 in the APC. Several

transcription factors were identified, that are expressed exclusively in specific Th cell subsets or involved in the regulation of gene expression in the APC (Rincon and Flavell, 1997). It would be of interest to determine the effect of TGFβ on the activation of those transcription factors to elucidate the molecular basis for the regulation by TGFβ of gene expression of a specific T cell subset-derived cytokine.

In addition to the effect of TGFβ on T cell function, the role of TGFβ in the regulation of macrophage function was also determined in NOD mice. I found that TGFβ inhibited the gene expression and production of cytotoxic molecules derived from macrophages, such as cytokines and free radicals. When I investigated the molecular mechanisms for the suppression by TGFβ of macrophage activation in LPS-stimulated macrophages, I found that TGFβ inhibited the activation of transcription factors NFκB and AP-1, resulting in the suppression of iNOS gene expression and NO production. Furthermore, I found that TGFβ inhibited IκB degradation and IKK kinase activity directing NFκB activation, and suppressed the kinase activity of ERK and

JNK inducing AP-1 activation in macrophages (manuscript in preparation). These results suggest that the suppression by TGFβ of macrophage-mediated cytotoxicity to pancreatic β cells contributes to the inhibition of autoimmune diabetes by TGFβ-producing CD4+ suppressor T cells in NOD mice.

Results gained from Chapter 5 investigating the molecular mechanisms for the suppression by TGFβ of immune cell function raise the question how the signaling pathways of TGFβ and IL-2 or LPS interact. TGFβ tranduces signals through type I and type II TGFβ receptors, which belong to a large family of receptor serine/threonine kinases (Heldin et al., 1997; Hu et al., 1998). Upon TGFβ binding to the type II receptor, the type I receptor is recruited, causing its phosphorylation and subsequent activation by constitutively active type II receptor kinase. It is currently thought that activated type I receptor kinase is sufficient to modulate most TGFβ downstream signals. Regardless of the existence of other signaling pathways, the recent identification of the SMAD family of signal transducer proteins has uncovered the mechanisms of signal

transduction of TGFβ from the cell membrane to the nucleus. SMAD2 and SMAD3 are phosphorylated by type I receptors, then associate with the common mediator SMAD4 and translocate to the nucleus where they activate target genes. It was recently shown that other signaling pathways could regulate SMAD signaling. For example, the MAPK pathways led to the phosphorylation of SMADs, affecting their transcriptional activity (Kretzschmar et al., 1997; Brown et al., 1999), and IFNγ-induced activation of the JAK/STAT pathway resulted in the gene expression of the inhibitory SMAD7 (Ulloa et al., 1999). In addition, SMAD signaling can be modulated by other signaling pathways at the level of the promoter to coordinately regulate transcription of target genes (Zhang and Derynck 1999).

In my study, the effect of TGFβ on signaling pathways induced by IL-2 or LPS was investigated. Since protein tyrosine phosphorylation is one of essential early events of IL-2 and LPS signaling, although the exact mechanism for TGFβ action observed in this study is not known, the possible explanation is the involvement of protein phosphatases. A study on TGFβ-treated keratinocytes showed a rapid increase in type 1 protein serine/threonine

phosphatase activity followed by growth arrest coincident with the increase of protein tyrosine phosphatase (PTP) activity (Gruppuso et al., 1991). The activation of phosphatases by TGFβ has also been reported in fibroblasts (Fontenay et al., 1992). TGFβ inhibited the receptor tyrosine kinase activity, while okadaic acid, an inhibitor of type 1 and 2A protein serine/threonine phosphatases, restored the receptor autophosphorylation, suggesting that dephosphorylation of serine/threonine residues of the receptor tyrosine kinase results in an inhibition of tyrosine kinase activity. Similarly, the restoration of protein tyrosine kinase JAK activation upon treatment with vanadate, a PTP inhibitor, in T lymphocytes suggested that the involvement of PTPs in TGFβ action (Bright et al., 1997). How protein phosphatases are regulated is not well known (Sun and Tonks 1994; Hunter 1995). Since TGFβ receptors themselves have serine/threonine kinase activity (Hu et al., 1998), serine/threonine phosphorylation by TGFβ receptors could affect the protein phosphatase activity, which may explain the early action of TGFβ. However, since the TGFβ action was clearly observed after treatment with TGFβ for hours in my study, and cycloheximide or actinomycin

D, a protein synthesis inhibitor or transcription inhibitor, respectively, recovered tyrosine phosphorylation of cellular proteins inhibited by TGFβ in macrophages (data not shown), I speculate that TGFβ induces the expression of protein phosphatases involved in TGFβ action. The exact identity of protein phosphatases sensitive to or induced by TGFβ remains to be established.

Aside from the results I found, it is possible that TGFβ may exert additional effects on the immune system of NOD mice, involved in pancreatic β cell destruction, such as the expression of MHC molecules (Czarniecki et al., 1988) and FasL molecules (Genestier et al., 1999), and the cytotoxic activity of CTL (Ranges et al., 1987). It would be of interest to determine the effect of TGFβ on those aspects in NOD mice.

On the basis of the results obtained through my studies, I conclude that TGFβ-mediated suppression of T cell proliferation, type 1 immune responses and macrophage activation may contribute to the prevention of immune cell-mediated pancreatic β cell destruction by TGFβ-producing CD4+ suppressor T cells in NOD mice. In addition, I

elucidated the molecular mechanisms by which TGFβ inhibits T cell proliferation and macrophage activation.

It is known that there is a long prediabetic period before the onset of clinical IDDM and that this onset occurs only after destruction of a majority of β cells. Therefore, if the prediabetic state is diagnosed sufficiently early, it may be possible to prevent subsequent β cell destruction by blocking further autoimmune processes and to prevent the clinical onset of the disease. The information obtained through this study may be invaluable for the development of immunological strategies to prevent IDDM at the early stages of the disease in humans and to prevent recurrent IDDM in patients who receive syngeneic islet transplants.

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