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The effects of sex hormones on the cytokine production of T cells in the NOD mouse, an animal model for type I diabetes

by

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ABSTRACT

Insulin-dependent diabetes mellitus (IDDM) in the non-obese diabetic (NOD) mouse, an animal model for human type I diabetes, is a T cell-mediated autoimmune disease, and $CD4^+$ T cells play a critical role in its pathogenesis through the Th1/Th2 cytokine secretion. It is known that the onset of autoimmune diabetes is earlier and with higher incidence in female NOD mice compared to male mice. Long-term administration of sex hormones suggested that sex hormone-mediated immune modulation might be responsible for the sexual dimorphism in the pathogenesis of autoimmune diabetes in NOD mice. However, it is unclear whether sexual dimorphism is associated with different cytokine profiles produced by autoreactive T cells in female and male NOD mice, and whether sex hormones, such as estrogen and testosterone, can directly modify the functions of $CD4^+$ T cells in NOD mice.

To investigate the mechanisms of sexual dimorphism in NOD mice, the cytokine gene expression of islet infiltrating T cells from different age groups of female and male NOD mice was first examined. The results showed that infiltrating lymphocytes in female NOD mice expressed higher level of IFN-γ and lower level of IL-4; more importantly, this sexual disparity was most significant between young (4-week-old) female and male NOD mice in which insulitis just started. When purified $CD4^+$ T cells were activated with mitogen *in vitro*, the cells from young female NOD mice also displayed significantly higher expression and production of IFN-γ but lower IL-4, compared with the cells from young males. However, identical gene expression of sex

hormone receptors was detected in the $CD4^+$ T cells from different-aged female and male NOD mice. In addition, the treatment with sex hormones *in vitro* showed no effect on $CD4^+$ T cell proliferation. When $CD4^+$ T cells from NOD mice were pretreated with estrogen, IL-12-induced IFN-γ production was enhanced; in contrast, pretreatment with testosterone reduced the IFN-γ secretion of $CD4^+$ T cells. Furthermore, the phosphorylation of transcription factor Stat4, a critical step required for IFN-γ gene expression, was significantly increased in activated $CD4^+$ T cells in the presence of estrogen, but decreased in the presence of testosterone. In conclusion, the results of this study showed that sexual dimorphism of autoimmune diabetes in NOD mice strongly correlates with different cytokine profiles produced by the activated $CD4^+$ T cells in female and male NOD mice. More importantly, this study also provides the first evidence that sex hormones can directly modify cytokine production of $CD4^+$ T cells by increasing or decreasing the phosphorylation of transcription factors, such as Stat4.

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LIST OF ABBREVIATIONS

APC	antigen presenting cells
Ag	antigen
BSA	bovine serum albumin
°c	degrees Celsius
CD	cluster of differentiation
cDNA	complementary DNA
CIA	collagen-induce arthritis
ConA	concanavalin A
cpm	count per minute
CFA	complete Freund adjuvant
CTL	cytotoxic T lymphocytes
DNA	deoxyribonucleic acid
DHT	dihydrotestosterone
DHEA	dehydroepiandrosterone
dNTPs	deoxyribonucleoside triphosphates
DTH	delayed type hypersensitivity
ECL	enhanced chemiluminescence
EAE	experimental allergic encephalomyelitis
EDTA	ethylenediaminetertraacetate
ELISA	enzyme-linked immunosorbent assay
E1	estrone

E2	estradiol
E3	estriol
ER	estrogen receptor
FCS	fetal calf serum
FITC	fluoresein isothiocyanated
GAD	glutamic acid decarboxylase
HBSS	Hank's balanced salt solution
HRP	horse-radish peroxidase
hrs	hours
IDDM	insulin-dependent diabetes mellitus
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
JAK	Janus kinase
LCMV	lymphocytic choriomeningitis virus
MHC	major histocompatibility complex
mRNA	messenger RNA
MS	mutiple sclerosis
NOD	nonobese diabetic
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PE	phycoerythrin
PMSF	phenylmethylsulfonyl flouride
PVDF	polyvinylidene difluoride
RA	rheumatoid arthritis
RNA	ribonucleic acid
RT-PCR	reverse transcirptase-polymerase chain reaction
SCID	severe combined immunodeficiency
SD	standard deviation
SDS	sodium dodecyl sulfate
SLE	systemic lupus erythematosus
STAT	signal transducers and activators of transcription
TCR	T cell receptor
Th1	T helper 1
Th2	T helper 2
TNF	tumor necrosis factor
TS	testosterone
TSR	testosterone receptor

A. INTRODUCTION

A.1 NOD mouse as an animal model for IDDM

Insulin-dependent diabetes mellitus (IDDM) is a multifactorial autoimmune disease caused by progressive destruction of insulin-producing pancreatic β-cells, which leads to hypoinsulinaemia and hyperglycaemia. Although the pathogenesis of autoimmune diabetes has been extensively studied for many years, the precise mechanisms involved in the initiation and progression of the disease are still not clear. To elucidate the etiology of IDDM, several animal models have been established. Among them, non-obese diabetic (NOD) mouse, which exhibits most of the salient features of human IDDM, is the most used animal model.

NOD mice spontaneously develop diabetes and the diabetic syndrome is similar to that of humans, except for the sex differences. NOD mouse is derived from a subline of outbred ICR mice (Makino et al., 1978) and was established in 1980 at Shionogi Research Laboratories in Japan as an animal model for IDDM (Makino et al., 1980). NOD mice spontaneously develop a diabetic syndrome, which is characterized by polydypsia (>20ml/day), polyuria (>18ml/day), severe glycosuria (Testape>1/4%), hyperglycemia (>250mg/dl), insulin deficiency (<1.0μU/ml), and rapid weight loss (Makino et al., 1980; Fushimi et al., 1980). However, not like humans, a clear sex difference with respect to the onset of diabetes was observed in NOD mice. Females start to develop diabetes at about 10 weeks of age, and the incidence of diabetes reaches 70-80% until 30 week-old. In contrast, the onset of the disease in male NOD mice is observed to begin at 20 weeks of age, and only 20% of males develop overt diabetic syndrome at 30 week-old (Makino et al., 1980).

Immunohistological examination of the pancreas of NOD mice reveals the infiltration of mononuclear cells into the pancreatic islets (termed insulitis). Insulitis appears in both female and male NOD mice at 3 weeks of age, and is well established until the animals are 10 week-old. The cumulative incidence of animals exhibiting insulitis reaches almost 100% by 30 weeks of age, but not all of them develop diabetes (Makino et al., 1980). Most of the mononuclear cells infiltrating the islets are Thy-1 positive T cells, and the majority is $CD4^+$ T cells as well as a small number of $CD8^+$ T cells. B cells, macrophages, dendritic cells, and NK cells are also detected in the periphery of pancreatic islets, but not inside the islets. Many islets of the diabetic mice show atrophy, and no infiltrated cells could be detected within the interior of these atrophied islets (Makino et al., 1980). β-cells, which secrete insulin, have disappeared in these islets; however, no notable change in α, δ, and pancreatic polypeptide (PP) cells, which make up pancreatic islets, is observed (Fujita et al., 1982).

Similar to human type I diabetes, multiple genes, including MHC and non-MHC genes, are involved in the development of diabetes in NOD mice. The MHC haplotype of NOD mice is designated as $H\text{-}2^{g7}$. The MHC class I molecule of NOD mice is identical to H-2 K^d and H-2 D^b; however, these mice have several unique characteristics in the class II region. First, their I-Aα molecule has the same coding sequence as $A\alpha^d$, but the Aβ gene is unique (Acha-Orbea et al., 1987). Particularly, in NOD mice the aspartic acid at position 57 of the β-chain is replaced by serine (Acha-Orbea et al., 1987; Prochazka et al., 1987; Acha-Orbea et al., 1991). Secondly, the MHC class II molecule in NOD mice is lack of I-E expression because of the defect in the promoter region of the

Eα gene (Hattori et al., 1986). On the other hand, not only MHC genes, but at least 15-20 non-MHC susceptibility loci called *Idd* genes have been identified linked to the disease development (Gonzalez et al., 1997) as well. The studies in MHC congenic strains of mice have shown that the NOD MHC molecule is essential, but is not sufficient to develop diabetes by itself (Wicker et al., 1995).

A.2 The development of autoimmune diabetes in NOD mice is T cell-mediated

Considerable evidence has shown that the development of IDDM in NOD mice is mediated by T cells. Immunohistological studies of pancreatic islets have revealed that most of the infiltrating cells are T lymphocytes, although a few dendritic cells, macrophages, and B cells could also be detected in the infiltrated islets (Kanazawa et al., 1984; Miyazaki et al., 1985; Signore et al., 1989). NOD/*scid* (severe combined immunodeficiency disease) mice, which have no mature T and B cell populations, do not develop diabetes (Prochazka et al., 1992). Neonatal thymectomized or athymic NOD mice significantly reduced insulitis and diabetes (Ogawa et al., 1985; Makino et al., 1986). Moreover, suppression of the onset of diabetes was observed in mice injected with anti-Thy1.2 antibody or cyclosporin A, which is known to be a suppressor of T lymphocytes (Harada and Makino, 1986; Mori et al., 1986). More directly, in adoptive transfer experiments, purified T cells from diabetic donors were sufficient to transfer insulitis and diabetes to young irradiated NOD or NOD/*scid* mice (Bendelac et al., 1987; Miller et al., 1988; Christianson et al., 1993; Rohane et al., 1995). Thus, T cells are essential for the development of diabetes in NOD mice, although other immunocytes,

such as B cells, macrophages, and dendritic cells, also play important roles in this disease process.

A.2.1 The roles of Antigen presenting cells (APCs) in the development of IDDM in NOD mice

The initial event in the development of autoimmune diabetes in NOD mice is the uptake and processing of autoantigen by antigen presenting cells (APCs). B cells, macrophages, and dendritic cells are all involved in the pathogenesis of diabetes. Similar results were obtained by different groups that B lymphocyte-deficient NOD mice are resistant to IDDM (Serreze et al., 1996; Akashi et al., 1997; Noorchashm et al., 1997). The finding that diabetes is inhibited rather than accelerated in B cell-deficient NOD mice indicated that B cells played a distinct diabetogenic role in the development of β cell-autoreactive T cells in NOD mice. It suggests that B cells are crucial APCs in the pathogenic autoimmune response in NOD mice, and that they may have a unique ability to process and present certain β cell antigens to autoreactive T cells (Falcone et al., 1998). Alternatively, B cells may contribute to IDDM in NOD mice through their ability to secrete autoantibodies that bind to pancreatic β cells and trigger autoreactive T cells through an antibody-dependent cell-mediated cytotoxic response.

Besides B lymphocytes, macrophages and dendritic cells also play important roles in the autoimmune process in NOD mice. The early inactivation of macrophages resulted in prevention of the development of β cell-specific effective T cells (Ihm et al., 1990). Furthermore, T cells in a macrophage-depleted environment lost their ability to differentiate into β cell cytotoxic T cells, leading to the prevention of IDDM in NOD

mice (Jun et al., 1999). On the other hand, the role of dendritic cells in the pathogenesis of diabetes in the NOD mouse remains to be defined.

A.2.2 The role of CD4$^+$ T cells in the development of IDDM in NOD mice

T lymphocytes play an obligatory role in the development of IDDM, and both CD4$^+$ and CD8$^+$ T cells are required for islet infiltration and subsequent β cell destruction; however, numerous studies showed that CD4$^+$ T cells alone are far more efficient in the adoptive transfer of disease than CD8$^+$ T cells. A diabetogenic CD4$^+$ T cell clone BDC2.5, which was derived from a diabetic NOD mouse, accelerated the disease in young NOD mice (Haskins et al., 1990). Moreover, the BDC2.5 T-cell receptor (TCR) transgenic NOD mouse, which carries rearranged TCR-α and β genes derived from BDC2.5 CD4$^+$ T cell clone, recognizes an islet-specific antigen presented by the NOD MHC class II allele I-A^{g7} (Katz et al., 1993). Furthermore, these mice develop diabetes earlier than normal NOD mice. Similarly, another group's study showed that a glutamic acid decarboxylase (GAD)-reactive CD4$^+$ T cells generated from NOD mice could induce diabetes in NOD/*scid* mice (Zekzer et al., 1998). More recently, a CD4^{high} T cell population has been identified from the islets of prediabetic NOD mice (Ridgway et al., 1998), and these cells are extremely potent in transferring diabetes to CD8 reconstituted NOD/*scid* mice (Lejon et al., 1999). The effectiveness of CD4$^+$ T cells in transferring disease is most likely due to their secretion of cytokines, such as IFN-γ and TNF-α, which are directly toxic to pancreatic β cells and recruit more effective cells to amplify the response.

A.2.3 The role of CD8$^+$ T cells in the development of IDDM in NOD mice

Although $CD4^+$ T cells are essential to IDDM development in NOD mice, the MHC class I-restricted $CD8^+$ T cells may also carry out a role at the initial and the final stages of the disease process. Under appropriate conditions, $CD8^+$ T cell clones isolated from young NOD islets can transfer rapid onset of diabetes in NOD mice in the absence of $CD4^+$ T cells (Wong et al., 1996). NOD mice transgenic for the TCRβ-chain of a $CD8^+$ T cell clone isolated from diabetic NOD mice have an earlier onset of IDDM (Utsugi et al., 1996; Verdaguer et al., 1996). Furthermore, NOD mice depleted of $CD8^+$ T cells by treatment with anti-CD8 antibody do not develop disease (Wang et al., 1996). More strikingly, β_2-microglobulin-deficient NOD mice, which lack of MHC class I and are therefore $CD8^+$ T cell deficient, are free of insulitis and diabetes (Wicker et al., 1994; Kay et al., 1996; Serreze et al., 1997). One recent study demonstrated that $CD8^+$ T cell clones isolated from the earliest insulitic lesions showed similar TCRα-chain CDR3 usage, which suggested that a single peptide/MHC class I complex may initiate disease pathogenesis as well as mediate final β-cell destruction (DiLorenzo et al., 1998).

A.2.4 Autoantigens targeted in IDDM in NOD mice

IDDM is a T cell-mediated autoimmune disease, and several autoantigens have been implicated as markers of the autoimmune assault in the disease process in NOD mice. The immune response to glutamic acid decarboxylase (GAD) develops at an early age in female NOD mice, which suggests that GAD may play an important role in the initiation and/or progression of T cell-mediated autoimmune diabetes (Kaufman et al., 1993; Tisch et al., 1993). More importantly, antisense GAD transgenic NOD mice generated in our group prevented diabetes whereas the persistent expression of GAD in β

cells in other lines of antisense GAD transgenic mice resulted in diabetes similar to NOD mice (Yoon et al., 1999). This result indicated that β cell-specific GAD expression was required for the development of IDDM in NOD mice. Insulin is another β cell autoantigen that appears to have a critical role in the diabetogenic response. Insulin B chain-specific $CD4^+$ T cell clones can accelerate diabetes in young NOD mice or adoptively transfer disease in NOD/*scid* mice (Daniel et al., 1995). Furthermore, oral or parental treatment of young NOD mice with whole insulin or insulin B chain may protect animals from diabetes (Zhang et al., 1991; Muir et al., 1995). Besides GAD and insulin, a number of other β cell autoantigens are targeted during the diabetogenic process. Thus, the precise role of these antigens in the initiation and progression of autoimmune diabetes remains unclear.

A.3 The roles of Th1/Th2 cytokines in the promotion and prevention of IDDM in NOD mice

$CD4^+$ T cells are essential in the pathogenesis of IDDM in the NOD mouse, and their effectiveness is based on their cytokine production. In general, $CD4^+$ T cells are divided into three subsets, Th0, Th1, and Th2 cells, according to the cytokines they produce. Th1 cells secrete IFN-γ, IL-2, and TNF-β, and participate in cell-mediated immune response such as delayed-type hypersensitivity (DTH) immune response and macrophage activation. Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, and these cytokines induce B cells to secrete antibodies (Constant et al., 1997). The third subset, called Th0 cells, produce a mixture of both cytokine patterns. The presence of IL-12, a product of macrophages or dendritic cells, favors Th1 differentiation; while the presence

of IL-4, a product of mast cells and more importantly, NK1.1 bearing CD4+ T cells (Yoshimoto et al., 1994) favors the differentiation of Th2 cells. Currently, in NOD mouse model, it is generally accepted that Th1 cells are responsible for the induction of diabetes while Th2 cells prevent the disease through the different cytokines they produce.

A.3.1 The roles of Th1 cytokines in the development of IDDM in NOD mice

Abundant evidence has suggested that Th1 cytokines, especially IFN-γ, are mediators of β cell destruction in the development of IDDM in NOD mice. In studies on cytokine gene expression in pancreatic islet-infiltrating immunocytes of NOD mice, IFN-γ mRNA expression was found to correlate with β cell destructive insulitis (Rabinovitch et al., 1994; Muir et al., 1995). T cell clones, which are able to accelerate the onset of diabetes in young NOD mice, are proved to produce Th1 type cytokines when challenged with islets and APCs *in vitro* (Haskins et al., 1990). Furthermore, splenic T cells from young NOD mice developed a unipolar Th1-cell response, with only IFN-γ but no IL-4 in response to autoantigen GAD (Tian et al., 1998). In T cell transfer experiments, Th1 cells, which produce IFN-γ and IL-2, actively promoted diabetes in neonatal NOD mice (Katz et al., 1995). In addition, monoclonal antibodies to IFN-γ protected against diabetes development in NOD mice (Campbell et al., 1991; Debray et al., 1991). Further evidence for IFN-γ being a β cell destructive cytokine comes from the studies on transgenic mice. Transgenic expression of IFN-γ by β cells in normal mice leads to an autoimmune, lymphocytic infiltration of the islets (insulitis), β-cell destruction, and diabetes (Sarvetnick et al., 1988; Sarvetnick et al., 1990). IFN-γ receptor knock out NOD mice are free of insulitis and diabetes; likewise, in IFN-γ receptor knock out BDC2.5

transgenic mice, insulitis can be detected but never evolved into diabetes (Wang et al., 1997). Additionally, the systemic treatment of NOD mice with IL-12, a potent inducer of Th1 cells, led to accelerated diabetes (Trembleau et al., 1995).

A.3.2 The roles of Th2 cytokines in the prevention and development of IDDM in NOD mice

In contrast to Th1 cytokines, Th2 cytokines, especially IL-4, have been associated with protection against diabetes in NOD mice. First, systemic administration of IL-4 to young NOD mice reduced the incidence of diabetes (Rapoport et al., 1993; Tominaga et al., 1998). This protection is due to a reversal in $CD4^{+}$ T cell hyporesponsiveness and the capacity of IL-4 production (Rapoport et al., 1993; Zipris et al., 1991). Second, analysis of the cytokine expression in the islets of NOD mice at different ages showed that IL-4 expression at the onset of islet inflammation predicted nondestructive insulitis in NOD mice (Fox et al., 1997). Additionally, regulatory Th2 cell lines against insulin and GAD peptides were recently generated, and these cells could block the development of IDDM when transferred into NOD or NOD/*scid* mice (Tisch et al., 1999; Maron et al., 1999). Protection against β-cell destructive insulitis and diabetes in NOD mice provided by injection of complete Freunds adjuvant (CFA), was reported to be associated with a relative increase in IL-4-producing T cells and as well as a decrease in IFN-γ-producing cells (Shehadeh et al., 1993). Furthermore, transgenic NOD mice that expressed IL-4 in their pancreatic β cells were free of insulitis and diabetes in both female and males (Mueller et al., 1996). Importantly, this absence of disease resulted from Ag-specific Th2 type cells, although Th1 cells were also present and found to be functional (Gallichan et

al., 1999). Thus, the expression of IL-4 in the islets of NOD mice did not directly prevent the generation of pathogenic islet response but induced an Ag-specific Th2 population that blocked the destruction by diabetogenic T cells in the pancreas.

IL-10, like IL-4, is a product of Th2 cells. Systemic administration of human IL-10 to NOD mice conferred antidiabetogenic effects (Pennline et al., 1994); while the administration of an IL-10/Fc fusion protein, which has long lasting biological activity completely prevents diabetes in NOD mice. In addition, splenic cells from IL-10/Fc treated NOD mice showed antidiabetogenic effect in recipient young NOD mice when these cells were co-transferred with cells from acute diabetic mice (Zheng et al., 1997). Furthermore, transgenic expression of IL-10 in NOD T cells also provided significant protection from the disease (Pauza et al., 1999). Therefore, Th2 cytokines, such as IL-4 and IL-10, provide protection to NOD mice from autoimmune diabetes in general. However, unlike IL-4, the effect of IL-10 remains controversial since local overexpression of IL-10 in pancreatic β cells accelerated the onset and increased the incidence of diabetes in NOD mice (Wogensen et al., 1994). Taken together, these results indicate that systemic expression of IL-10 provides protection, whereas local expression of IL-10 in pancreatic islets accelerates the pathogenic process. Therefore, individual Th2 cytokine may exert its effect through different mechanisms.

A.3.3 IL-12 as a major mediator inducing the production of Th1 cytokines

Since Th1 and Th2 cytokines play pivotal roles in the development of autoimmune diabetes, the mechanisms that regulate the cytokine profiles of immune responses have been extensively studied in recent years. It has been shown that IL-12 is

the major cytokine for Th1 differentiation and promotes the IFN-γ production of $CD4^+$ T cells. IL-12, which is composed of p35 and p40 subunits, is mainly produced by activated macrophages and dendritic cells. The critical role of IL-12 in cell-mediated immunity is examplified by the IL-12 knockout mice, which have an impaired Th1 immune response (Magram et al., 1996). Like other cytokines, IL-12 exerts its biological activities by engaging specific receptor molecules expressed on the surface of target cells (T cells). Similar to the most of other cytokine receptors, IL-12 receptor subunits do not have intrinsic tyrosine kinase activity, but they function by association with the JAK2 and TYK2, two members of Janus family of kinases (JAKs) (O'Shea, 1997). Engagement between IL-12 and the receptor induced tyrosine phosphorylation of JAK2 and TYK2, which leading to the phosphorylate of a transcription factor Stat4 (signal transducers and activators of transcription 4) (Bacon et al., 1995). Subsequently, dimerized Stat4 translocates into nucleus and bind to IFN-γ promoter, thereby regulate gene expression (Xu et al., 1996). Thus, Stat4 is a key factor which bridges IL-12 and IFN-γ expression. The critical role of Stat4 in the intracellular signal transduction cascade activated by IL-12 has been clearly demonstrated in Stat4-deficient mice reported recently (Kaplan et al., 1996; Thierfelder et al., 1996). Interestingly, the lymphocytes from Stat4-deficient mice showed an impaired IL-12-induced proliferation, and IFN-γ production; in contrast, the production of Th2 type cytokines was increased. These results demonstrated that Stat4 was absolutely required for IL-12-induced Th1 immune response. The role of Stat4 signaling pathway on the pathogenesis of lymphocytic choriomeningitis virus (LCMV)-transgene induced diabetes has been investigated recently (Holz et al., 1999). Infection

of mice with LCMV triggers a T cell-mediated autoimmune response in the mice transgenically expressing LCMV antigen in their pancreatic islets. Although the development of diabetes in this animal model is not spontaneous, it is also promoted by Th1 cytokine production. The absence of Stat4 signaling provided protection from $CD4^+$ T cell-mediated diabetes, and the protection was associated with a significant reduction of IFN-γ production by T cells. This result implicates the importance of Stat4 signaling pathway in T cell-mediated autoimmune diabetes. It will be of interest to determine if the phosphorylation of Stat4 induced by IL-12 can be modified during the immune responses.

A.4 Sexual dimorphism in NOD mice

As an animal model for human IDDM, NOD mice spontaneously develop a diabetic syndrome and share several genetic and immunogical features with human type I diabetes, except for the sex difference. In NOD mice, insulitis begins at 3-4 weeks of age and is present in 100% of females and more than 90% of males at 30 weeks of age (Kikutani et al., 1992). However, there is a clear sexual dimorphism in the incidence of diabetes: first, females exhibit a higher percentage of islets with more destructive lesions than observed in males. Secondly, in females the disease onset occurs earlier starting at 8-10-week-old and the incidence of diabetes reaches 70-80% until 30 weeks of age. In contrast, male NOD mice start to develop the disease at 20-week-old and the incidence only reaches about 10% -20% until 30 weeks of age.

For a long time, the different incidence of autoimmune diabetes between male and female NOD mice has attracted a great deal of attention. However, there is a very limited

understanding of the cellular and molecular basis of this sexual dimorphism. An initial study showed that castration at weaning significantly increased the incidence of diabetes in male NOD mice, whereas there was a tendency of a decrease in females (Fitzpatrick et al., 1991). Neonatal gonadectomy increased the incidence of diabetes in males but decreased in females (Hawkins et al., 1993). The results of these early studies indicated that sex steroids might play some roles in the pathogenesis of IDDM in the NOD mouse model. Thereafter, the effects of sex hormones and their derivatives were tested in many studies. Androgen administration, initiated after the onset of insulitis, was found to prevent islet destruction and diabetes without eliminating the islet infiltration in female NOD mice; however, the disease could still be transferred into those hormone-treated recipients (Fox, 1992). This result clearly indicated that androgen administration did not increase the resistance of NOD mice to diabetes but modulated the autoimmune attack on pancreatic islets. Furthermore, neonatal hormonal manipulation had a significant influence on the incidence of diabetes in NOD mice (Hawkins et al., 1993), since testosterone treatment of female NOD mice decreased the incidence of diabetes. However, they also found that ovariectomy plus testosterone administration increased the incidence to 100% in females, which is hard to explain so far. Androgen derivative 5-α-dihydrotestosterone (5DHT) also exerted a significant effect on the pathogenesis of autoimmune diabetes (Toyoda et al., 1996, Pearce et al., 1995). It was shown that none of the female NOD mice which received 5DHT developed insulitis while all control mice developed diabetes. These studies provided strong evidence for the roles of sex steroids in pathogenesis of autoimmune diabetes. Male hormones may provide certain protection

from the disease while female hormones may promote the progress of the disease. Importantly, sex hormone manipulation strongly influenced the development of insulitis and diabetes if the treatment was started as early as at the time of weaning. Thus, it is likely that male and female sex hormones modulate the autoimmune response in NOD mice in a reciprocal way. In addition, it was reported that administration of 5DHT decreased the percentage of $CD4^+$ T cells but not $CD8^+$ T cells in NOD mice (Toyota et al., 1996). More interestingly, an increased IL-4 mRNA expression was observed in T cells of 5DHT-treated mice. Therefore, systemic administration of 5DHT appears to have multiple effects on T cell immunity.

Additional evidence that supports the important role of sex hormones in the development of diabetes in NOD mice is from the measurement of the concentration of circulating sex hormones. It was reported that in 2-month-old prediabetic female NOD mice, the basal estrogen levels were found to be twice as high as those reported in other mouse strains (Durant et al., 1998). However, the amount of testosterone in NOD mice was similar to those detected in other mouse strains. Therefore, the higher levels of estrogens may contribute to the progression of diabetes in female NOD mice. It should be noted that it is difficult to accurately measure the levels of sex steroids in mice because the value is very low (0.1ng/ml – 300ng/ml).

A.5 The roles of sex hormones on the immune system

Androgens, estrogens and progesterone, in terms of sex hormones, all belong to a group of small lipophilic compounds derived from a common precursor, cholesterol. They differ in the number of carbon atoms they contain, the receptors they

bind, and the biological activities they possess. Androgens include testosterone and its derivatives, such as dihydrotestosterone (DHT) and dehydroepiandrosterone (DHEA). The main hormonal product of the testis is testosterone. The effects of androgen are usually interpreted as the sum of the effects of testosterone and its conversion products. On the other hand, estrogens are a group of female hormones, which include estrone (E1), 17β-estradiol (E2), and estriol (E3). Estradiol is in equilibrium with estrone, which can be converted by the liver and placenta to estriol. Estriol is believed to be the principal estrogen secreted by the placenta during pregnancy. All of them have estrogenic effects, 17β-estradiol being the most active. Progesterone, also a female steroid, plays an important role in the maintenance of pregnancy. It is produced by the corpus luteum in mature and nonpregnant individuals; however, during pregnancy, it is largely secreted by the placenta.

The phenomenon that gender influences the immune system has been noticed for many years. In general, females have enhanced immune responses compared to males (Butterworth et al., 1967; Eidinger et al., 1972), and they are more prone to develop autoimmune diseases. The difference between the sexes could have several explanations, such as the influence by sex chromosomes, sex hormones and/or a complex of neuronal and endocrine pathways. However, sex hormones (androgens, estrogens and progesterone) apparently play important roles on the immune system.

A.5.1 Sex hormones can modulate immune responses

The influence of sex hormones on the immune system was first discovered by the detection of hormone receptors in immunocompetent cells. The sex steroid receptors had

been found not only in the classical reproductive tissues but also in many other tissues including the immune system. These receptors have been detected in murine $CD4^+$ and $CD8^+$ T cells (Budd et al., 1987; Bebo et al., 1999; Viselli et al, 1995), and macrophages (Bebo et al., 1999) by different methods. Although the mechanisms by which sex hormones are able to regulate immune cell function still remain to be defined, it is conceivable that the sex hormones exert their effects in the immune system by interacting with specific steroid receptors expressed on immune cells. Not only the end products, 17β-estrodiol and testosterone, but also the intermediate metabolites, such as 16α-estradiol and DHEA, have been proposed to interact with the immune system (Jansson et al., 1998).

A.5.1.1 The effects of estrogens on immune responses

In general, females have higher immune response and are more prone to autoimmune diseases than males. Growing evidence has shown that estrogens play major roles in these heightened immune responses; however, despite extensive studies, the precise mechanisms are not understood.

Estrogen modulation of immune cells and the influence on immune responses have been investigated and described in both *in vitro* and *in vivo* systems. Some early studies have shown that estrogen exerted dual effects on the immune system. Estrogen administration suppressed T cell response, but enhanced B-cell activation and autoantibody production (Carlsten et al., 1990). Various T cell-dependent immune response, both *in vivo* and *in vitro*, such as transplant rejection, delayed type hypersensitivity (DTH) reaction and T cell proliferation, were found to be suppressed by

estrogen treatment (Holmdahl., 1989). In contrast, estrogen has stimulatory effects on T cell-independent B cell response (Holmdahl., 1989). However, more recent studies demonstrated that the effects of estrogen on T cell immune response were not simply suppressive, and the results of these studies indicated that estrogen and its derivatives had profound influence on the cytokine profiles produced by T cells. It was shown that in the presence of estrogen and its derivatives, estradiol (Gilmore et al., 1997), estrone and estriol (Gorreale et al., 1998), some human T cell clones displayed a dose-dependent enhancement of IL-10 and IFN-γ secretion in response to Ag-stimulation. On the other hand, one study showed that progesterone, another female hormone, favored the development of human Th2 cells producing Th2 type cytokines, and even promoted IL-4 production in established Th1 cell clones (Piccinni et al., 1995). The discrepancy between the studies may be due to different dosage, treatment time or T cell clones. However, these data suggested that estrogens were capable of modulating immune response by exerting a direct effect on the antigen-specific human $CD4^+$ T cells. More importantly, estrogen and its derivatives have selective effects on the production of individual cytokine regardless of T cell subsets or antigen specificity. In support of this notion, one study reported that estrogen markedly increased the activity of the IFN-γ promoter in human lymphoid cells that expressed the appropriate hormone receptor (Fox et al., 1991). However, the mechanisms of enhanced or suppressed cytokine production under the influence of estrogen and its derivatives remain to be elucidated.

A.5.1.2 The effects of androgens on immune responses

Compared with estrogens, fewer studies were related to the effects of androgens on the immune system; however, some evidence indicated that androgen, like estrogen, is also able to modulate immune responses. Data suggested that *in vivo* administration of testosterone and its derivative 5-α dihydrotestosterone (5DHT) enhanced production of Th2 type cytokines IL-4 or IL-10 by activated T cells from female mice (Toyoda et al., 1996, Dalal et al., 1997). Furthermore, androgen-selected T cell lines secreted less IFN-γ and more IL-10 than untreated cells while androgen treatment of naive TCR transgenic T cells resulted a shift in the balance of Th1/Th2 cytokines (Bebo et al., 1999). Although most of the data indicated that androgen treatment correlated with more production of Th2 type cytokines by T cells, there was also contradictory data. One study showed that a short time exposure of splenocytes from female NOD mice with 5DHT seemingly decrease IL-4 production but increase IL-2 secretion (Pearce et al., 1995). It was also found that 5DHT treatment may affect the development of $CD4^{+}$ T cell population (Toyota et al., 1995). Apparently, like estrogen, androgen may have very complex effects on the immune system. Whether androgens directly modify T cell functions and by what mechanisms remain to be studied in detail.

A.5.2 The sexual dimorphism in other autoimmune diseases

Besides diabetes development in NOD mice, gender affects the susceptibility to many other autoimmune diseases, such as rheumatoid arthritis (RA), multiple sclerosis (MS), and systemic lupus erythematosus (SLE). Women have an increased risk of developing these diseases compared with men. The female preponderance is believed to

depend, at least in part, on the influence of sex hormones on the immune system. Many researchers are working on the effects of sex hormones on the immune system using different models; however, the mechanisms involved are far from clear.

Human SLE is an autoantibody-mediated autoimmune disease characterized by a strong female preponderance. The sex ratio for the incidence of SLE is about 13:1 for females and males, respectively (Rothfield, 1981). Both pregnancy (Mund et al., 1963) and consumption of estrogen-contraceptive pills (Jungers et al., 1982) will exacerbate the disease. In lupus-prone mouse strains, estrogen treatment also accelerates autoantibody production and therefore increases the mortality (Roubinian et al., 1978). Therefore, the effect of estrogen in the development of this disease seems to occur through the enhancement of B cell activation and further stimulation of autoantibody production.

Contrary to SLE, RA is a T cell-mediated autoimmune disease that also shows female preponderance. Pregnancy ameliorates the disease while during postpartum there is an increased risk of disease flare (Ostenson et al., 1983). In the animal model of RA, type II collagen-induce arthritis (CIA), estrogen treatment could overcome disease development (Mattsson et al., 1991). Estrogen treatment of castrated female mice induced a suppression of Ag-specific T cell proliferation, decreased the DTH reaction in a dose-dependent manner and also lowered the levels of anti-CII-specific IgG antibodies (Ansar et al., 1985). Thus, in the animal model of RA, estrogen appears to have a suppressive effect on T cell-mediated immune response and exerts an inhibitory influence on the disease process.

MS is another T cell-mediated female preponderant autoimmune disease. In both human and animal studies, pregnancy suppressed the onset of disease until post-partuition (Abramsky et al., 1984; Brenner et al., 1991). It was found that in rats, treatment with estrogens suppressed the development of experimental autoimmune encephalomyelitis (EAE), which is an animal model for human MS disease (Trooster et al., 1993; Jansson et al., 1994). A different result was reported in a recent study. It was clearly shown that testosterone-treatment inhibited the onset of EAE in female mice, and that the administration of testosterone greatly enhanced IL-10, a Th2 type cytokine, by autoantigen-specific T cells (Dalal et al., 1997).

Taken together, the results of these studies in SLE, RA and MS were consistent with the theory that estrogen inhibits T cell-mediated immune response but enhances humoral immune response. However, the effects of sex hormones on these diseases are different from that on autoimmune diabetes in NOD mice. Unlike autoimmune diabetes, RA and MS in animals are induced by immunization of a single antigen. The pathogenesis of these single antigen-specific-induced autoimmune diseases may be different from those involved in autoimmune diabetes, in which pathogenic mechanisms are apparently far more complicated. Therefore, the influence of sex hormones on autoimmune diabetes in NOD mice may be very different from that seen in other autoimmune diseases.

Since most known autoimmune diseases show sexual dimorphism, it is important to understand the cellular and molecular basis of sex hormones involved in immune modulation. This will not only reveal pathogenic mechanisms of the autoimmune

diseases, but may also enhance the development of new therapeutic strategies. The sexual dimorphism in the NOD mouse model provides a unique opportunity for the systemic characterization of the influence of sex steroids on the functions of immune cells, such as $CD4^+$ T cells.

B. RESEARCH OBJECTIVES

B.1 General Objective:

The phenomenon of sexual dimorphism on the development of diabetes in the NOD mouse model and other autoimmune diseases has been recognized for decades; however, knowledge of the mechanisms involved is far from complete. Recent studies have suggested interesting mechanisms of action of the sex hormones on the target immunocytes involved in immune response. Moreover, the influence of sex hormones on the immune response gains additional support by the detection of sex hormone receptors on the immune cells. There are several lines of evidence suggesting that sex hormones modulate the cytokine production of $CD4^+$ T cells in other autoimmune diseases, for instance, human MS and its animal model EAE. $CD4^+$ T cells are critical cells in immune regulation since they can differentiate into Th1 or Th2 type of T cells, secrete different cytokines and subsequently determine the function of effectors in immune responses. However, it remains an open question that whether sexual dimorphism of autoimmune diabetes in NOD mice is due to, at least in part, the direct modulation on the $CD4^+$ T cell function by sex hormones. If sex hormones significantly influence the functions of $CD4^+$ T cells in NOD mice, the cytokine profiles produced by $CD4^+$ T cells isolated from male and female NOD mice may be different following activation. The difference in cytokine profiles of $CD4^+$ T cells may correlate with age and sex maturation. Therefore, the overall objective of my research project is to investigate the effects of sex hormones on the function of $CD4^+$ T cells in NOD mice, and to identify the potential molecular mechanisms involved in the sex hormone-mediated modulation of cytokine production by $CD4^+$ T cells in NOD mice.

B.2 Specific objectives:

B.2.1 Identification of Th1/Th2 cytokine expression in the islets of female and male NOD mice at different ages.

Many recent studies have been focused on the contribution of Th1/Th2 type cytokines on the pathogenesis of IDDM in NOD mice. Despite some minor contradictory results from different groups, it is generally believed that Th1 type cytokines, especially IFN-γ, is pathogenic for the disease while Th2 type cytokines, such as IL-4, is associated with protection from the disease development. In NOD mice, a clear sexual dimorphism with respect to the incidence of the disease has been known for more than twenty years. Limited evidence suggested that this gender difference was directly associated with the Th1/Th2 cytokine production of T cells from NOD mice. The insulitic lesions of every NOD mouse contain many activated autoantigen-specific $CD4^+$ T cells. Therefore, if sexual dimorphism of diabetes in NOD mice is associated with the Th1/Th2 cytokine production in $CD4^-$ T cells, then islet infiltrating antigen-specific $CD4^-$ T cells may produce different cytokine profiles in male and female NOD mice. Although insulitis contains $CD4^-$ and $CD8^-$ T cells, as well as some macrophages and dendritic cells, IL-4 is mainly produced by $CD4^+$ T cells. Therefore, islet-infiltrating cells are the best candidates for identifying the difference in cytokine production by autoantigen-specific T cells in male and female NOD mice. Thus, the first specific objective of my research project was to determine the Th1/Th2 cytokine gene expression by autoreactive T cells in the natural history of autoimmune diabetes in both female and male NOD mice. To achieve this objective, I examined IFN-γ and IL-4 gene expression at the infiltrated islet

lesions in different age groups of female and male NOD mice using a semi-quantitative RT-PCR method.

B.2.2 Identification of Th1/Th2 cytokine production by CD4$^+$ T cells from female and male NOD mice at different ages upon mitogen activation *in vitro*.

To further determine whether CD4$^+$ T cells from male and female NOD mice produce different cytokine profiles when they are activated, CD4$^+$ T cells would be isolated from both female and male NOD mice and activated by mitogens. In this study, two groups of mice at different ages would be chosen. One group of NOD mice would be young and sexually premature, while the other group would be adult mice for examination of T cell function. Since the cytokine production by CD4$^+$ T cells is initiated by mitogen activation *in vitro*, the isolated CD4$^+$ T cells from female and male NOD mice of different age groups would be activated by anti-CD3 antibody and anti-CD28 antibody, which provides a costimulatory signal. The function of the T cells would be examined using proliferation assay, ELISA and RT-PCR methods.

B.2.2.1 Examination of the cytokine production of CD4$^+$ T cells from young female and male NOD mice upon mitogen activation *in vitro*.

CD4$^+$ T cells would be isolated from 3-4 week-old female and male NOD mice, cultured and subsequently activated with anti-CD3 and anti-CD28 for 3 days. The mRNA level of cytokine gene expression in CD4$^+$ T cells would be measured using semi-quantitative RT-PCR while cytokine production would be examined using ELISA method. A proliferation assay would be used as an indicator of T cell activation, and also

as a standard for the comparison of cytokine expression and production between female and male mice.

B.2.2.2 Examination of the cytokine production of CD4$^+$ T cells from adult female and male NOD mice upon mitogen activation *in vitro*.

CD4$^-$ T cells would be isolated from 8-10-week-old female and male NOD mice, which are sexual mature. The cells would be cultured and activated as described above. The methods used were the same as above.

B.2.3 Examination of the effects of estrogen and testosterone on the function of CD4$^+$ T cells from NOD mice *in vitro*.

For decades, many studies have shown the effects of sex hormones on the immune system using different animal models of autoimmune diseases. The results of these studies indicate that sex hormones might affect several different aspects of immune system. T cell development may be affected by sex hormones, and estrogen and androgen may selectively elevate or suppress the production of individual cytokines (Gilmore et al., 1997, Bebo et al., 1999). The development of autoimmune diabetes is strongly affected by sex hormone administration; although, there is little clear evidence showing whether sex hormones modify the functions of CD4$^-$ T cells directly. To answer this question, I will examine the direct influence of estrogen and testosterone on the function of CD4$^+$ T cells from NOD mice using a proliferation assay, ELISA and RT-PCR methods.

B.2.3.1 Examination of the expression of sex hormone receptors in CD4$^+$ T cells of NOD mice.

Sex hormone-mediated immune regulation relies on the engagement between the hormones and their receptors. It is unclear whether $CD4^+$ T cells isolated from male or female NOD mice express different levels of receptors for androgen and estrogen. Therefore, to investigate the direct effects of estrogen and testosterone on $CD4^+$ T cells of NOD mice, I initially examined the expression of both androgen and estrogen receptors in $CD4^+$ T cells isolated from female and male NOD mice using RT-PCR method.

B.2.3.2 Examination of the effects of estrogen and testosterone on the proliferation of $CD4^+$ T cells from NOD mice *in vitro*.

It was reported that sex hormones might affect the development of $CD4^+$ T cell population in NOD mice. It is possible that sex hormones may affect the proliferation of activated $CD4^+$ T cells. Therefore, to test this possibility, I will pre-treat the cultured $CD4^+$ T cells from female or male NOD mice with estrogen or testosterone for a certain time period. The proliferation of pretreated $CD4^+$ T cells will be assayed following stimulation with anti-CD3 and anti-CD28 antibodies for 3 days.

B.2.3.3 Examination of the effects of estrogen and testosterone on the Th1/Th2 cytokine expression and production of $CD4^+$ T cells from NOD mice *in vitro*.

To investigate the direct effects of sex hormones on the cytokine production by the activated $CD4^+$ T cells of NOD mice, the best way is to compare the cytokine production from $CD4^+$ T cells after treatment with sex hormones *in vitro*. In particular, I am interested in whether the pretreatment of sex hormones can modify Th1 or Th2 cytokine expression. It is known that IL-12 is a potent inducer of Th1 cytokine production, an interesting question is whether IL-12-induced Th1 cytokine production

can be modified by pretreatment with different sex hormones. Therefore, I plan to examine the IL-12-induced cytokine expression of cultured NOD CD4$^+$ T cells with or without pretreatment of sex hormones using ELISA and RT-PCR methods.

B.2.4 Examination of the effects of estrogen and testosterone on IL-12-induced phosphorylation of Stat4 in CD4$^+$ T cells from NOD mice.

There are limited reports indicating that sex hormones may influence the Th1/Th2 cytokine production of T cells. However, the mechanism by which sex hormones modify cytokine profiles of activated T cells remains unknown. Since Stat4 has been suggested to play an essential role in IL-12-mediated signaling in Th1 cell differentiation (Kaplan et al., 1996; Thierfelder et al., 1996), a possible mechanism for the influence of sex hormones on cytokine production of T cells is that sex hormone-treatment may promote the increase or decrease on the phosphorylation level of Stat4 protein. To test this hypothesis, I plan to examine the effects of estrogen and testosterone on IL-12-induced phosphorylation of Stat4 in CD4$^+$ T cells isolated from NOD mice.

C. MATERIALS AND METHODS

C.1 Animals

NOD mice were purchased from Taconic (Germantown, NY), and maintained in the animal facilities at the University of Calgary (Calgary, Canada). C57BL/6 mice were purchased from Charles River (Montreal, Canada), and maintained under the same conditions.

C.2 Islet isolation from NOD mice

Islets were isolated from female and male NOD mice in different age groups. Three females or males for each age group were sacrificed and their islets were isolated and pooled. Briefly, after clamping the both ends of the common bile duct, 3ml-syringe was punctured into the duct with 30G1/2 bended needle. Three ml ice-cold Hanks' Balanced Salt Solution (HBSS) containing collagase (470u/ml, Worthington Biochemical Corporation, Freehold, NJ) was injected into the duct. The distended pancreas was removed and incubated for digestion in a 37°c water bath for 21 minutes. The digested pancreas was placed on ice and cold HBSS was added to stop the digestion. After pipetting 20-30 times with a special glass pipett, the tube was placed on ice for 5-10 minutes. Then the supernatant was discarded; the pellet was resuspended in cold HBSS and passed through mesh with 600μm. The tissue was washed and resuspended in 4ml 25% Ficoll (Sigma Chemical co., St. Lousi, MO), vortexed, and overlayed in 2ml each of 23%, 21.5%, 20.5% and 11% Ficoll solution, and centrifuged at 4°c (1800 x g) for 10 minutes. Islets were recovered from the 1st and 2nd interface, and placed in HBSS. Finally, the islets were picked by hand free of other tissues using a dissecting microscope.

C.3 RT-PCR analysis of cytokine gene expression

Total RNA was isolated from islets or cultured cells using TRIzol reagent (GIBCO-BRL Life Technologies, Germany) according to the manufacturer's instructions. Briefly, samples were homogenized, and incubated at room temperature for 5-10 minutes to permit the complete dissociation of nucleoprotein complexes. After phase separation by chloroform, the mixture separated into two phases, and then the upper colorless aqueous phase was transferred to a new tube. Following precipitation of the RNA with isopropanol, the RNA containing pellet was washed with 75% ethanol and air-dried. Total RNA was suspended in DEPC-treated distilled water.

Three μg of total RNA from each sample was subjected to first-strand cDNA synthesis in 20μl of reaction mixture (final volume) containing 50mM Tris-HCL(pH 8.3), 75mM KCL, 3mM $MgCL_2$, 10μg/ml oligo-dT primer (GIBCO-BRL Life Technologies, Germany), 10mM DTT, 0.5 mM of each dNTP, 2u/μl Rnase-out Rnase inhibitor (Life Technologies, Germany), and 2.5u SuperScriptII reverse transcriptase (Life Technologies, Germany) at 37°c for 60 minutes. After the reaction, the tubes were heated at 95°c for 5 minutes to inactivate the enzymes. The samples were diluted to 100μl with distilled water, and 5μl of each sample was used for PCR. The PCR was run using specific primers for several cytokines (Table 1) under conditions optimized for each set of primers. β-actin was used as an internal standard. The reaction was

Table 1 Oligonuleotide sequences:

β-actin	5'	GTTACCAACTGGGACGACA
	3'	TGGCCATCTCCTGCTCGAA
IFN-γ	5'	AGCTCTGAGACAATGAACGC
	3'	GGACAATCTCTTCCCCACCC
IL-4	5'	TCTTTCTCGAATGTACCAGG
	3'	CATGGTGGCTCAGTACTACG
E2R	5'	GAGACTGTCCAGTAACGAGAA
	3'	GGACAAGGCAGGGCTATTC
TSR	5'	TCTCAAGAGTTTGGATGGCTCC
	3'	GAGATGATCTCTGCCATCATTTC

performed using a different number of cycles to ensure that there was a linear relationship between the cDNA amount (serial dilution) subjected to PCR amplification and the PCR signal. The PCR mixture (20μl) contained 0.2mM of each dNTP, 1 mM of specific primers, 1.5 mM $MgCL_2$, 50 mM KCL, 10mM Tris-HCL (pH9.0), and 1 u of *Tag* DNA polymerase (Pharmacia). Following amplification, 10 μl of each PCR product was subjected to electrophoresis on a 1.5% agarose gel and detected by ethidium bromide staining. The bands were scanned and the density was measured using Adobe PhotoShop software.

C.4 Purification, activation and proliferation of $CD4^+$ T cells

Lymphocytes from spleens and lymph nodes were obtained by macerating spleens and lymph nodes from the mice between the frosted ends of glass slides in RPMI 1640 (Gibco, Gaithersburg. MD), containing 2mM glutamine, $5x10^{-5}$μM 2-mercaptoethanol, 100 u/ml penicillin, and 100μg/ml streptomycin. After lysis of RBC in ammounium chloride, cells were washed twice and ready for purification of $CD4^+$ T cells.

$CD4^+$ T cells were purified using MACS CD4 microbeads (Miltenyi Biotec, Germany) according to the instructions supplied by the manufacturer. In brief, the cells were incubated with anti-mouse CD4 microbeads for 15 minutes at 4°c. After washing, the cell suspension was applied in appropriate amount of buffer onto the positive selection column (Miltenyi Biotec, Germany). The negative cells were allowed to pass through and the column was rinsed with appropriate amount of buffer. Finally, after removing the column from the magnetic field, the positively selected $CD4^+$ T cells were flushed out firmly using the plunger supplied with the column.

A 96-well round bottom culture-plate was precoated with anti-CD3 antibody at different concentrations for 7 hours at 37°c and washed with PBS twice prior to plating the cells. The purified $CD4^-$ T cells were washed twice with RPMI medium and diluted to 2.5×10^6 cells/ml, and 200μl aliquots were delivered to each well of 96-well-culture plate. Anti-CD28 antibody (Pharmingen, San Diego, CA) was used at 2.5μg/ml and added into cell suspension directly. 17-β-estradiol was purchased from Sigma in Canada. It was resolved in DMSO and the solution was kept at concentration of 50mg/ml at room temperature. 17-β-estradiol was further diluted into 25ng/ml ($1{:}2 \times 10^6$ dilution) with RPMI medium. Testosterone was purchased through prescription. The concentration of original reagent was 200mg/ml and 0.5% chlorobutanol was used as preservative in sesame oil. Since the concentration used for culturing was only 25ng/ml ($1{:}8 \times 10^6$ dilution), the preservative was considered to have a negligible effect on the culturing system. Five wells were used for each condition with each well containing 0.5×10^6 cells in 200μl medium in 96-well-culture plate. After culturing for 72hours, 110μl of supernatant from each of three wells and 400μl from the rest of two wells of each condition was collected and stored at -20°c until used for ELISA assay. The cells from two wells of each condition were collected and lysed in an appropriate amount of TRIzol reagent and kept at -70°c for subsequent RNA isolation. Each of the remaining three wells of each condition were pulsed with 1μCi [^{3}H]-thymidine. After an additional 16-hour incubation, the cells were harvested onto fiber filter mats and the amount of ^{3}H incorporation was determined by liquid scintillation counting.

C.5 Flow cytometric analysis

Purified $CD4^{+}$ T cells (1×10^{6}) or cultured T cells were washed with staining buffer (PBS containing 2% FCS), and incubated with FITC conjugated anti-TCR and PE-conjugated anti-CD4 antibodies for 30 min at 4°c. Then the cells were washed with staining buffer twice and analyzed by FACScan (Becton Dickinson, Sunnyvale, CA).

C.6 Cytokine detection by ELISA

Measurement of cytokines, IFN-γ and IL-4, was performed by using DuoSet ELISA development system (R&D systems, Minneapolis, MN) according to the manufacturer's protocol. In brief, 100μl of each sample was added to the plates precoated with specific capture antibodies, and each sample was assayed in duplicate. After incubation at room temperature for two hours, the samples were washed, and the detection antibodies for the specific cytokines were added to the wells using the dilution recommended by the manufacturer. The plates were washed, and 100μl of the working dilution of Streptavidin-HRP (1:200) was added to each well. The color was developed by adding 100μl substrate solution (R&D systems, Minneapolis, MN), and immediately read using an automated microplate reader set to 450nm. The wavelength correction was set to 540nm. A standard curve was generated for each set of samples assayed.

C.7 IFN-γ production of $CD4^{+}$ T cell by IL-12 stimulation

Lymphocytes were isolated from spleens and lymph nodes of 8-week-old female or male NOD mice. The cells were first activated with ConA (2.5μg/ml) in RPMI 1640 medium with 10% FCS and rIL-2 (10u/ml) at 37°c for 2-3 days. Then the cells were washed three times with medium and reincubated in some RPMI medium without ConA

for an additional 4 days. After 4-day-culture, the purity of $CD4^+$ T cells was examined by the flow cytometric analysis described as before.

The $CD4^+$ T cells were rested and pretreated with either 17β-estradiol (E2, 25ng/ml) or testosterone (TS, 25ng/ml) in IL-2-free RPMI medium 20 hours. After that, IL-12 (20ng/ml) was added to the medium and the cells were incubated for additional 48 hours. Each condition was done in triplicates. Finally, the supernatant from each condition was collected for ELISA and the cells were lysed for RT-PCR.

C.8 Immunoprecipitaion and immunoblot analysis

Cultured $CD4^+$ T cells were rested and pretreated by E2 (25ng/ml) or TS (25ng/ml) in IL-2-free RPMI 1640 medium for 20 hours. The cells (50 $\times 10^6$/condition) were washed and resuspended in 1ml of the same medium, then IL-12 (PeproTech, Rocky Hill, NJ, 20ng/ml) was added to the medium and incubated at 37°c for 20 minutes. Following washing, the cells were lysed by ice-cold lysis buffer containing 1% Triton x-100, 0.5% sodium deoxycholate, 0.1% SDS, 10mg/ml phenylmethysulfonyl fluoride (PMSF), 30μl Aprotinin, and 100mM sodium orthovanadate. The lysates were left on ice for 20 minutes, and then were cleared of debris by centrifugation at 12,000 x g at 4°c for 20 minutes. The lysates were immunoprecipitated with anti-Stat4 antibody, and were resolved on 10% SDS-PAGE transferred to PVDF membranes (Bio-Rad, Hercules, CA). The membrane was probed with anti-phosphotyrosine antibody (4G10, Upstate, Lake Placid, NY). After washing, the membrane was blotted with secondary antibody conjugated with horseradish peroxidase (HRP). The detection was performed by the use of enhanced chemiluminescence (ECL, Amersham Life Science Inc., Arlington Heights,

IL). The membranes were subsequently stripped using stripping buffer (100mM 2-mercaptoethanol, 2% sodium dodecyl sulphate, and 62.5mM Tris-HCL) and reprobed with anti-Stat4 antibody (Upstate, Lake Placid, NY).

C.9. Statistical analysis

Student's t test was used to calculate statistical significance for difference in all the experiments between different groups. P value <0.05 was considered to be statistically significant.

D. RESULTS AND DISCUSSION

D.1 Identification of Th1/Th2 cytokine expression of the islets from female and male NOD mice at different ages.

Sexual dimorphism is a clear pathogenic characteristic of autoimmune diabetes developed in NOD mice. Since cytokines expressed by autoreactive T cells are known to play critical roles in the pathogenesis of autoimmune diabetes, sexual dimorphism may be a consequence of different cytokine profiles produced by autoreactive T cells in female and male NOD mice because of the sex hormone modulation. Plenty of evidence has shown that Th1 cytokines, especially IFN-γ, play an important role in the progress of islet antigen-specific autoimmunity; while Th2 cytokines, such as IL-4, may delay or prevent the development of insulitis and the onset of diabetes. To investigate whether the pattern of Th1/Th2 cytokines expressed by islet antigen-specific T cells correlates with gender and age of NOD mice, islets were isolated from both female and male NOD mice from 4 weeks to 20 weeks of age, since these islets contained islet antigen-specific infiltrating T cells. Total RNA was extracted from these islets and IFN-γ and IL-4 mRNA expression were examined using semi-quantitative RT-PCR method (Figure 1). Because RT-PCR is a sensitive method, RNA was also prepared from pancreatic islets isolated from C57BL/6 mice as a negative control since C57BL/6 mice do not spontaneously develop insulitis.

The results showed that IFN-γ gene expression from the islets of 4-week-old female NOD mice was significantly higher than age-matched males (Figure 2; Figure

Figure 1. Detection of islet cytokine gene expression by RT-PCR.

cDNA produced from the islets of NOD mice at different ages was serially diluted (1:4 dilution), and the PCR reactions specific for IFN-γ and IL-4 cDNA were performed using serially diluted islet cDNA as template. The specific primers for IFN-γ and IL-4 were described in "Materials and Methods". As an internal standard, the PCR reaction of β-actin was performed. The PCR products were analyzed on 1.5% agarose gel and visualized by ethidium bromide staining. The results showed that the yield of PCR products decreased proportionally to the diluted template, so RT-PCR analysis identified different levels of gene expression. Lane M: 100bp ladder; lane 1: 5μl of cDNA; lane 2: 5/4μl of cDNA; lane 3: 5/16μl of cDNA; lane 4: 5/64μl of cDNA.

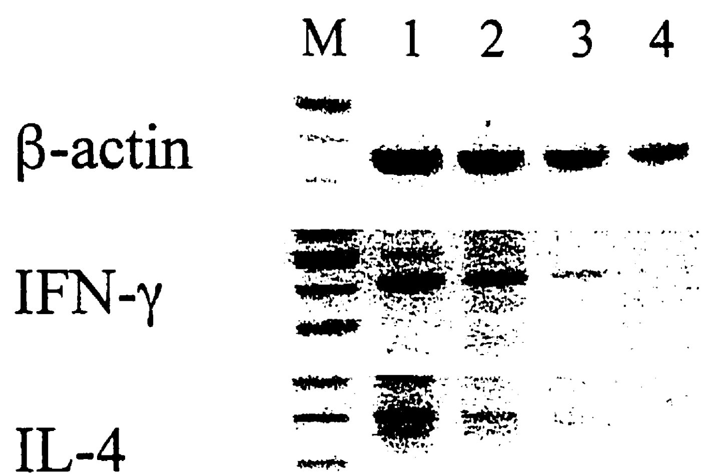

M 1 2 3 4
β-actin
IFN-γ
IL-4

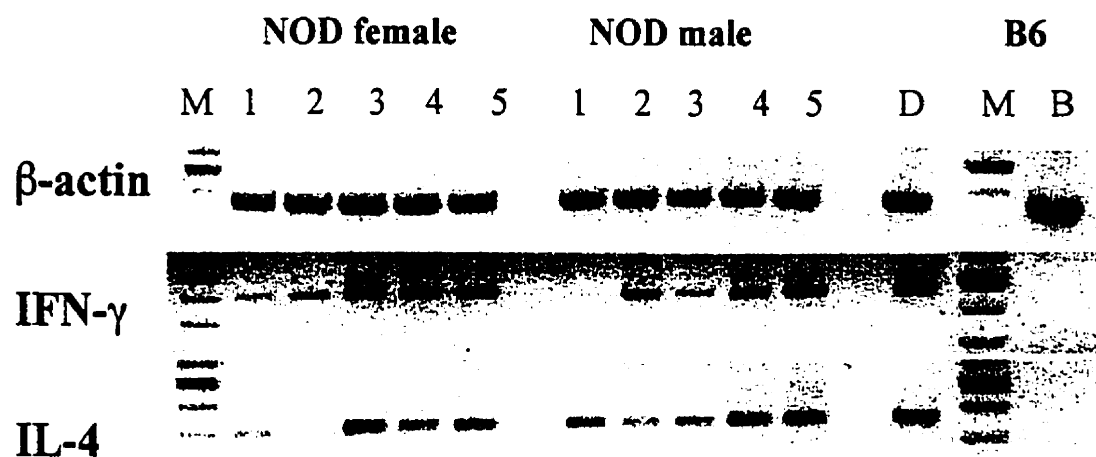
Figure 2. A. RT-PCR analysis of cytokine gene expression of islet infiltrating cells in female and male NOD mice at different ages.

Pancreatic islets were isolated from female and male NOD mice at different ages. Three mice were used in each age group, and the same experiments were repeated three times. In each set of experiment, equal amount of islet RNA (3μg of each sample) was used as template for RT reactions. RT-PCR reactions specific for IFN-γ (30 cycles), IL-4 (45cycles) and β-actin (30 cycles) gene expression were performed for each islet cDNA sample. Identical amounts (5μl from total 100μl) of cDNA were used in all PCR reactions. Islet cDNA was also prepared from diabetic NOD mice and C57BL/6 mice as positive and negative controls, respectively. All PCR products were analyzed on 1.5% agarose gel and visualized by ethidium bromide staining. Lane M: 100bp ladder; Lane 1: 4-week-old NOD mice; Lane 2: 8-week-old NOD mice; Lane 3: 12-week-old NOD mice; Lane 4: 16-week-old NOD mice; Lane 5: 20-week-old NOD mice; Lane D: diabetic NOD mice; Lane B: C57BL/6 mice. The result shown here was the representative of three similar independent experiments.

B. RT-PCR analysis of IFN-γ gene expression of islet infiltrating cells in 4-week-old female and male NOD mice.

The PCR reactions of IFN-γ gene using cDNA from 4-week-old female and male NOD islets were performed by increased PCR reaction cycles, 32 and 34 cycles. At 34 cycles, IFN-γ expression could be detected in both female and male NOD islets of 4-week-old, but the expression level of female islets was much stronger than that of male islets. The result shown here was the representative of three similar independent experiments.

A.



B.

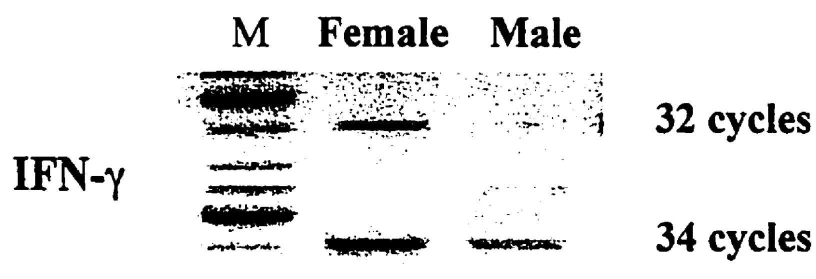
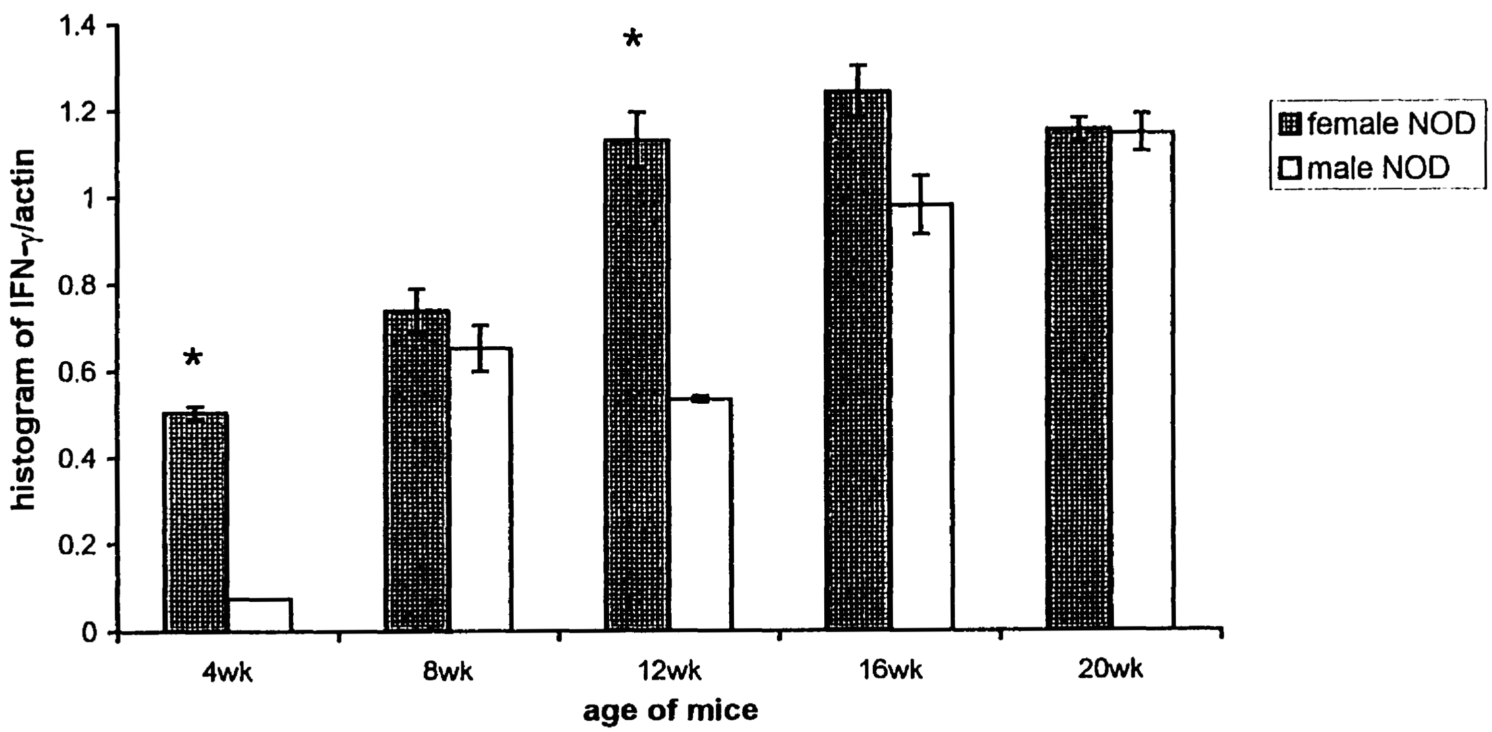


Figure 3. Analysis of IFN-γ gene expression in the islets of female and male NOD mice at different ages.

The density of PCR products of IFN-γ gene expression from NOD islets was measured by scanning and further analyzed by Adobe PhotoShop software. The levels of IFN-γ gene expression detected by RT-PCR reactions were normalized with β-actin gene expression measured in each sample. The relative levels of IFN-γ gene expression were then grouped according to the age of mice. The values shown here were the mean of three independent experiments. The values of SD shown here were the standard deviation of the values obtained from three independent experiments.

* $P<0.01$ as compared to the value of age-matched male NOD mice.


histogram of IFN-γ/actin
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
4wk
8wk
12wk
16wk
20wk
age of mice
female NOD
male NOD

3), when insulitic development was still at an early stage. IFN-γ gene expression was clearly detected in the islets of 4-week-old young female mice, but not in the islets of same-aged male NOD mice although when the PCR reaction cycle was increased the IFN-γ mRNA could be seen weakly. At the age of 8 weeks, the expression of IFN-γ gene was found in the islets from both female and male NOD mice. The expression levels of IFN-γ gene increased along with the age up to 12 weeks. However, by 20 weeks of age when severe insulitis has developed in every NOD mouse, similar levels of IFN-γ gene expression were detected in the islets from both female and male mice. In control C57BL/6 mice which do not have insulitis, IFN-γ mRNA was not detected in the islets by RT-PCR (Figure 2), indicating that RT-PCR specifically detects cytokine gene expression of infiltrating T cells in NOD mice.

In contrast to IFN-γ, the intra-islet mRNA level of IL-4 in female NOD mice at 4-8-week-old was found to be lower than that in age-matched males (Figure 2; Figure 4). In male NOD mice, significant IL-4 gene expression was detected in the islets from a very young age (4-week-old). Therefore, similar to IFN-γ gene expression, the most pronounced gender difference of IL-4 gene expression was also found during early stage of insulitic development. No IL-4 mRNA could be detected from the islets of C57BL/6 mice (Figure 2).

The results of RT-PCR showed that both IFN-γ and IL-4 genes were expressed in the islets of female and male NOD mice, and the expression levels increased with the progression of insulitic development. IFN-γ gene expression was significantly higher, but IL-4 gene expression was lower in young female mice than that in age-matched male

mice. It is more distinct when the ratios of IFN-γ and IL-4 expression were compared between female and male NOD mice at different ages. Female NOD mice have a higher ratio of IFN-γ/IL-4 gene expression than that of males (Figure 5). More importantly, the ratio of IFN-γ/IL-4 expression clearly indicated that the most significant gender difference in islet cytokine profile was in young NOD mice (Figure 6). This gender difference was diminished in older mice, and by 20 weeks of age, cytokine profiles of insulitis were similar in female and male NOD mice.

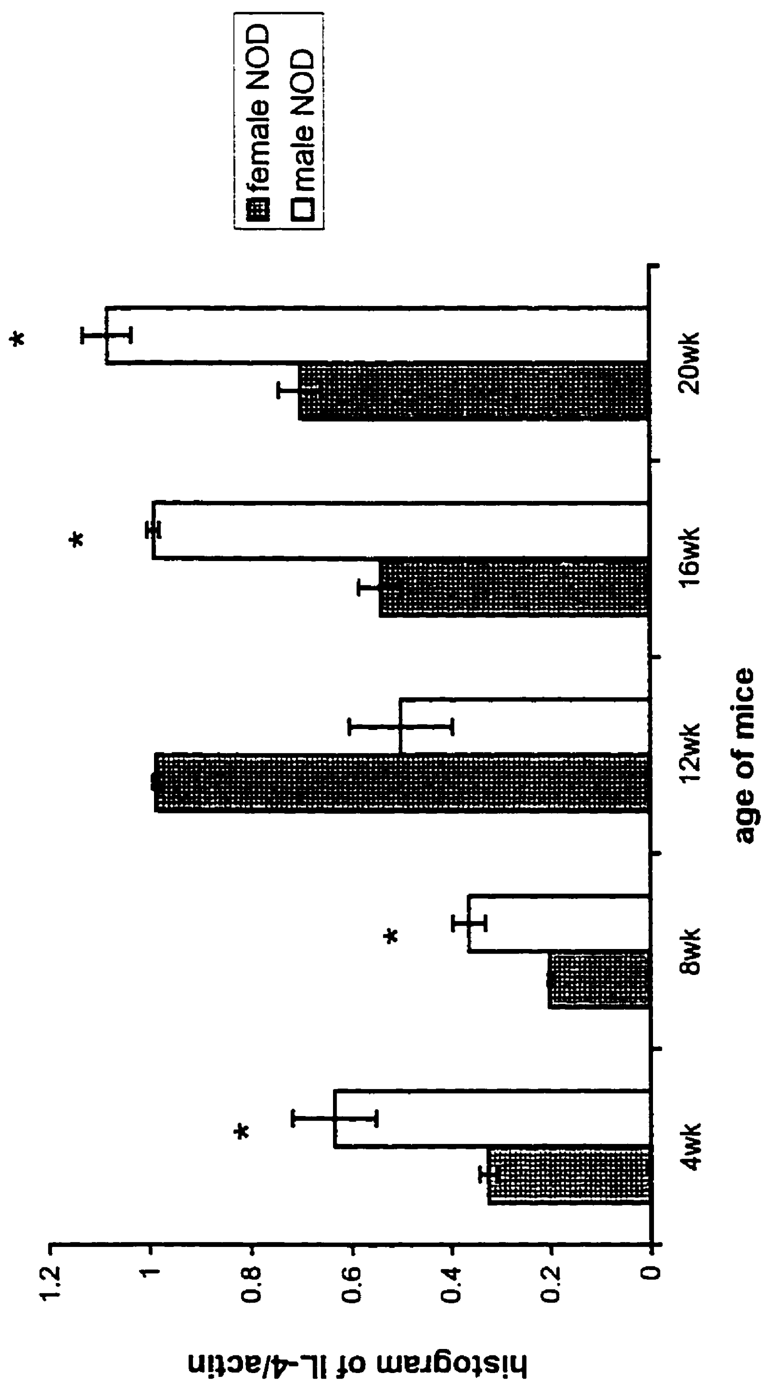
D.2 Discussion

The pathogenic process of autoimmune diabetes in NOD mice initiates with mononuclear lymphocyte infiltration of pancreatic islets (insulitis) in both female and male young NOD mice. However, the progress of the pathogenic process is different between male and female NOD mice. Among female mice, the onset of diabetes starts at about 12 week-old, and the incidence can reach to 70-80% at 30 weeks of age. Male NOD mice typically develop diabetes after 20 weeks of age, and the incidence of the disease remains as low as 10-20% at 30 weeks of age. Since it is known that type I and type II cytokines, such as IFN-γ and IL-4, produced by autoreactive T cells play key roles in the pathogenic process in NOD mice, whether differentiated cytokine production results in sexual dimorphism in autoimmune diabetes in NOD mice has been an important question in recent studies. Several studies suggested that the capacity of female NOD mice to produce more IFN-γ but less IL-4 within the islets than that of males contributed to the higher incidence of diabetes. An early study reported

Figure 4. Analysis of IL-4 gene expression in the islets of female and male NOD mice at different ages.

The density of PCR products for gene expression of IL-4 from NOD islets was measured by scanning and further analyzed by Adobe PhotoShop software. The levels of IL-4 gene expression detected by RT-PCR reactions were normalized with β-actin gene expression measured in each sample. The relative levels of IL-4 gene expression were then grouped according to the age of mice. The values shown here were the mean of three similar experiments. The values of SD shown here were the standard deviation of the values obtained from three similar experiments.

* $P<0.05$ as compared to the value of age-matched female NOD mice.



female NOD
male NOD
histogram of IL-4/actin
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
*
4wk
8wk
12wk
16wk
20wk
age of mice

Figure 5. Analysis of IFN-γ/IL-4 gene expression ratio from the islets of female and male NOD mice at different ages.

The IFN-γ gene expression from the islets of female and male NOD mice was normalized with the density of IL-4 expression of the same cDNA sample. The IFN-γ/IL-4 ratio was grouped between age-matched female and male NOD mice. The values shown here were the mean of three independent experiments. The values of SD shown here were the standard deviation of the values obtained from three independent experiments.

* $P<0.005$ as compared with the ratio obtained from age-matched male NOD mice.

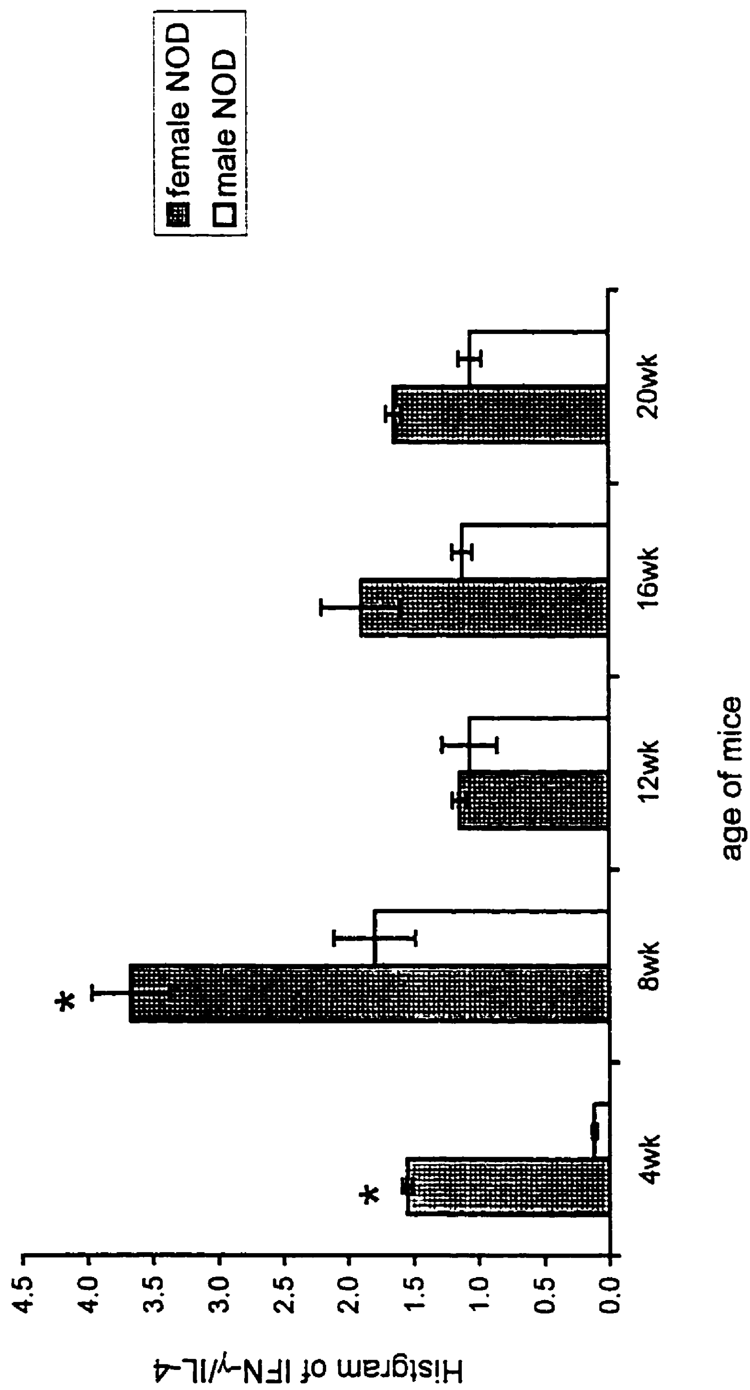
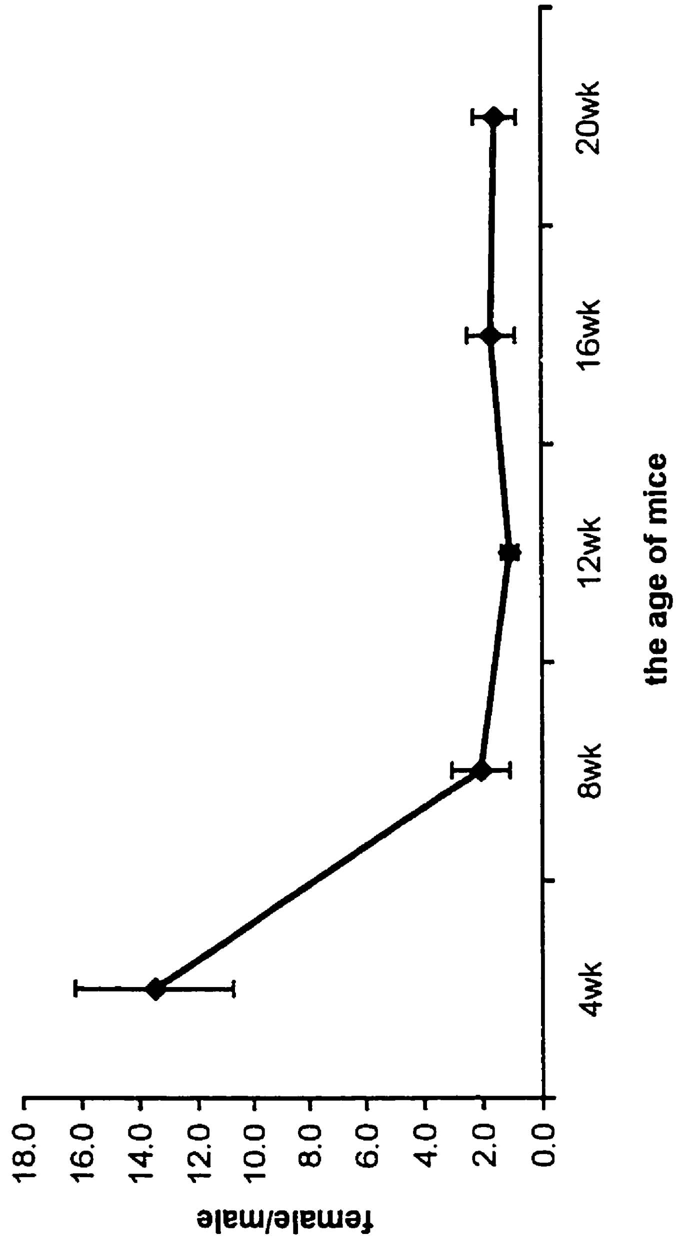

female NOD
male NOD
Histgram of IFN-γ/IL-4
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
4wk
8wk
12wk
16wk
20wk
age of mice

Figure 6. Comparison of the expression of islet IFN-γ/IL-4 between age-matched female and male NOD mice.

IFN-γ/IL-4 gene expression determined in Figure 5 was compared between age-matched female and male NOD mice by normalizing IFN-γ/IL-4 ratio of female islets with that of male islets. The significant difference of IFN-γ/IL-4 ratio was found between young female and male NOD mice. In adult mice (8 weeks of age), female NOD mice still had a higher islet IFN-γ/IL-4 expression ratio than that of age-matched male NOD mice, but the difference (1-2 folds) between adult female and male NOD mice was much smaller than that (14 folds) of 4-week-old female and male NOD mice. The values shown here were the mean ± SD of three independent experiments.


female/male
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
4wk
8wk
12wk
16wk
20wk
the age of mice

(Rabinovitch et al., 1995) that only IFN-γ mRNA levels were significantly higher in the islets from 12-week-old female NOD mice than that of age-matched male NOD mice. In contrast, the IL-4 mRNA levels were lower in females than in males. No significant difference could be found on the cytokine expression of splenocytes among these groups. When cytokine expression of insulitis from 20-180-day-old female and male NOD mice was analyzed by semi-quantitative RT-PCR (Fox et al., 1997), it was reported that at 1 to 2 month of age, islet-infiltrating T cells displayed a Th1 cytokine bias in females, and a Th2 bias in males. In later stages, the data suggested that a stronger T cell recruitment into islet lesions and a higher level of IFN-γ gene expression in female NOD mice than that in male mice were found. These results implied that high Th1 cytokine expression in early insulitis exerts substantial impact on β-cell destruction and overt diabetes.

In this study, I analyzed Th1/Th2 cytokine gene expression levels from the islets of female and male NOD mice at different ages, using C57BL/6 mice as a negative control. In young age group, IFN-γ mRNA levels from insulitic lesions of female NOD mice were significantly higher than that of age-matched males. In contrast, IL-4 mRNA levels in the islets of young females were reduced compared to same-aged males except for one age group. No cytokine gene expression could be detected in the islets of diabetes-free strain C57BL/6 mice, which have no insulitis. The results obtained from this study were consistent with previous studies in regarding different expression levels of IFN-γ and IL-4 genes of islet infiltrating cells between female and male NOD mice (Fox et al., 1997). It is important to note that the most striking gender difference was in young NOD mice (about 4-week-old) when insulitis equally developed in both female

and male NOD mice. The differentiated cytokine expression between female and male NOD mice was demonstrated more clearly when the ratio of IFN-γ/IL-4 expression was analyzed. This observation suggested that the cytokine profiles in the earliest insulitic lesion of NOD mice had profound effects on the late events of autoimmune reactions. Additionally, IFN-γ/IL-4 ratio was more critical to the final stage of islet destruction than the yield of individual cytokine gene expression since in both female and male NOD mice, the individual cytokine gene expression levels increased dramatically at later stage.

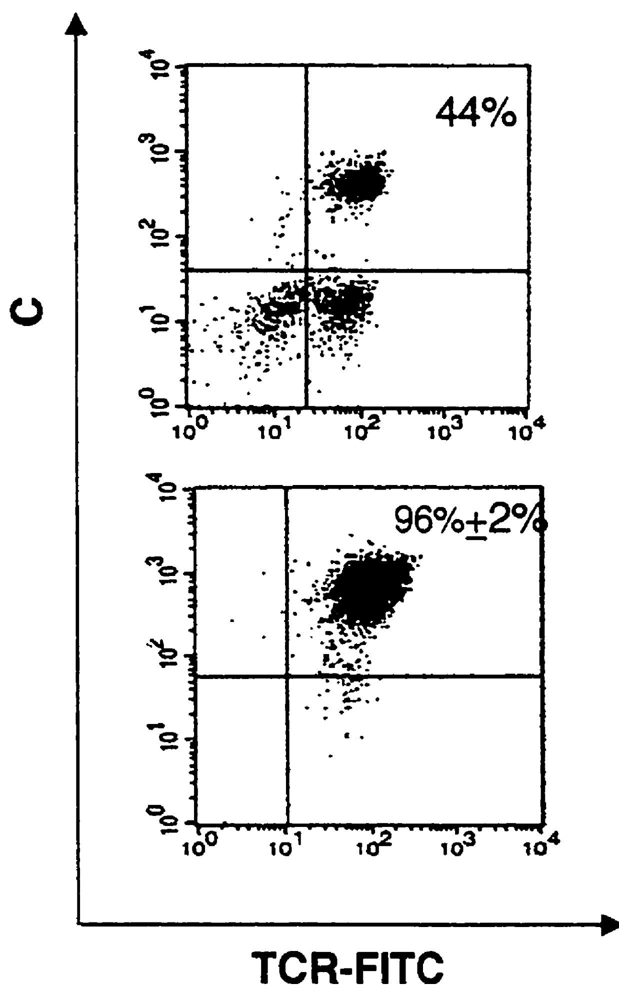
The results of RT-PCR study demonstrated a strong correlation between gender difference (sexual dimorphism) and differentiated Th1/Th2 cytokine gene expression in the islets of NOD mice. Therefore, the gender-specific factors, especially sex hormones, may have a powerful influence on the cytokine expression and production of T cells. However, whether sex hormones can directly regulate cytokine gene expression of T cells remains unknown. In addition, it is intriguing why the most strikingly differentiated cytokine expression in the islets correlated with gender difference was found in young, but not in older and sexually mature NOD mice. In other words, if gender factors directly modify the cytokine gene expression of T cells, the T cells isolated from female or male NOD mice may express different cytokine profiles when they are activated regardless where and how they are activated. Therefore, *in vitro* activation experiments were performed to investigate the gender-related cytokine gene expression of T cells from both female and male NOD mice at different ages.

D.3 Examination of IFN-γ and IL-4 cytokine production by activated $CD4^+$ T cells from female and male NOD mice *in vitro*.

Analysis of the cytokine profiles of islet infiltrating cells indicated that autoreactive T cells expressed differentiated levels of IFN-γ and IL-4 in young female and male NOD mice. To determine whether gender and age related differentiation in cytokine production could be detected from *in vitro* activated $CD4^+$ T cells, lymphocytes were isolated from young (3-4-week-old) and adult (10-week-old) NOD mice of both sexes. $CD4^+$ T cells were purified from lymph node cells using magnetic beads conjugated anti-CD4 antibody. The purified $CD4^+$ T cells (Figure 7) were activated by immobilized anti-CD3 antibody in the presence of anti-CD28 antibody, which provided costimulatory signal required for T cell activation. Three different concentrations (ranging from 1.25-5μg/ml) of anti-CD3 antibody while an identical concentration of anti-CD28 antibody were used for activation. After 72-hour activation, culture supernatants were collected to determine cytokine production by ELISA analysis, and the cells were collected for RNA extraction and RT-PCR analysis. The proliferation of the activated T cells was determined by ^{3}H-thymidine incorporation. The results of proliferation assay indicated that: 1. The activation of $CD4^+$ T cells became stronger along with activation strength (Figure 8-9); 2. The $CD4^+$ T cells of female and male NOD mice were activated equally well under all different activation conditions (Figure 8-9). However, the results of ELISA clearly showed that $CD4^+$ T cells from young female NOD mice consistently produced significantly higher amount of IFN-γ but less IL-4 compared to those produced by $CD4^+$ T cells from age-matched male mice (Figure 10-11). The differences in the IFN-γ and IL-4 production by T cells from female and male young NOD mice were more pronounced when they were

Figure 7. Examination of the purity of $CD4^+$ T cells from NOD mice.

Lymphocytes were isolated from female or male NOD mice (n=3), and further purified using magnetic beads conjugated anti-CD4 antibody. The percentage of $CD4^+$ T cells before or after purification was examined by flow cytometric analysis. A. Lymphocytes (before purification); B. Purified $CD4^+$ cells. Data shown here were the representative of five independent experiments.



A.
B.
44%
96%±2%
C
TCR-FITC

Figure 8. Equal proliferation of activated $CD4^+$ T cells from 3-week-old female and male NOD mice.

$CD4^+$ T cells were isolated from 3-week-old female (n=3) and male (n=3) NOD mice. The cells (0.5×10^6 cells/well) were activated with plate-bound anti-CD3 (1.25μg/ml, 2.5μg/ml, and 5.0μg/ml) and soluble anti-CD28 (2.5μg/ml) antibodies for 72 hours. The cells were then pulsed with 0.5μCi ^3H-thymidine for additional 16 hours before harvest. As a control, $CD4^+$ T cells were cultured without antibodies. Each condition was studied in triplicate. The data shown here were the mean ± SD from three independent experiments.

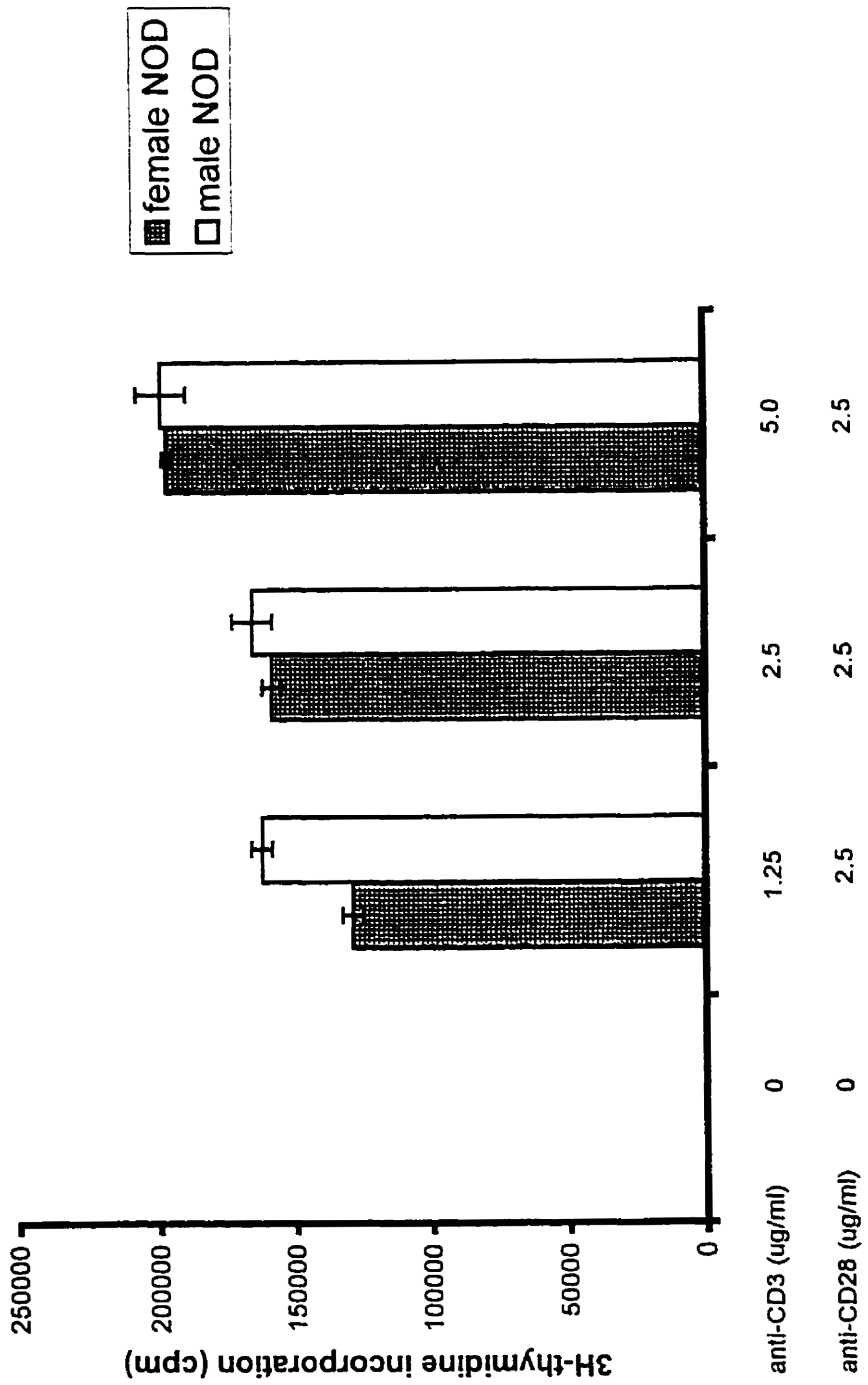
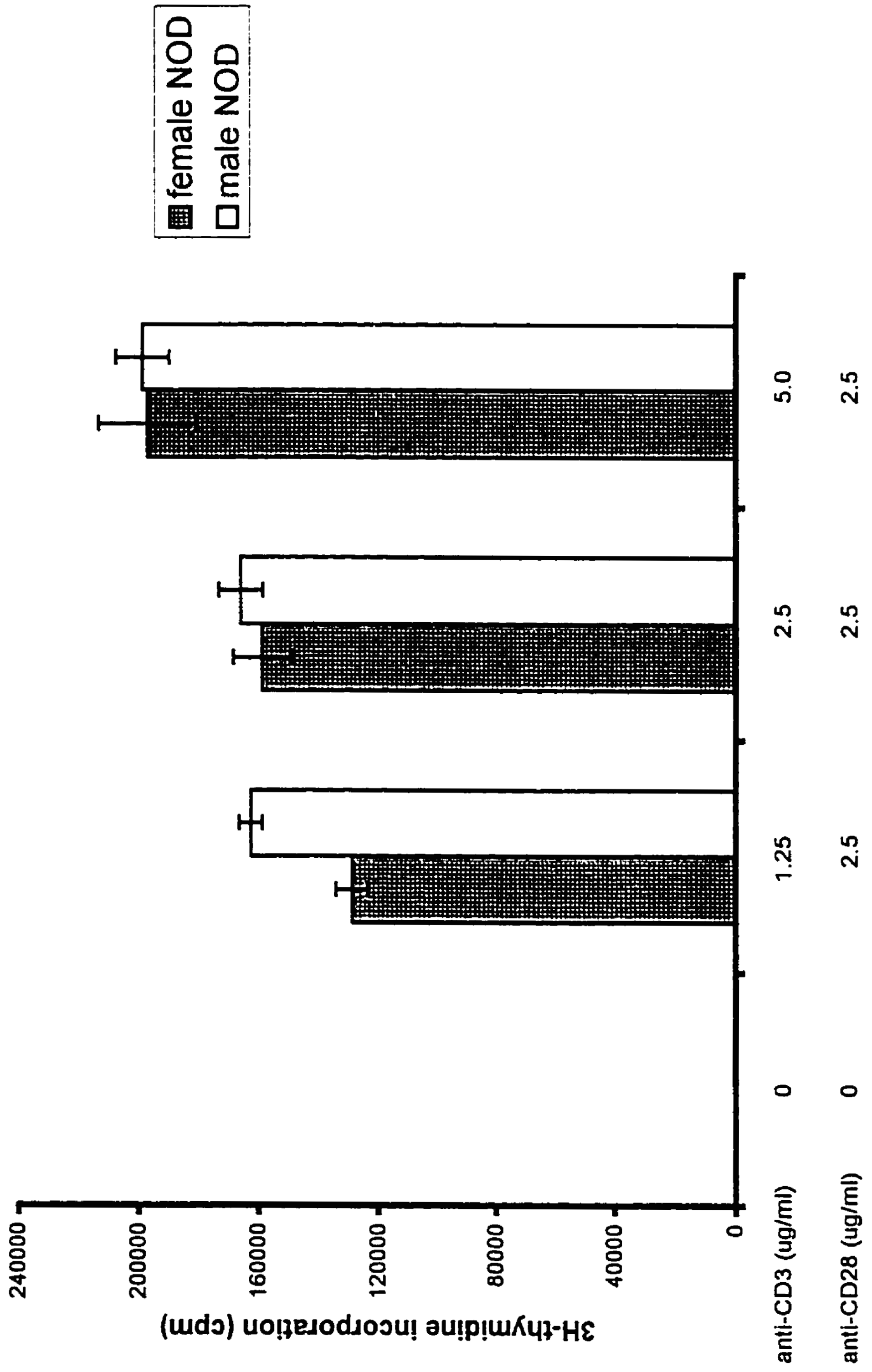

female NOD
male NOD
3H-thymidine incorporation (cpm)
250000
200000
150000
100000
50000
0
anti-CD3 (ug/ml)
anti-CD28 (ug/ml)
0
0
1.25
2.5
2.5
2.5
5.0
2.5

Figure 9. Equal proliferation of activated $CD4^+$ T cells from 10-week-old female and male NOD mice.

$CD4^-$ T cells were isolated and purified from 10-week-old female (n=3) and male (n=3) NOD mice. The cells (0.5 x 10^6 cells/well) were activated with plate-bound anti-CD3 (1.25μg/ml, 2.5μg/ml, and 5.0μg/ml) and soluble anti-CD28 (2.5μg/ml) antibodies for 72 hours and then pulsed with 0.5μCi ^{3}H-thymidine for additional 16 hours. As a control, $CD4^-$ T cells were cultured without antibodies. Each condition was performed in triplicate. The cells were harvested and the incorporation of ^{3}H-thymidine was measured. The data shown here were the mean ± SD from three independent experiments.


female NOD
male NOD
3H-thymidine incorporation (cpm)
240000
200000
160000
120000
80000
40000
0
anti-CD3 (ug/ml)
0
1.25
2.5
5.0
anti-CD28 (ug/ml)
0
2.5
2.5
2.5

activated with higher concentration of anti-CD3 antibody. The stronger the activation, the more IFN-γ and less IL-4 produced by female $CD4^+$ T cells than that produced by male $CD4^+$ T cells (Figure 10-11). $CD4^+$ T cells isolated from adult females also produced higher IFN-γ than that produced by male $CD4^+$ T cells under the same activation conditions (Figure 12-13). However, gender-related difference in the cytokine production by T cells of adult NOD mice was much less significant than that of young NOD mice (Figure 14). When the gene expression levels of IFN-γ and IL-4 in activated $CD4^+$ T cells were analyzed, a significant gender-associated differentiation of cytokine profile was easily identified in young NOD mice (Figure 15-16). Higher levels of IFN-γ (Figure 17) and lower levels of IL-4 (Figure 18) gene expression were seen in $CD4^+$ T cells of young female NOD mice compared with that in $CD4^+$ T cells of young male mice under all activation conditions (Figure 19). The cytokine gene expression in the $CD4^+$ T cells of adult NOD mice did not show a significant gender-related difference (Figure 20-23). Taken together, the analysis of cytokine gene expression strongly suggested that gender factors directly affect the transcription of cytokine genes in activated $CD4^+$ T cells from NOD mice.

D.4 Discussion

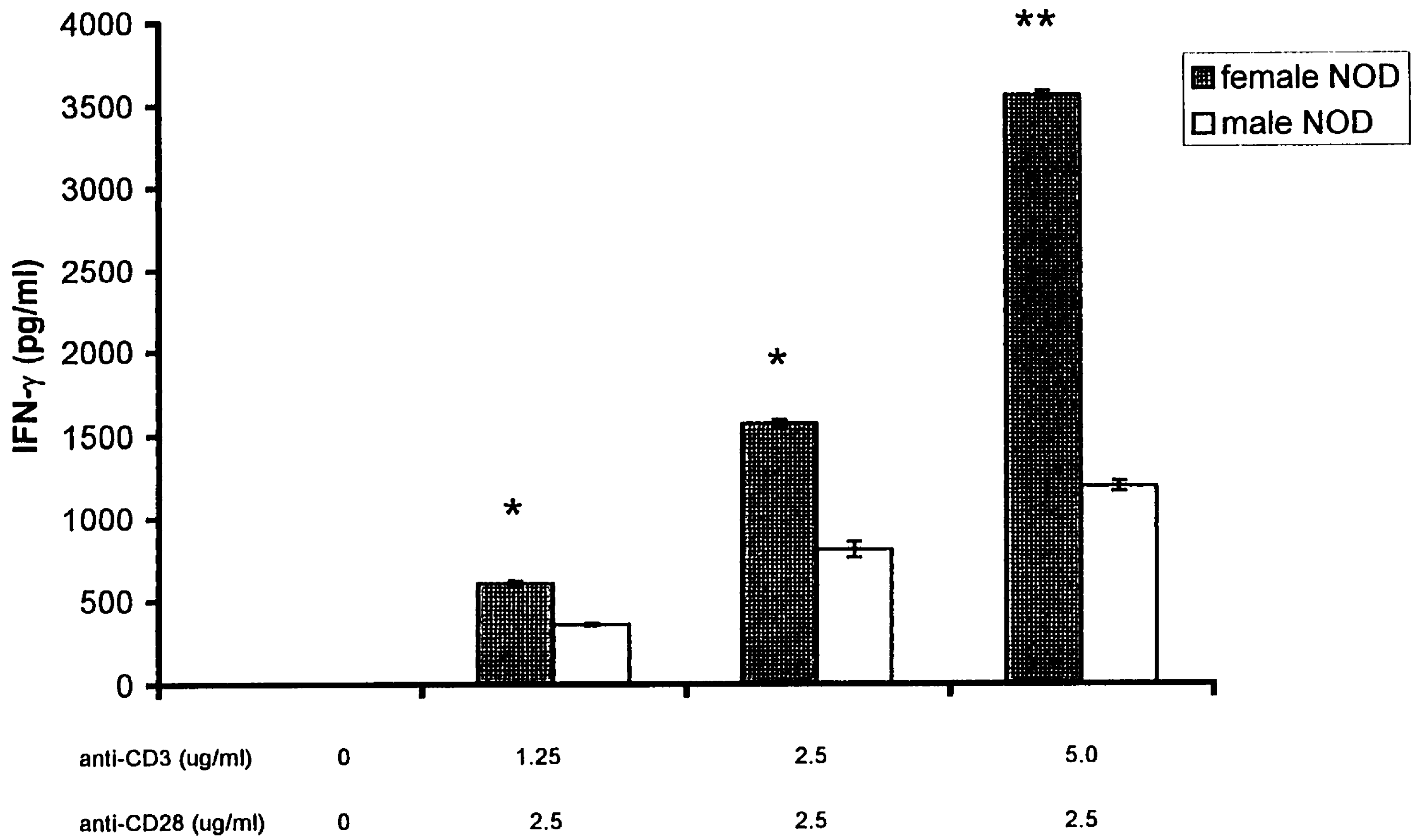
Cytokine gene expression and production by $CD4^+$ T cells upon mitogen activation is one of the most important properties of T cells. In this study, $CD4^+$ T cells were isolated and further purified from either female or male NOD mice. After activation by anti-CD3 and anti-CD28 antibodies *in vitro*, $CD4^+$ T cells from young female NOD mice (4-week-old) produced significantly higher amount of IFN-γ but less

Figure 10. IFN-γ production by activated $CD4^+$ T cells from 3-week-old female and male NOD mice.

$CD4^+$ T cells from 3-week-old female (n=3) and male (n=3) NOD mice were activated by plate-bound anti-CD3 (1.25μg/ml, 2.5μg/ml, and 5.0μg/ml) and soluble anti-CD28 (2.5μg/ml) antibodies for 72 hours. In control experiment, $CD4^+$ T cells were cultured without antibody. Each condition was performed in triplicate. The culture supernatants were collected, and the production of IFN-γ was measured by ELISA. The data shown here were the mean ± SD from three independent experiments.

* $P<0.05$ as compared with the value obtained from male $CD4^+$ T cells under the same activation condition.

** $P<0.01$ as compared with the value obtained from male $CD4^+$ T cells under the same activation condition.



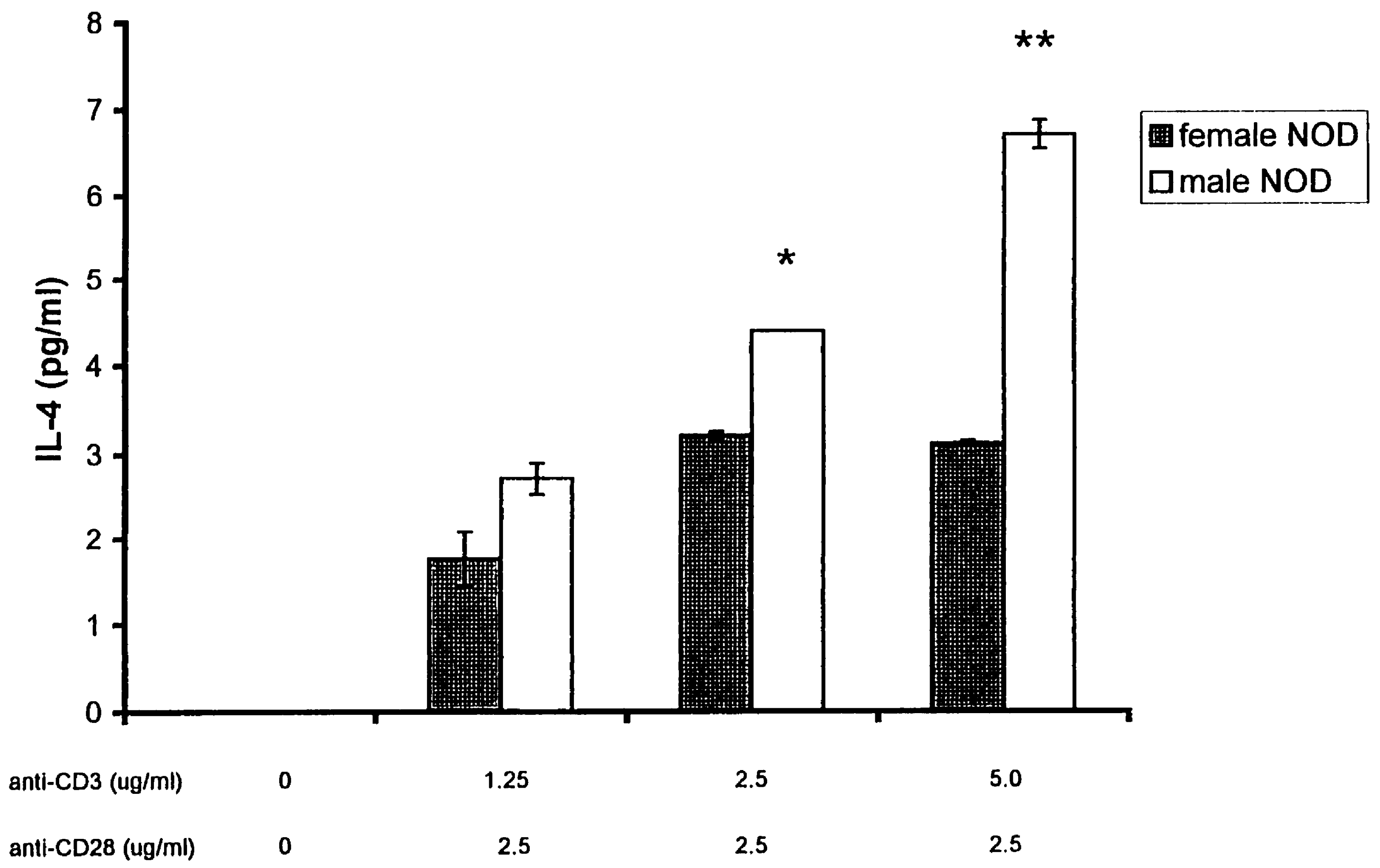
IFN-γ (pg/ml)
4000
3500
3000
2500
2000
1500
1000
500
0
*
*
**
female NOD
male NOD
anti-CD3 (ug/ml) 0 1.25 2.5 5.0
anti-CD28 (ug/ml) 0 2.5 2.5 2.5

Figure 11. IL-4 production by activated CD4$^+$ T cells from 3-week-old female and male NOD mice.

CD4$^+$ T cells from 3-week-old female (n=3) and male (n=3) NOD mice were activated by plate-bound anti-CD3 (1.25μg/ml, 2.5μg/ml, and 5.0μg/ml) and soluble anti-CD28 (2.5μg/ml) antibodies for 72 hours. As a control, CD4$^+$ T cells were cultured without antibody. Each condition was performed in triplicate. The culture supernatants were collected, and the production of IL-4 under different activation conditions was measured by ELISA. The data shown here were the mean ± SD from three independent experiments.

* $P<0.01$ as compared with the value obtained from female CD4$^+$ T cells under the same activation condition.

** $P< 0.05$ as compared with the value obtained from female CD4$^+$ T cells under the same activation condition.

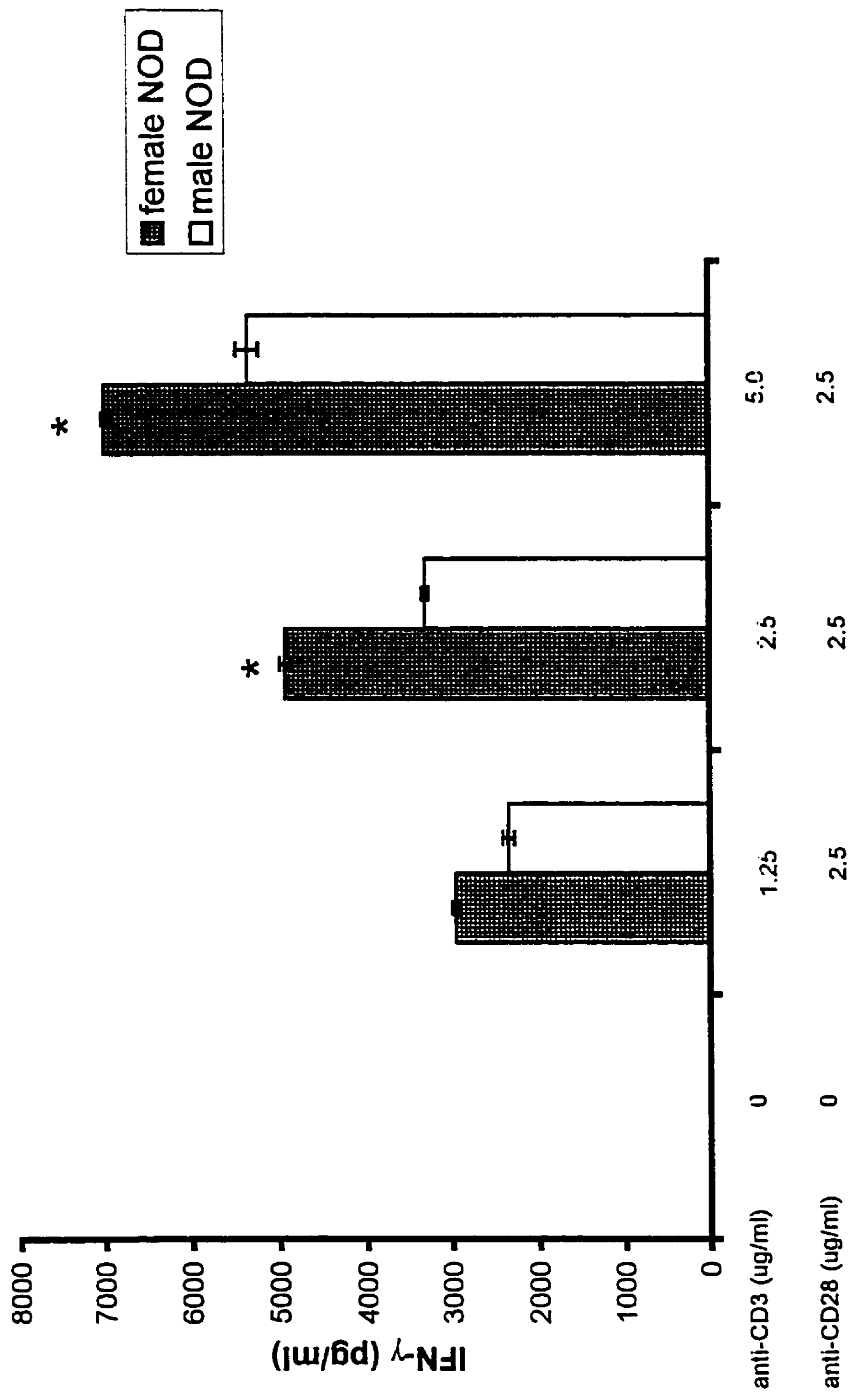


IL-4 (pg/ml)
8
7
6
5
4
3
2
1
0
**
*
female NOD
male NOD
anti-CD3 (ug/ml)
0
1.25
2.5
5.0
anti-CD28 (ug/ml)
0
2.5
2.5
2.5

Figure 12. IFN-γ production by activated $CD4^+$ T cells from 10-week-old female and male NOD mice.

$CD4^+$ T cells from 10-week-old female (n=3) and male (n=3) NOD mice were activated by plate-bound anti-CD3 (1.25μg/ml, 2.5μg/ml, and 5.0μg/ml) and soluble anti-CD28 (2.5μg/ml) antibodies for 72 hours. As a control, $CD4^+$ T cells were cultured without antibody. Each condition was performed in triplicate. The culture supernatants were collected, and the production of IFN-γ under different activation conditions was measured by ELISA. The data shown here were the mean ± SD of three independent experiments.

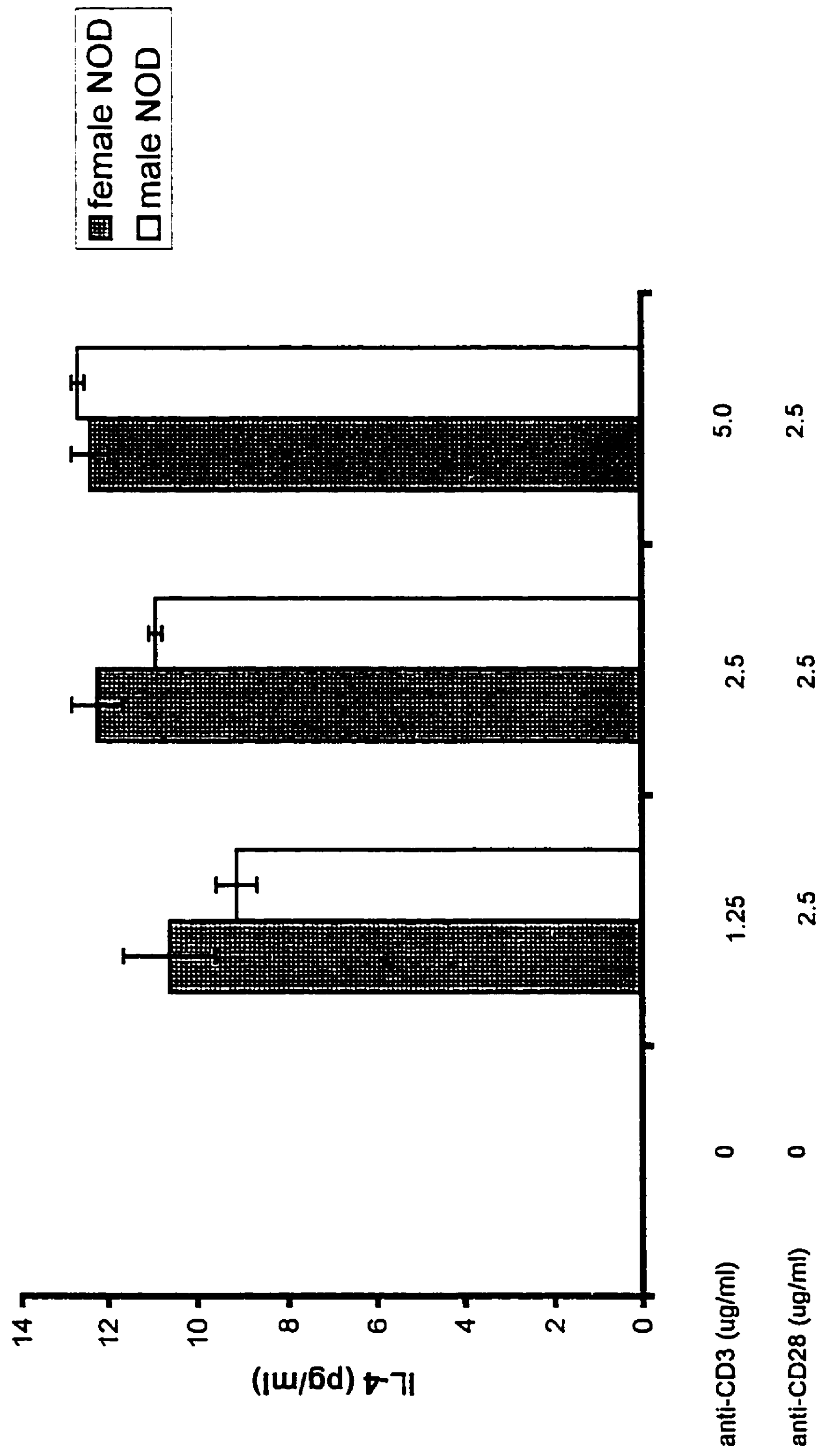
* $P<0.05$ as compared with the value obtained from male cells under the same activation condition.



female NOD
male NOD
IFN-γ (pg/ml)
8000
7000
6000
5000
4000
3000
2000
1000
0
anti-CD3 (ug/ml)
anti-CD28 (ug/ml)
0
0
1.25
2.5
2.5
2.5
5.0
2.5
*
*

Figure 13. IL-4 production by activated CD4^{+} T cells from 10-week-old female and male NOD mice.

CD4^{+} T cells from 10-week-old female (n=3) and male (n=3) NOD mice were activated by plate-bound anti-CD3 (1.25μg/ml, 2.5μg/ml, and 5.0μg/ml) and soluble anti-CD28 (2.5μg/ml) anti-bodies for 72 hours. As a control, CD4^{+} T cells were cultured without antibody. Each condition was performed in triplicate. The culture supernatants were collected, and the production of IL-4 under different activation conditions was measured by ELISA. The data shown here were the mean ± SD of three independent experiments. There was no significant difference between CD4^{+} T cells from female and male NOD mice.



female NOD
male NOD
IL-4 (pg/ml)
14
12
10
8
6
4
2
0
anti-CD3 (ug/ml)
anti-CD28 (ug/ml)
0
0
1.25
2.5
2.5
2.5
5.0
2.5

Figure 14. Analysis of the ratio of IFN-γ/IL-4 production by activated $CD4^+$ T cells from female and male NOD mice.

IFN-γ and IL-4 production by activated $CD4^+$ T cells described in Figure 10-13 are summarized as IFN-γ/IL-4 production ratio under different activation conditions and grouped as age-matched female and male NOD. The data shown here were the mean ± SD of three independent experiments.

* $P<0.05$ as compared to the value of age-matched male mice.

** $P<0.001$ as compared to the value of age-matched male mice.

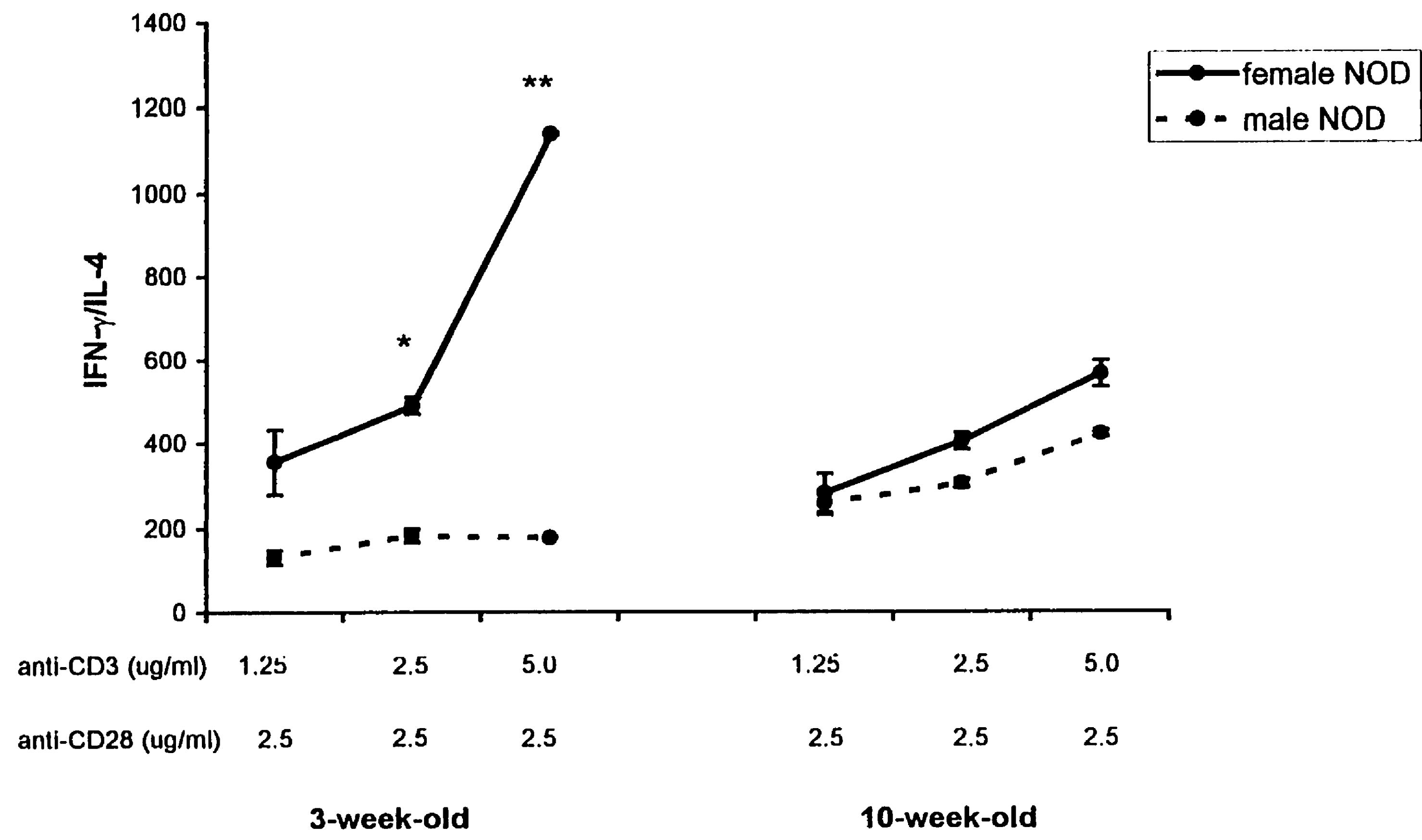

IFN-γ/IL-4
1400
1200
1000
800
600
400
200
0
**
*
female NOD
male NOD
anti-CD3 (ug/ml) 1.25 2.5 5.0 1.25 2.5 5.0
anti-CD28 (ug/ml) 2.5 2.5 2.5 2.5 2.5 2.5
3-week-old
10-week-old

Figure 15. The cytokine gene expression by activated $CD4^+$ T cells of NOD mice. $CD4^+$ T cells isolated from NOD mice were activated with plate-bond anti-CD3 (1.25μg/ml, 2.5μg/ml, 5.0μg/ml) and soluble anti-CD28 (2.5μg/ml) antibodies for 72 hrs. The total RNA was extracted from the activated $CD4^+$ T cells, and 2μg of total RNA from each sample was subjected to cDNA synthesize. Serially diluted cDNA was used in PCR reactions for IFN-γ and IL-4. As an internal control, β-actin cDNA was also amplified by PCR reaction. PCR products were analyzed on 1.5% agarose gel and visualized with ethidium bromide staining. The result showed that the yield of PCR product decreased proportionally to the diluted template. Thus, RT-PCR analysis identified different levels of cytokine gene expression. Lane M: 100bp ladder; Lane 1: 5μl of cDNA; Lane 2: 5/4μl of cDNA; Lane 3: 5/16μl of cDNA; Lane 4: 5/64μl of cDNA.

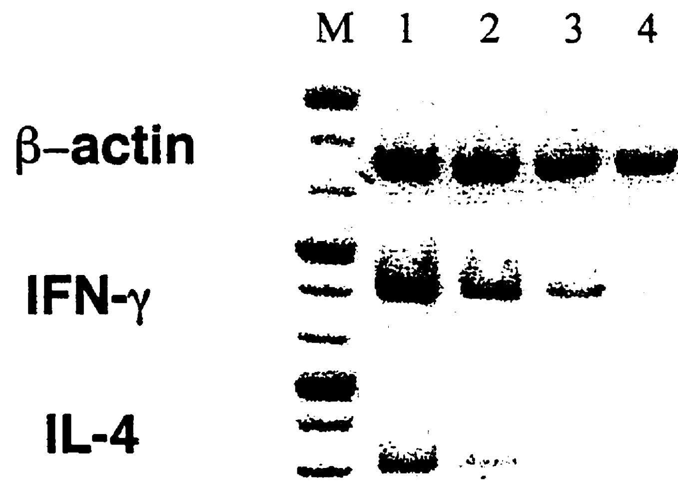

M 1 2 3 4
β–actin
IFN-γ
IL-4

Figure 16. Cytokine gene expression by activated $CD4^+$ T cells of 3-week-old female and male NOD mice.

$CD4^+$ T cells from 3-week-old female (n=3) and male (n=3) NOD mice were activated by plate-bound anti-CD3 (1.25µg/ml, 2.5µg/ml, and 5.0µg/ml) and soluble anti-CD28 (2.5µg/ml) anti-bodies for 72 hours. As a control, $CD4^+$ T cells were cultured without antibody. Total RNA was extracted from the activated $CD4^+$ T cells and 2µg RNA of each sample was subjected for cDNA synthesize. PCR reaction for IFN-γ and IL-4 were performed, and the PCR products were analyzed on 1.5% agarose gel. The representative data of three independent experiments were shown here.

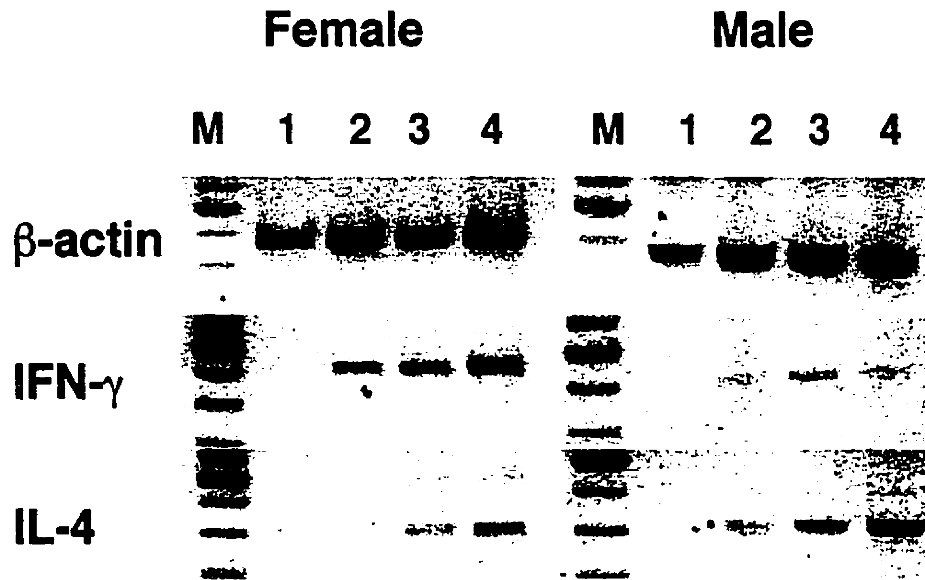

Female
Male
M 1 2 3 4
M 1 2 3 4
β-actin
IFN-γ
IL-4

Figure 17. Analysis of IFN-γ gene expression by $CD4^+$ T cells of 3-week-old female and male NOD mice.

The density of PCR products of IFN-γ and β-actin of $CD4^+$ T cells from 3-week-old NOD female (n=3) and male (n=3) mice was measured by Adobe PhotoShop software. The value of IFN-γ expression was normalized with that of β-actin. The normalized IFN-γ gene expression levels were then grouped according to the activation conditions. The data shown here were the mean ± SD of three independent experiments.

* $P<0.05$ as compared to the value of age-matched male NOD mice.

** $P<0.01$ as compared to the value of age-matched male NOD mice.

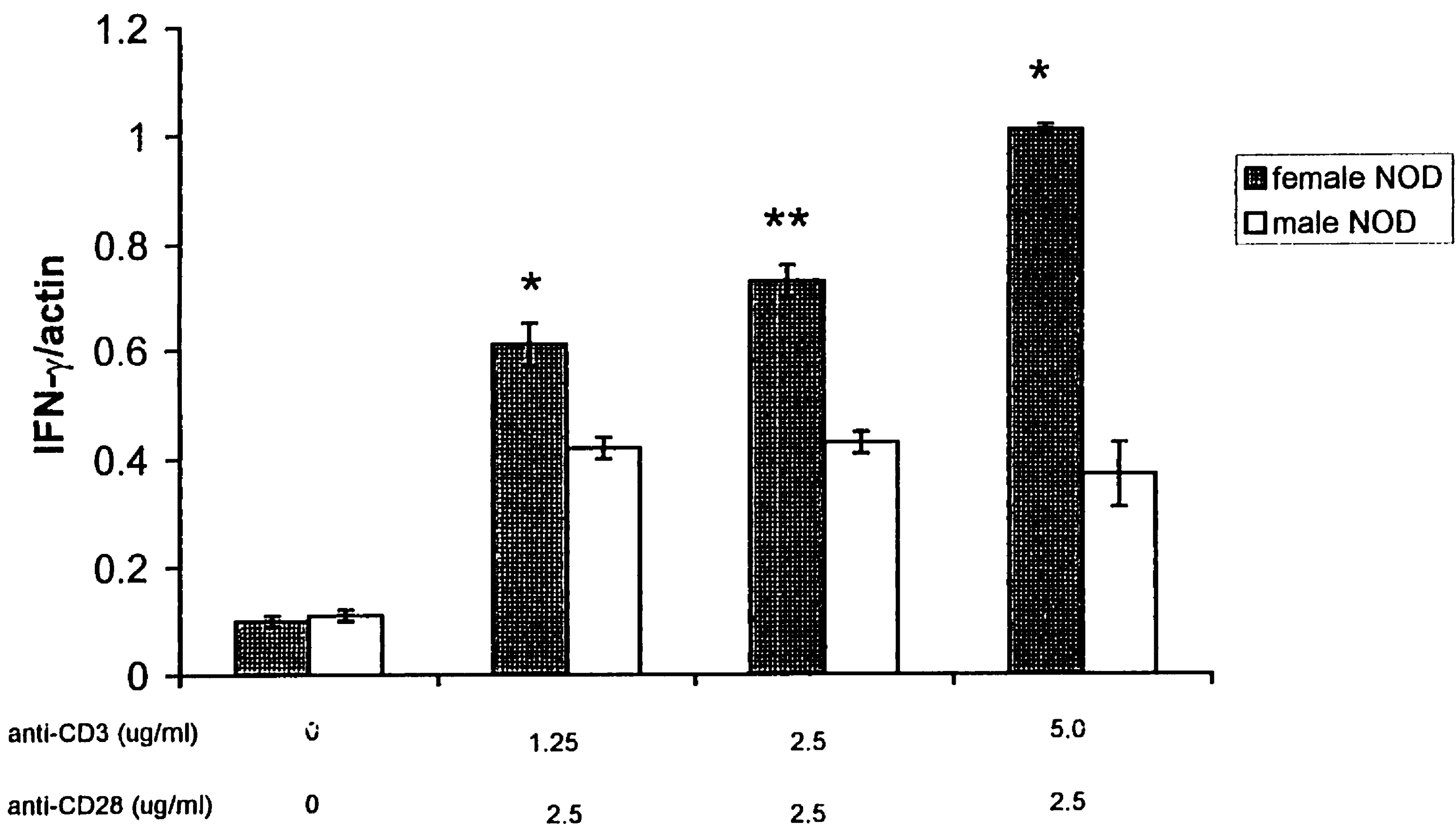

1.2
1
0.8
0.6
0.4
0.2
0
IFN-γ/actin
*
**
*
female NOD
male NOD
anti-CD3 (ug/ml)
0
1.25
2.5
5.0
anti-CD28 (ug/ml)
0
2.5
2.5
2.5

Figure 18. Analysis of IL-4 gene expression from $CD4^+$ T cells of 3-week-old female and male NOD mice.

The density of PCR products of IL-4 and β-actin of $CD4^+$ T cells from 3-week-old NOD female (n=3) and male (n=3) mice was measured by scanning and Adobe PhotoShop software. The value of IL-4 expression was normalized with that of β-actin. The normalized IL-4 gene expression levels were then grouped according to the activation conditions. The data shown here were the mean ± SD of three independent experiments.

* $P<0.001$ as compared to the value of age-matched male NOD mice.

** $P<0.05$ as compared to the value of age-matched male NOD mice.

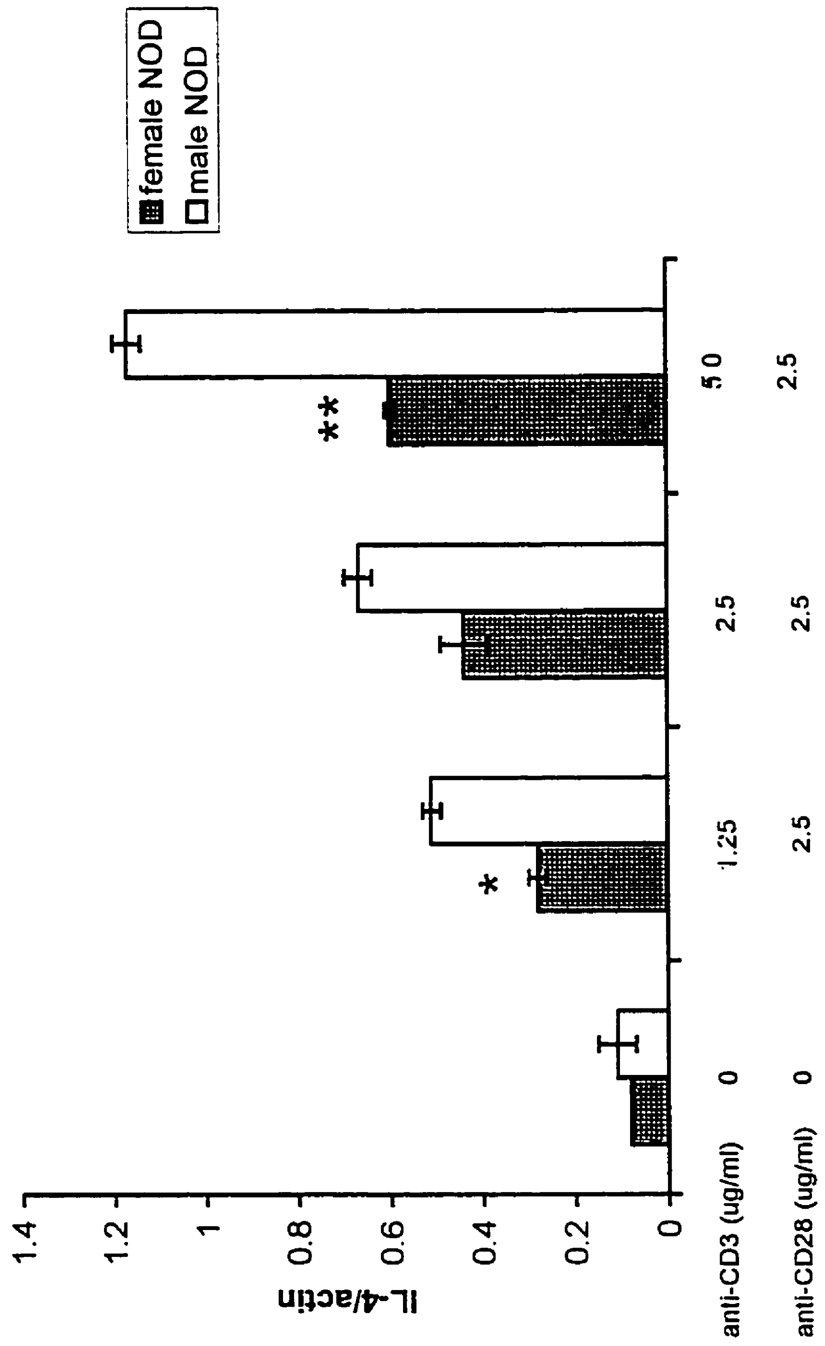

female NOD
male NOD
IL-4/actin
1.4
1.2
1
0.8
0.6
0.4
0.2
0
anti-CD3 (ug/ml) 0 1.25 2.5 5.0
anti-CD28 (ug/ml) 0 2.5 2.5 2.5
*
**

Figure 19. Analysis the ratio of IFN-γ/IL-4 gene expression by $CD4^{+}$ T cells from 3-week-old female and male NOD mice.

The ratios of IFN-γ and IL-4 gene expression level in the activated $CD4^{-}$ T cells from 3-week-old female (n=3) and male (n=3) NOD mice were compared and then grouped according to the activation conditions. The data shown here were the mean ± SD from three independent experiments

* $P<0.01$ as compared to the value of age-matched male NOD mice.

** $P<0.05$ as compared to the value of age-matched male NOD mice.

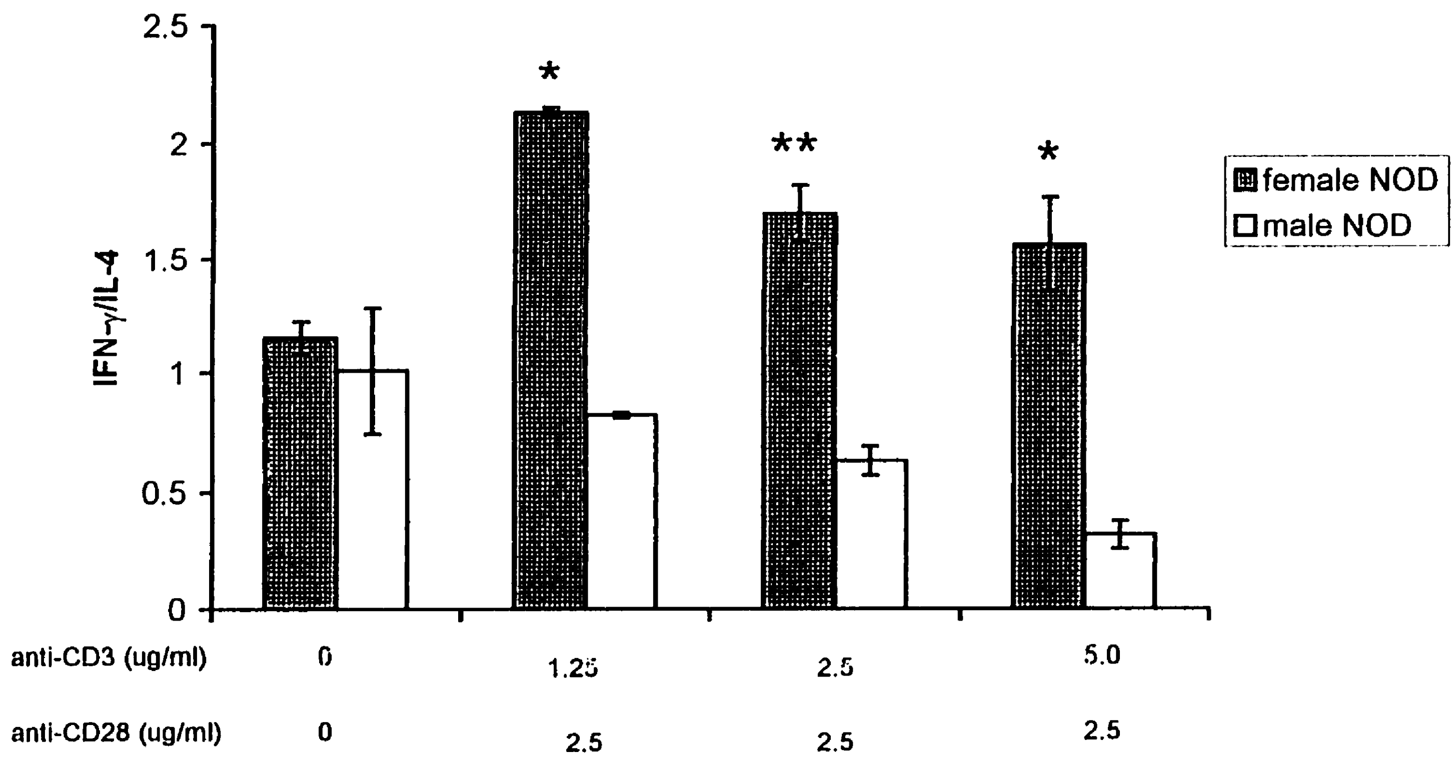
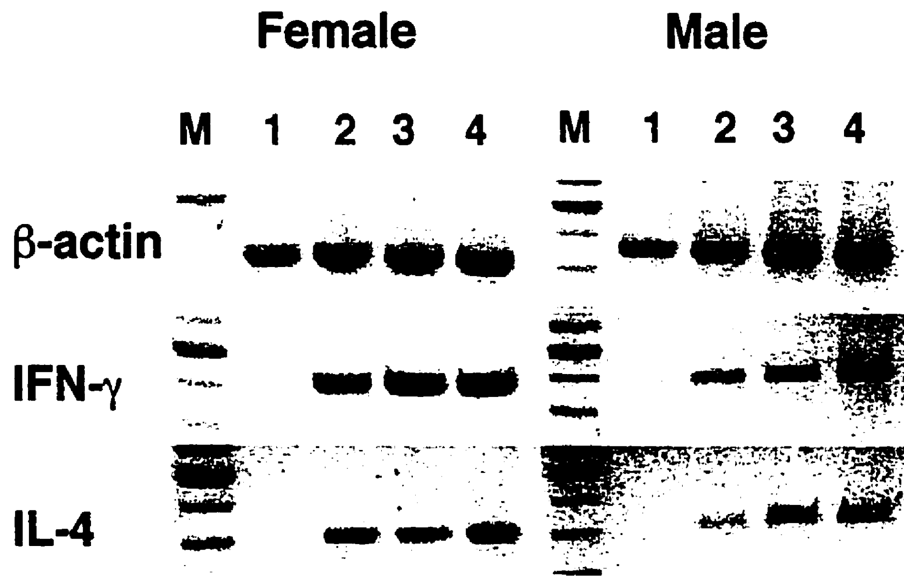

2.5
2
1.5
1
0.5
0
IFN-γ/IL-4
*
**
*
female NOD
male NOD
anti-CD3 (ug/ml)
0
1.25
2.5
5.0
anti-CD28 (ug/ml)
0
2.5
2.5
2.5

Figure 20. Cytokine gene expression of activated CD4$^+$ T cells from 10-week-old female and male NOD mice.

CD4$^+$ T cells from 10-week-old female (n=3) and male (n=3) NOD mice were activated by plate-bound anti-CD3 (1.25μg/ml, 2.5μg/ml, and 5.0μg/ml) and soluble anti-CD28 (2.5μg/ml) anti-bodies for 72 hours. As a control, CD4$^+$ T cells were cultured without antibody. Total RNA was extracted from activated CD4$^+$ T cells and 2μg RNA of each sample was subjected for cDNA synthesize. PCR reactions of IFN-γ and IL-4 were performed, and the PCR products were analyzed on 1.5% agarose gel. The representative data of three similar independent experiments were shown here.



Female
Male
M 1 2 3 4 M 1 2 3 4
β-actin
IFN-γ
IL-4

Figure 21. Analysis of IFN-γ gene expression of $CD4^+$ T cells from 10-week-old female and male NOD mice.

The density of PCR products of IFN-γ and β-actin of $CD4^-$ T cells from 10-week-old NOD female (n=3) and male (n=3) mice was measured by Adobe PhotoShop software. The value of IFN-γ expression of each sample was normalized with that of β-actin. The normalized IFN-γ gene expression levels were then grouped according to the activation conditions. The data shown here were the mean ± SD of three independent experiments.

* $P<0.01$ as compared to the value of age-matched male NOD mice.

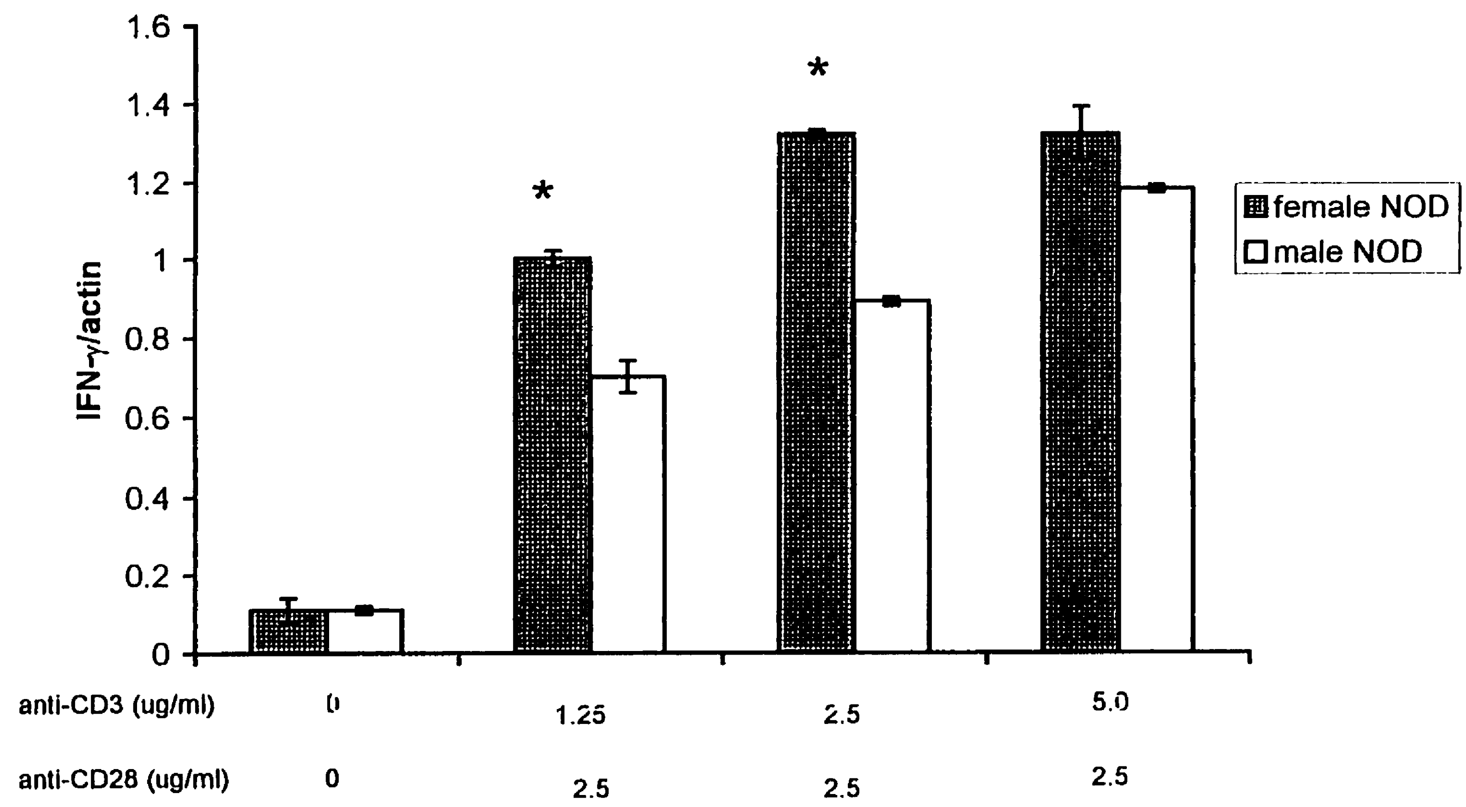
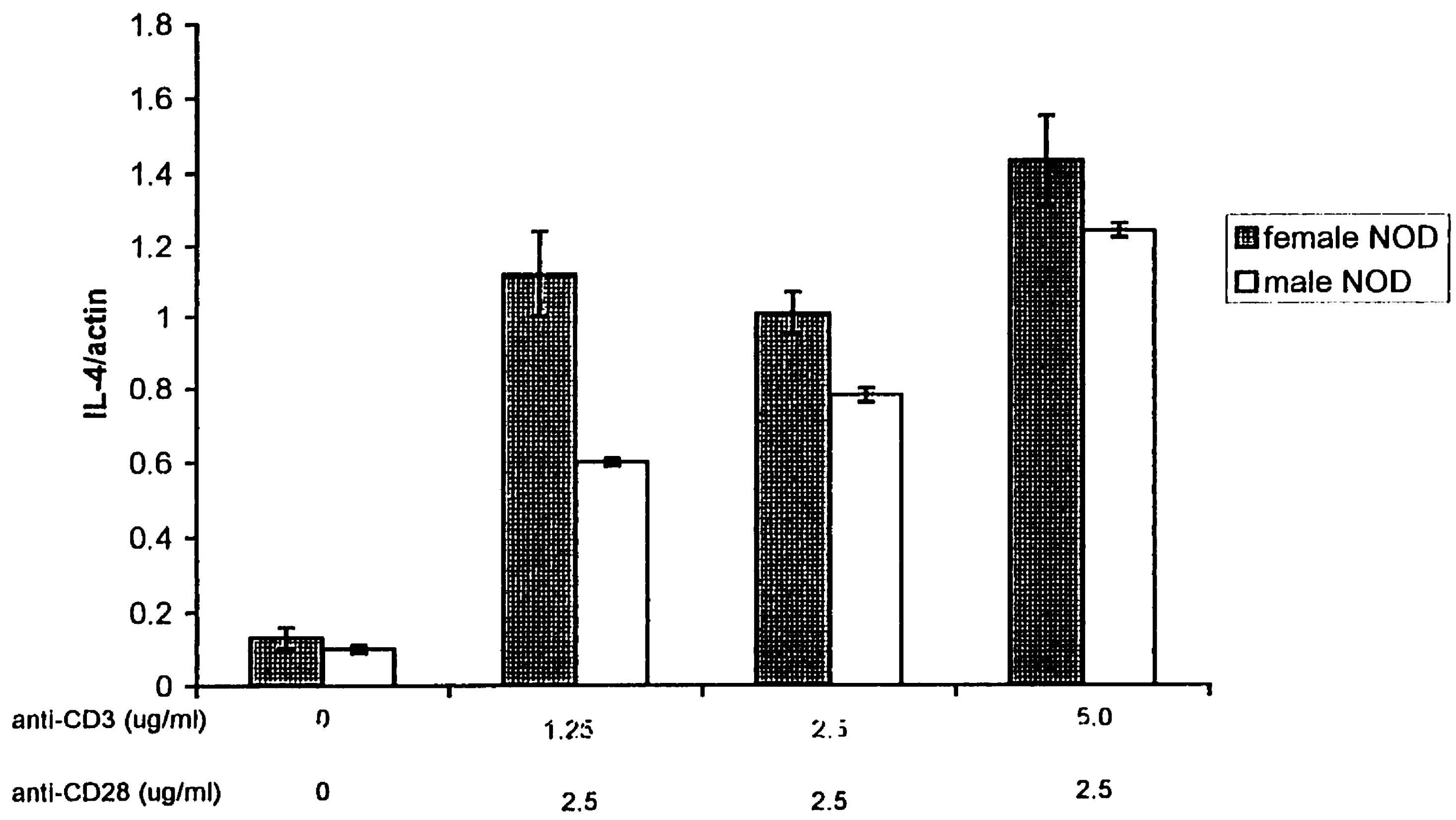

IFN-γ/actin
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
female NOD
male NOD
anti-CD3 (ug/ml)
0
1.25
2.5
5.0
anti-CD28 (ug/ml)
0
2.5
2.5
2.5

Figure 22. Analysis of IL-4 gene expression of $CD4^+$ T cells from 10-week-old female and male NOD mice.

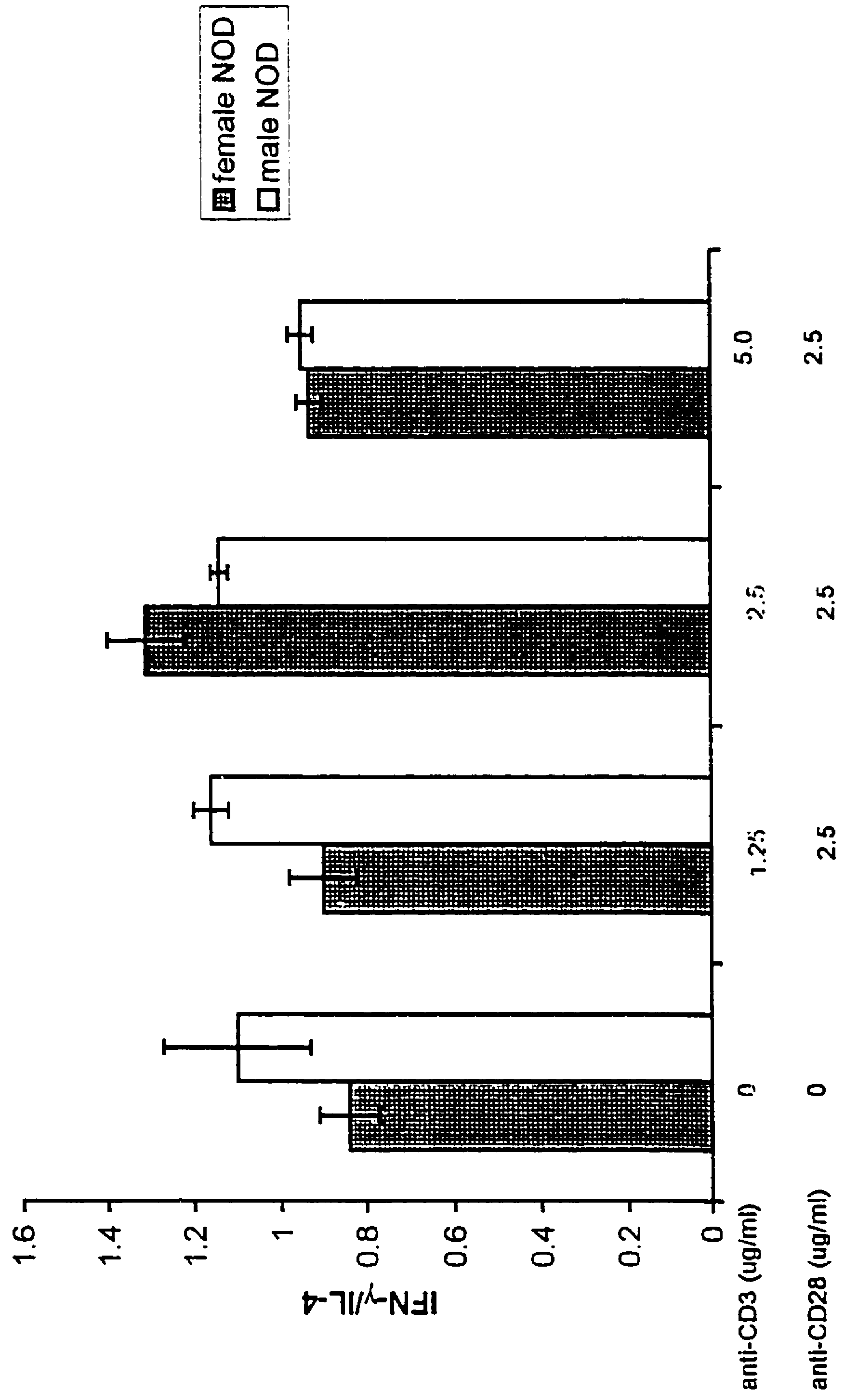
The density of PCR products of IL-4 and β-actin of $CD4^+$ T cells from 10-week-old NOD female (n=3) and male (n=3) mice was measured by Adobe PhotoShop software. The value of IL-4 expression was normalized with that of β-actin. The normalized IFN-γ gene expression levels were then grouped according to the activation conditions. The data shown here were the mean ± SD of three independent experiments. No significant difference was found between age-matched female and male mice.



1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IL-4/actin
female NOD
male NOD
anti-CD3 (ug/ml)
0
1.25
2.5
5.0
anti-CD28 (ug/ml)
0
2.5
2.5
2.5

Figure 23. Analysis the ratio of IFN-γ/IL-4 gene expression by $CD4^+$ T cells of 10-week-old female and male NOD mice.

The ratios of IFN-γ and IL-4 gene expression in the activated $CD4^+$ T cells from 10-week-old female (n=3) and male (n=3) NOD mice were compared and then grouped according to the activation conditions. The data shown here were the mean ± SD of three independent experiments. No significant difference was found between age-matched female and male mice.


female NOD
male NOD
IFN-γ/IL-4
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
anti-CD3 (ug/ml)
0
1.25
2.5
5.0
anti-CD28 (ug/ml)
0
2.5
2.5
2.5

IL-4 than the cells from age-matched male mice even when they proliferated equally well. However, there was much less significant difference between adult mice. Therefore, the results obtained from *in vitro* activation study were consistent with the analysis of cytokine gene expression from islet infiltrating cells. It was surprising and important to note that the sexual disparity on the cytokine production was much greater in young mice, in which insulitis just developed, but less in adult NOD mice. However, the sexual dimorphism of autoimmune diabetes in NOD mice only can be seen in the later stage of life. Taken together, it was suggested that gender factors had a strong effect on the T cells of young NOD mice, and this early event brought an important impact on the progress of pathogenic process. However, the important question remaining is that why a significant sexual disparity on the cytokine production was only seen in sexually immatured young, but not in adult NOD mice.

D.5 Examination of the effects of estrogen and testosterone on the function of $CD4^+$ T cells from NOD mice *in vitro*.

To investigate whether sex hormones can directly modulate the function of T cells, the gene expression of estrogen and testosterone receptors in $CD4^+$ T cells was first examined. $CD4^+$ T cells were isolated and purified from young (4-week-old) and adult (10-week-old) NOD mice of both sexes. Total cellular RNA of each group was extracted from 5×10^6 $CD4^+$ T cells, and RT-PCR was further performed using β-actin, estrogen and testosterone receptor-specific PCR primers. Gene expression of both estrogen and testosterone receptors was easily detected in $CD4^+$ T cells (Figure 24) of NOD mice regardless the age and sex. In addition, no detectable difference on the gene expression

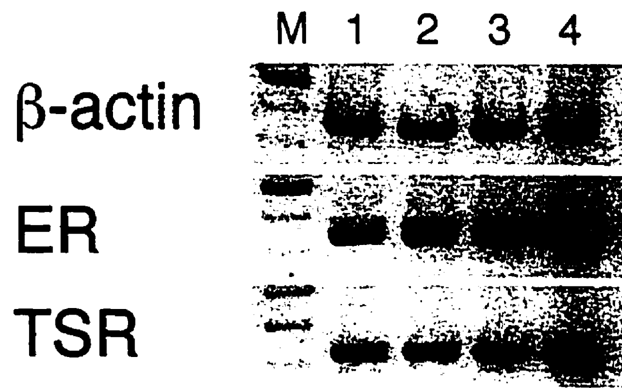
levels of hormone receptors was found between female and male mice in both age groups. This result suggested that $CD4^+$ T cells from female or male NOD mice were able to interact with both sex hormones similarly through their receptors.

To determine whether sex hormones have direct effects on the cytokine production by NOD T cells, $CD4^+$ T cells were isolated from young female and male NOD mice and activated *in vitro* with anti-CD3 and anti-CD28 antibodies in the presence of different concentrations of estrogen and testosterone. However, the presence of sex hormones did not significantly modify the cytokine production of the $CD4^+$ T cells isolated from either female or male NOD mice (data not shown). The possible explanation could be that T cells had been exposed to gender factors *in vivo* for long time, and the exposure of sex hormones *in vivo* had orientated the cytokine gene activity before T cells were isolated and activated *in vitro*. If it was the case, a prolonged culture may release sexual orientation of T cells and allow a redirection in the presence of sex hormones *in vitro*. To test this hypothesis, lymph node cells were isolated from young NOD mice of both sexes, activated with ConA for 3 days and further cultured in the presence of IL-2 for additional 7 days. $CD4^+$ T cells were harvested from culture and their response to the stimulator with or without sex hormone-treatment were further determined.

Possible effects of sex hormones on T cell proliferation were first examined. $CD4^+$ T cells were activated with anti-CD3 and anti-CD28 antibodies in the presence of estrogen or testosterone. ^{3}H-thymidine incorporation was measured after 72-hour culture. The results showed that there was no detectable difference on the proliferation between

Figure 24. The gene expression of estrogen and testosterone receptors in $CD4^+$ T cells from female and male NOD mice.

$CD4^+$ T cells were isolated and purified from 3-week-old and 10-week-old female and male NOD mice individually. Total RNA was extracted from $5x10^6$ cells of each sample, and 2μg RNA of each sample was subjected for cDNA synthesis. The PCR reactions of estrogen and testosterone receptors were performed using specific primers described in "Materials and methods". PCR products were analyzed on 1.5% agarose gel and the representative results of three independent experiments was shown here. Lane M: 100bp ladder; Lane 1: 3-week-old female NOD mice; Lane 2: 3-week-old male NOD mice; Lane 3: 10-week-old female NOD mice; Lane 4: 10-week-old male NOD mice. No significant difference of the expression levels of hormone receptors was identified by densitometric analysis among the cells isolated female and male NOD mice of different ages.


M 1 2 3 4
β-actin
ER
TSR

sex hormone-treated and non-treated cells (Figure 25). In addition, same results were obtained from the T cells isolated from female or male NOD mice regardless the age. Therefore, sex hormone-treatment had no effect on the proliferation of $CD4^+$ T cells *in vitro*.

It is known that gene expression of Th1/Th2 cytokines is activated by different signals; therefore, sex hormones may have distinct effects on the expression of individual cytokine genes through different mechanisms. Thus, I have focused on the effects of estrogen or testosterone on a representative Th1 cytokine, IFN-γ, which can be specifically induced by IL-12. Purified $CD4^+$ T cells from young female or male NOD mice were pre-activated and cultured for 10-12 days, then were rested and treated with either estrogen or testosterone for additional 20 hours before exposure to IL-12. After 48-hour IL-12 stimulation, the supernatants of cell culture were collected for the measurement of IFN-γ production by ELISA, and the activated $CD4^+$ T cells were harvested for the analysis of IFN-γ gene expression by RT-PCR. The results showed that IL-12 activation induced a strong IFN-γ production (Figure 26). And the presence of estrogen at a concentration close to physiological concentration in circulation significantly enhanced IFN-γ production. In contrast, the presence of testosterone did not enhance IFN-γ production by IL-12 induction; instead, a moderate inhibitory effect on IFN-γ production was seen. Similar to the protein production, IFN-γ gene expression induced by IL-12 was increased in the presence of estrogen, but decreased in the presence of testosterone (Figure 27-28). These results clearly showed that both estrogen and

Figure 25. The effects of estrogen and testosterone on the proliferation of $CD4^+$ T cells of NOD mice.

$CD4^+$ T cells were purified from NOD female (n=3) or male mice (n=3). The cells (0.5×10^6 cells/well) were activated with plate-bound anti-CD3 (1.25μg/ml, 2.5μg/ml, and 5.0μg/ml) and soluble anti-CD28 (2.5μg/ml) antibodies for 72 hours, with or without the treatment of E2 (25ng/ml) or TS (25ng/ml). As a control, $CD4^+$ T cells were cultured without antibody. Each condition was triplicate. The cells were further pulsed with 0.5μCi ^{3}H-thymidine for additional 16 hours, then were harvested and the incorporation of ^{3}H-thymidine was measured. Similar results were obtained from female or male NOD mice regardless the age. Therefore, the data shown here were the mean ± SD of four independent experiments including female (2 groups) and male (2 groups) NOD mice.

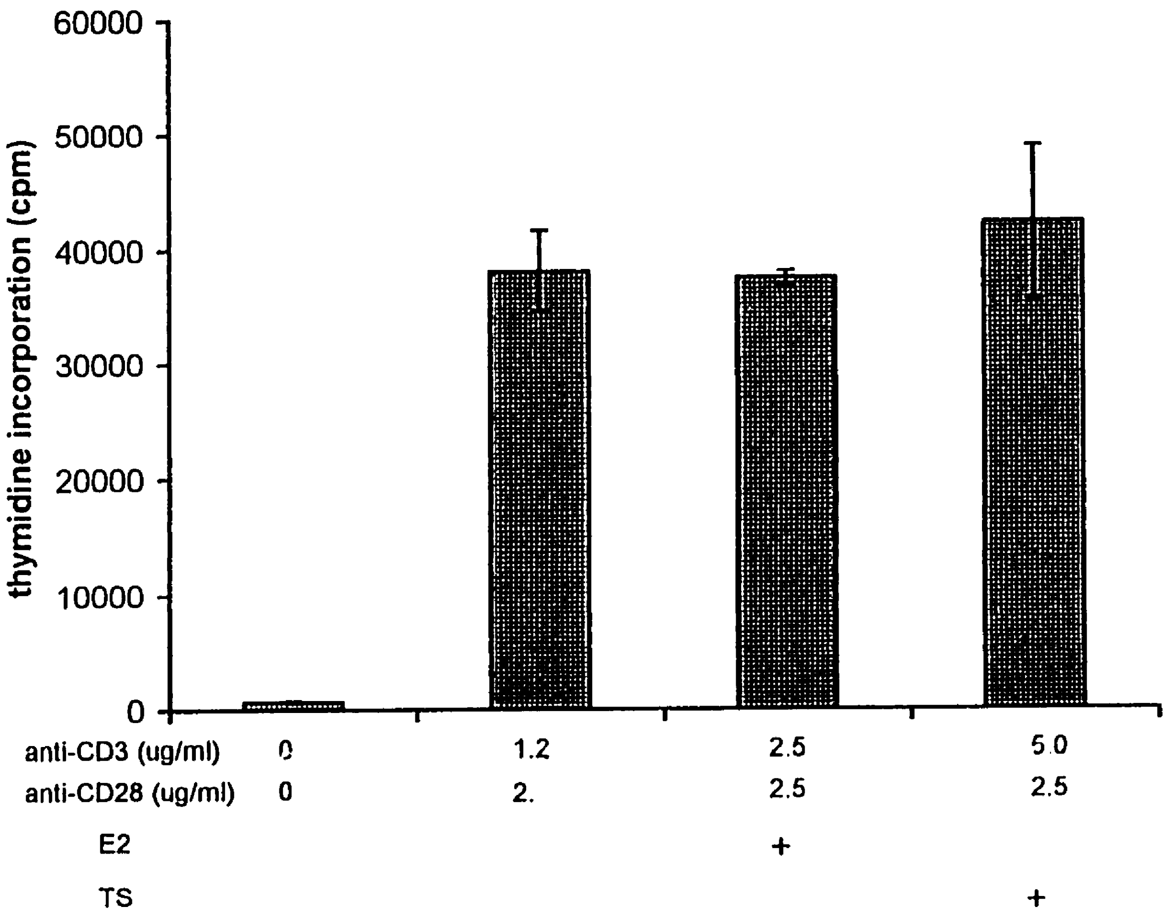
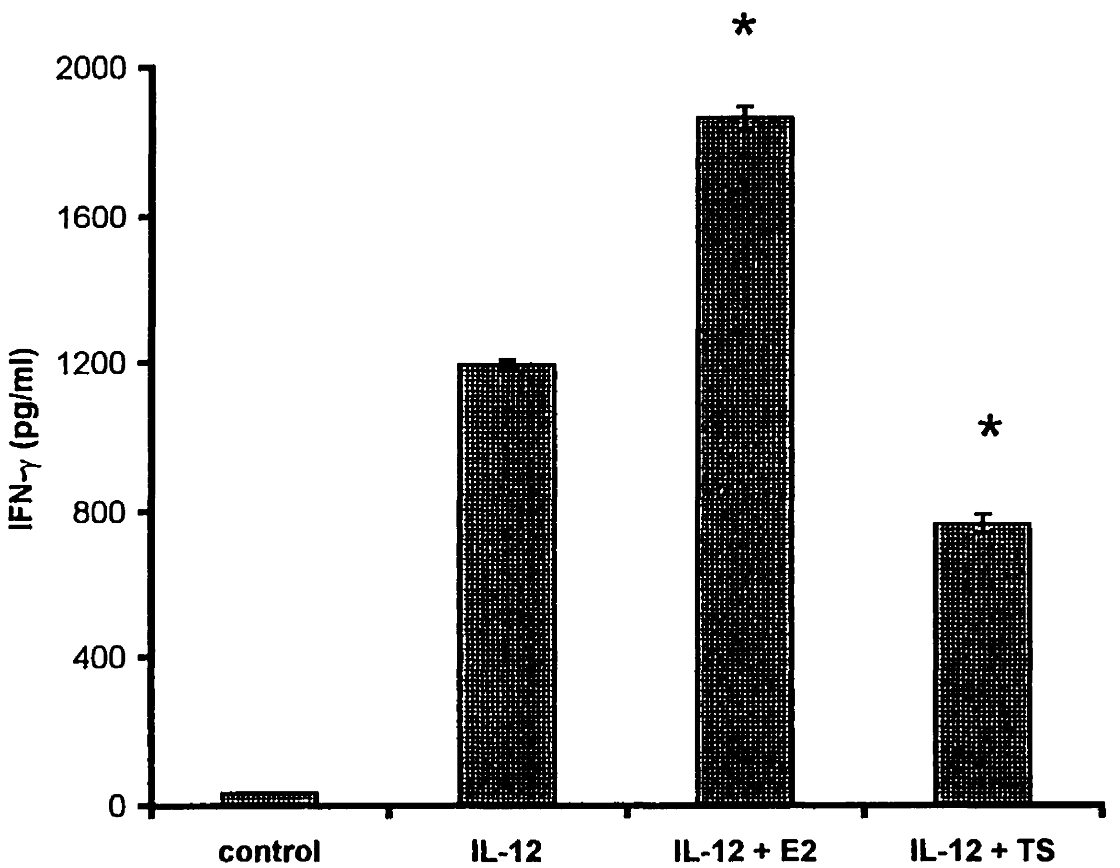

thymidine incorporation (cpm)
60000
50000
40000
30000
20000
10000
0
anti-CD3 (ug/ml) 0 1.2 2.5 5.0
anti-CD28 (ug/ml) 0 2. 2.5 2.5
E2 +
TS +

Figure 26. The effects of estrogen or testosterone on the IL-12-induced IFN-γ production by $CD4^+$ T cells from NOD mice.

Preactivated and cultured NOD $CD4^+$ T cells (4 x 10^6 cells/well in 48-well culture plate) were rested in 500μl IL-2-free culture medium and treated with or without E2 (25ng/ml) or TS (25ng/ml) for 20 hours. Then IL-12 (20ng/ml) was added to each well except the control. Each condition was performed in triplicate. After 48-hour culture, the supernatants were collected and the production of IFN-γ was measured by ELISA. Similar results were obtained from $CD4^+$ T cells of female or male NOD mice regardless the age. Therefore, the data shown here were the mean ± SD of four independent experiments including female (2 groups) and male (2 groups) NOD mice.

* $P < 0.01$ as compared to the value obtained from the condition of IL-12-treatment.


2000
1600
1200
800
400
0
IFN-γ (pg/ml)
control
IL-12
IL-12 + E2
IL-12 + TS
*
*

testosterone could directly affect IFN-γ gene expression of CD4$^+$ T cells and female and male hormones modulated the gene activity in a reciprocal ways. On the other hand, there were no detectable effects of sex hormones on the gene expression of hormone receptors in CD4$^+$ T cells (Figure 29). Interestingly, identical results were obtained from CD4$^+$ T cells isolated from either female or male NOD mice in the cytokine production and the gene expression of intra-cellular sex hormone receptors. Therefore, not only the representative data (Figure 27, 29) were shown, the results from both female and male CD4$^+$ T cells of NOD mice were pooled and further analyzed in this study (Figure 25, 26, and 28).

D.6 Discussion

It has been shown that sex hormones can modulate T cell-mediated immune responses; however, there is no clear evidence showing that T cell functions can be directly modified by sex hormones. Long-term *in vivo* administration of sex hormones redefined the pathogenic process in several T cell-mediated autoimmune disease models including the NOD mouse. Most of the *in vitro* studies were performed with T cell lines or clones. For instance, it was reported that in the presence of estrogen, most of antigen-specific human T cell clones showed a dose-dependent enhancement of IL-10 and IFN-γ secretion (Gilmore et al., 1997; Correale et al., 1998). Similarly, antigen-specific T cell lines developed in the presence of androgen produced less IFN-γ but more IL-10 than the control cell lines without androgen treatment (Bebo et al., 1999). It was also reported that short-time (8hrs) exposure of estrogen increased IFN-γ mRNA in activated splenocytes (Fox, et al., 1991). Since splenocytes contain a large numbers of

Figure 27. The effects of estrogen or testosterone on the IL-12-induced IFN-γ gene expression by $CD4^+$ T cells from NOD mice.

Pre-activated and cultured NOD $CD4^+$ T cells (4×10^6 cells/well in 48-well culture plate) were rested in 500μl IL-2-free culture medium and treated with or without E2 (25ng/ml) or TS (25ng/ml) for 20 hours. Then IL-12 (20ng/ml) was added to each well except the control. Each condition was triplicate. After 48-hour culture, $CD4^+$ T cells were harvested and total RNA was extracted from the activated $CD4^+$ T cells. Two μg RNA of each sample was subjected to cDNA synthesis. PCR reactions specific for IFN-γ and β-actin gene expression were performed using identical amount of cDNA (5μl) from each sample. Similar results were obtained from female or male NOD mice regardless the age. The data shown here were the representative of four independent experiments including female (2 groups) and male (2 groups) NOD mice. Lane M: 100bp ladder; Lane 1: untreated; Lane 2: IL-12 treatment; Lane 3: IL-12 + estrogen; Lane 4: IL-12 + testosterone.

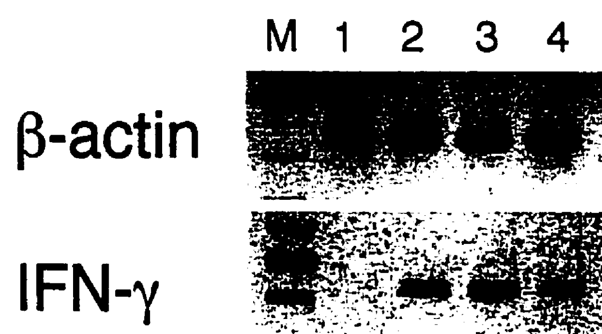
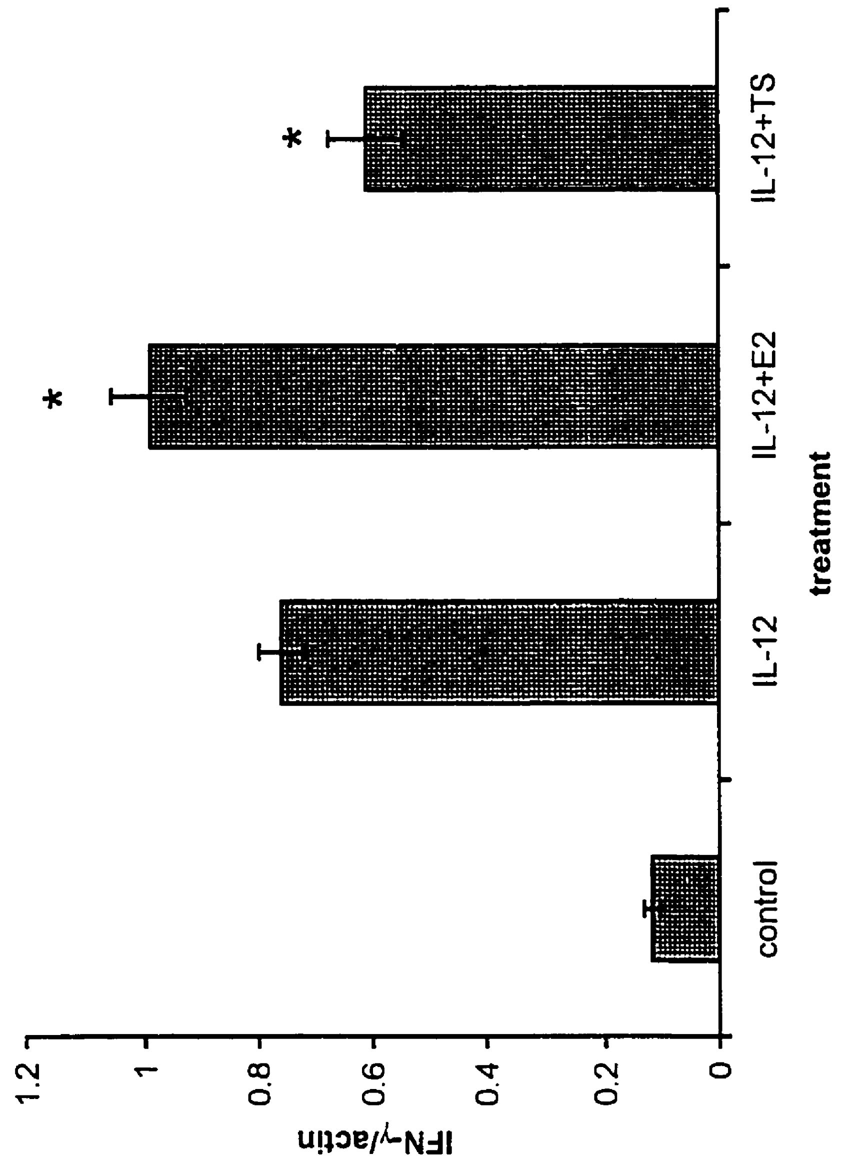

M 1 2 3 4
β-actin
IFN-γ

Figure 28. Analysis of the effects of estrogen or testosterone on the IL-12-induced IFN-γ gene expression by $CD4^+$ T cells from NOD mice.

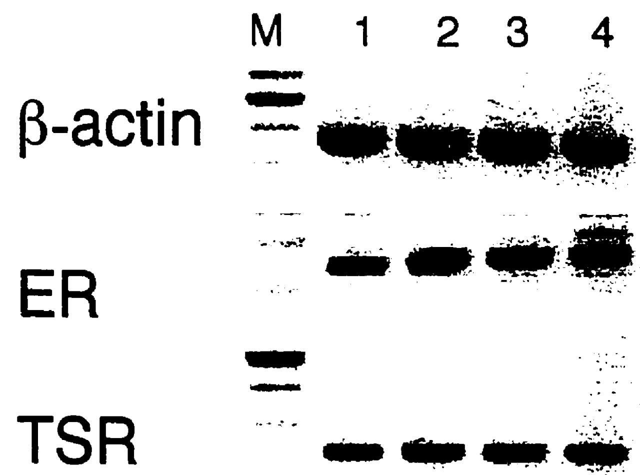
The level of IFN-γ gene expression by activated NOD $CD4^+$ T cells with or without E2 or TS treatment was normalized with β-actin gene expression measured in each sample. The data shown here were the mean ± SD of four independent experiments including female (2 groups) and male (2 groups) NOD mice.

* $P<0.01$ as compared to the value obtained from the condition of IL-12-treatment.



IFN-γ/actin
1.2
1
0.8
0.6
0.4
0.2
0
control
IL-12
IL-12+E2
IL-12+TS
treatment
*
*

Figure 29. The effects of estrogen or testosterone on the gene expression of sex hormone receptors on the activated CD4^{+} T cells from female and male NOD mice. Purified CD4^{-} T cells from NOD female (n=3) or male mice (n=3) were pretreated with E2 (25ng/ml) or TS (25ng/ml) for 20 hours in IL-2-free culture medium. Then IL-12 (20ng/ml) was added to each well except the control. After 48-hour culture, the activated CD4^{-} T cells were harvested for cellular RNA extraction. cDNA was synthesized from 2μg of total RNA of each sample. The PCR reactions specific for estrogen or testosterone receptors were performed using the primers described in "Materials and methods". Similar results were obtained from female or male NOD mice regardless the age. The data shown here were the representative of four independent experiments including female (2 groups) and male (2 groups) NOD mice. Lane M: 100bp ladder; Lane 1: untreated; Lane 2: IL-12 treatment; Lane 3: IL-12 + estrogen; Lane 4: IL-12 + testosterone.


M
1
2
3
4
β-actin
ER
TSR

macrophages, which may produce a large amount of IFNγ shortly upon activation. Therefore, it was hard to distinct the target cell populations of sex hormones in that study. In this study, I first examined sex hormone receptors in $CD4^+$ T cells isolated from NOD mice. The detection of the similar levels of mRNA of estrogen and testosterone receptors in $CD4^+$ T cells from either female or male NOD mice at different ages indicated that $CD4^+$ T cells could be the targets for the action of sex hormones. Furthermore, the equal proliferation between the sex hormone-treated and untreated $CD4^+$ T cells suggested that there was no direct effect of sex hormones on the proliferation of $CD4^+$ T cells from either female or male NOD mice.

To examine the direct effects of sex hormones on the cytokine production by T cells, I initially used freshly isolated $CD4^+$ T cells from NOD mice. Purified $CD4^+$ T cells were activated with or without sex hormones, but I was unable to identify a significant effect of either estrogen or testosterone on IFN-γ or IL-4 production, regardless of the gender and the age of the animals. However, when T cells were pre-activated, cultured and rested, the exposure to sex hormones could significantly modify the IFN-γ gene expression and production. In the presence of estrogen, $CD4^+$ T cells produced more IFN-γ; while in the presence of testosterone, the amount of IFN-γ induced by IL-12 was reduced. This result provided clear evidence that both estrogen and testosterone could directly modulate IFN-γ gene expression and production by $CD4^+$ T cells from NOD mice. This result also suggested that pre-culturing of the $CD4^+$ T cells was required for *in vitro* study of sex hormone-mediated immunomodulation. It has been shown that the structure of IL-4 gene containing chromosomal change during cell

divisions, which was required for IL-4 gene activation (Bird et al., 1998). Therefore, the preculture conditions might have changed the structure of the chromosomal fragment containing the cytokine genes. As a result, the genes may become more accessible for the signals from sex hormones. Another possibility is that the preculture condition is required to allow the cells to become responsive to sex hormones again if there was saturation of hormone receptors during long-term exposure to sex hormones *in vivo*.

In addition, since identical results of sex hormone-treatment experiments were obtained from the $CD4^-$ T cells isolated from either female or male NOD mice, it was clearly indicated that the $CD4^-$ T cells from NOD mice of both sexes could be modified similarly by female or male hormone.

IFN-γ gene expression can be regulated by sex hormones. This result is supported by a previous study showing that estrogen could augment the activity of the IFN-γ promoter (Fox et al., 1991). However, the mechanisms by which sex hormones regulate $CD4^-$ T cell function remain unclear. It is likely that the signals from sex hormones may influence the activities of some transcription factors required for cytokine gene expression, and thus exert the effects on gene expression.

D.7 The influence of estrogen and testosterone on the IL-12-induced phosphorylation of Stat4 in $CD4^+$ T cells from NOD mice.

IL-12 is a potent and specific inducer for Th1 cytokine gene, especially IFN-γ. It is known that IL-12 signaling triggers rapid phosphorylation of transcription factor Stat4, which is required for the activation of IFN-γ gene. Therefore, I next examined whether sex hormone-treatment could influence the phosphorylation level of Stat4 induced by IL-

12. Purified $CD4^+$ T cells were pretreated with estrogen or testosterone for 20 hours, and further induced by IL-12 for 20 minutes. The cells were then lysed and precipitated by anti-Stat4 antibody and subjected to immunoblot with anti-phosphotyrosine and anti-Stat4 antibodies. Interestingly, the results showed that the IL-12-induced phosphorylation of Stat4 was enhanced by estrogen pretreatment; whereas the testosterone-treatment inhibited the phosphorylation level of Stat4 (Figure 30). Similar results were obtained from the $CD4^+$ T cells of either female or male NOD mice. Therefore, the representative figure from the results of female and male mice was presented in this study (Figure 30)

D.8 Discussion

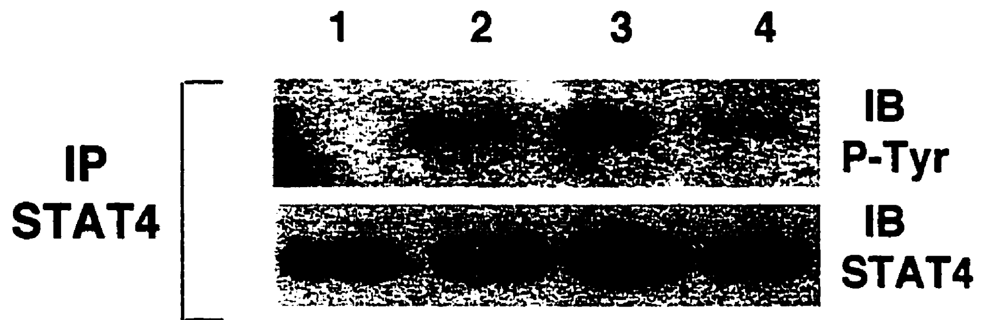
Recent studies have revealed that transcription factor Stat4 is a key molecule in the intracellular signal transduction cascade activated by IL-12. Deficiency in Stat4 signaling pathway causes defects in IFN-γ production, NK cell activity, IL-12- mediated proliferation, and Th1 differentiation (Thierfelder et al., 1996; Kapan et al., 1996). In the response to IL-12, Stat4 is phosphorylated and translocated into the nucleus and further activates genes that presumably promote Th1 differentiation (Bacon et al., 1995; Jacobson et al., 1995). Therefore, phosphorylation of Stat4 is a critical step for Th1 cytokine gene, such as IFN-γ gene activation and expression.

The results of this study showed that the treatment of sex hormones could enhance or decrease, depending on the sex hormone used, the IL-12-induced phosphorylation of Stat4. In addition, the phosphorylation of Stat4 in CD4+ T cells from female or male NOD mice may be modulated similarly by sex hormones. Therefore, this

study provides a strong evidence of the mechanisms by which sex hormones influence the cytokine gene expression. More importantly, the result of this study may lead us to understanding of the molecular basis of sexual dimorphism in autoimmune diabetes in the NOD mouse model.

Figure 30. The effects of estrogen or testosterone on the IL-12-induced phosphorylation of Stat4 in $CD4^+$ T cells from NOD mice.

Purified $CD4^+$ T cells (50×10^6) from female (n=3) or male NOD (n=3) mice were pretreated with E2 (25ng/ml) or TS (25ng/ml) for 20 hours in IL-2-free medium. Then, the cells were resuspended in 1ml medium, and IL-12 (20ng/ml) was added to each well except the control, which had no treatment. After 20-minute incubation, $CD4^+$ T cells were harvested, lysed for protein extraction. Five hundred μg protein of each sample was used for immunoprecipitation with anti-Stat4 antibody. The precipitated proteins were analyzed on 10% SDS PAGE gel and subjected to immunoblot with anti-phosphotyrosine antibody followed by immunoblotting with anti-Stat4 antibody. Lane 1: untreated; Lane 2: IL-12 treatment; Lane 3: IL-12 + estrogen; Lane 4: IL-12 + testosterone. Similar results were obtained from female or male NOD mice regardless the age. The data shown here were the representative of three independent experiments including female (2 groups) and male (1 group) NOD mice.


1
2
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STAT4

E. OVERALL DISCUSSION AND FUTURE PLAN

As an animal model for human IDDM, the NOD mouse spontaneously develops a diabetic syndrome similar to that of the human disease. Although multiple cell types are involved, the development of diabetes in NOD mice is thought to be a result of T cell-mediated destruction of pancreatic β cells. Moreover, accumulating evidence supports that Th1/Th2 cytokines produced by $CD4^+$ T cells play a key role in the pathogenesis of IDDM in NOD mice. Nowadays, a widely held belief is that Th1 cytokines, especially IFN-γ, are pathogenic; while IL-4, a representative Th2 cytokine, is protective against the disease development.

The prevalence of diabetes in NOD mice exhibits a clear female preponderance. Similar sexual dimorphism has also been observed in many other autoimmune diseases, including SLE, RA, and MS. This female preponderance could have several explanations. For instance, sex-correlated differences in chromosome structure may affect gene regulation in the immune system; sex hormones may exert a strong influence on the immune, neuronal and endocrine systems, or a combination of multiple factors elevates the level of immune response in females. Several studies have suggested that the female's high susceptibility to autoimmune diseases, at least in part, be due to the effects mediated by sex hormones. The involvement of sex hormones in the pathogenesis of autoimmune diseases was evidenced in the experiments involving sex hormone administration (Fox et al., 1992, Dalal et al., 1997). The detection of sex hormone receptors in immune cells, such as T cells and macrophages (Bebo et al., 1999), provides additional support for the roles of sex hormones in autoimmune diseases. Furthermore, studies of T cell clones (Gilmore et al., 1997; Gorreale et al., 1998) strongly suggested

that estrogen or testosterone might have selective effects on cytokine production by T cells. Whether sexual dimorphism in autoimmune diabetes in NOD mice is due to sex hormone-modified Th1/Th2 cytokine expression by T cells remains a question, and the mechanisms involved are currently unknown.

To understand the potential roles of sex hormones in the sexual dimorphism of autoimmune diabetes in NOD mice, I have studied whether sex hormones modulate the cytokine profiles produced by activated $CD4^+$ T cells. If sex hormones can influence the function of $CD4^+$ T cells and subsequently affect the pathogenic progress of autoimmune diabetes, we should be able to detect a difference in cytokine profiles produced by activated $CD4^+$ T cells between female and male NOD mice. In addition, we may potentially be able to alter the cytokine profile produced by female or male $CD4^+$ T cells by *in vitro* treatment with sex hormones. To test these hypotheses, I first conducted *in vivo* and *in vitro* experiments to examine the cytokines produced by the activated T cells of NOD mice. For *in vivo* experiments, I examined cytokine gene expression in the islets isolated from female and male NOD mice since these islets contained activated, autoantigen-specific T cells. For *in vitro* experiments, I isolated $CD4^+$ T cells from either female or male NOD mice, activated these cells by TCR engagement and then determined cytokine profiles produced by the activated $CD4^+$ T cells. Because autoimmune diabetes in NOD mice is a chronic disease, sex hormones may affect the pathogenic progress through long-term exposure, or sex hormones may input their influence at certain critical stage during the development of autoimmune diabetes. To elucidate the mechanisms by which sex hormones mediate pathogenic dimorphism, I

conducted multi-point experiments to determine whether there is any difference in cytokine production by activated T cells isolated from NOD mice at different ages from both sexes.

The results from both the *in vivo* and *in vitro* experiments showed a clear gender-associated difference in Th1/Th2 cytokine gene expression by activated T cells from NOD mice. Semi-quantitative RT-PCR analysis revealed differential IFN-γ and IL-4 gene expression patterns between female and male NOD mice. The majority of infiltrating immunocytes in insulitic lesions are T cells, and the gene expression of IFN-γ, especially IL-4 is mainly from activated $CD4^+$ T cells. Therefore, the results of RT-PCR from islets indicated that islet antigen-specific $CD4^+$ T cells from female NOD mice expressed higher levels of IFN-γ and lower levels of IL-4 than that of $CD4^+$ T cells from age-matched male islets. These results are consistent with previous studies in which higher expression level of IFN-γ gene in insulitic lesions of female NOD mice was also identified (Rabinovitch et al., 1995, Fox et al., 1997). The gene expression levels of both IFN-γ and IL-4 increased along with age. Presumably, the increase of cytokine gene expression is mainly due to the increase of islet antigen-specific T cells recruited and activated in the islets of older NOD mice. However, when the IFN-γ/IL-4 expression ratio was examined, it was found that the most striking gender related difference in IFN-γ/IL-4 ratio was detected in the islets of young NOD mice, when insulitis just started.

The analysis of insulitis indicated that autoreactive $CD4^+$ T cells from female and male NOD mice produced different cytokine profiles, and the differentiated cytokine production by $CD4^+$ T cell started at an early life stage of NOD mice. It is unclear why T

cells from young female and male NOD mice demonstrated the most significant difference in cytokine production. Since mice start to enter puberty at 3-4 weeks of age, sex hormone receptors in immune cells must start to interact with sex hormones in the circulation at this time. The initial exposure to sex hormones may have powerful influence on T cell function. However, when sex hormone receptors were saturated in the cells of adult mice, the effects of sex hormones may not be as strong as that occurring at the initial interaction. If this is the case, it was expected to detect the highly differentiated cytokine profiles produced by CD4$^+$ T cells from young NOD mice, but not from adult mice. To test this hypothesis, I next isolated and purified CD4$^+$ T cells from both young and adult NOD mice of both genders, and examined Th1/Th2 cytokine expression following activation by TCR ligation *in vitro*. The hypothesis was confirmed by the *in vitro* experiments of activation of CD4$^+$ T cells purified from young and adult NOD mice. ELISA analysis showed that CD4$^+$ T cells from adult (10 weeks of age) NOD mice produced higher level of both IFN-γ and IL-4 than that of CD4$^+$ T cells from young (3 weeks of age) NOD mice. However, when the IFN-γ/IL-4 ratio was examined, CD4$^+$ T cells from young female mice had the highest ratio while the cells from young male NOD mice had the lowest one although these CD4$^+$ T cells proliferated equally well. The results of RT-PCR analysis similarly showed gene expression patterns consistent with the results of the ELISA assay. Taken together, the results of present study demonstrated that CD4$^+$ T cells from female and male NOD mice differentiated significantly in respect of IFN-γ and IL-4 expression and production from a very young

age, although the differences diminished during later life stages when these T cells produced cytokines massively.

The finding of differential cytokine expression and production by CD4⁺ T cells of young female and male NOD mice is unexpected but may be important since the balance between IFN-γ/IL-4 expression is critical to the pathogenic progress in NOD mice. It is well known that the presence of IFN-γ during T cell activation strongly induces Th1 differentiation; whereas IL-4 induces Th2 immune response. Therefore, in young female NOD mice, cytokine expression in insulitis is relatively low, but the high IFN-γ/IL-4 ratio (high level of IFN-γ and low level of IL-4) induces more islet-specific T cells to differentiate into Th1 cells. IFN-γ is directly toxic to pancreatic β cells, and it also activates macrophages to release free radicals that may damage β cells. More importantly, IFN-γ enhances the maturation of autoantigen-specific cytotoxic T cells and subsequently these CTLs kill β cells through different mechanisms (Fig. 31). Conversely, a relative low IFN-γ/IL-4 ratio (less IFN-γ but more IL-4) in the islets of young male NOD mice promotes Th2-biased differentiation of islet infiltrating T cells, and the Th2-biased immune response induced in the islets of young male mice slows down the pathogenic process of islet β cell destruction. Taken together, the cytokine profiles produced by the insulitic lesions during early stage may have a substantial impact on the pathogenesis of autoimmune diabetes in NOD mice, and strongly correlate with the development of sexual dimorphism of the disease. The importance of gender influence on the early life stage was also supported by a castration study, in which castration at weaning (about 3-week-old) significantly increased the incidence of diabetes

in male NOD mice (Fitzpatrick et al., 1991). It is most likely that castration severely decreased the levels of male hormones that subsequently changed the balance of cytokines produced by insulitis in these mice. Furthermore, consistent with this study, IL-4 administration at early age (2-week-old) could prevent insulitis and diabetes in NOD mice (Cameron et al., 1997).

Next I investigated whether sex hormones directly affected the cytokine profiles by activated CD4$^+$ T cells, and by which mechanism sex hormones modify the cytokine production by CD4$^+$ T cells. For this purpose, purified CD4$^+$ T cells were pre-treated with estrogen and testosterone prior to activation. IL-12 was chosen for stimulation because IL-12 is a key element in the development of cell-mediated immune response, and it activates a specific signal transduction pathway in T cells to induce IFN-γ gene expression. Thus, using IL-12-mediated activation permitted the identification of the direct effects of estrogen and testosterone on IFN-γ gene expression and to further study the possible mechanisms of sex hormone-mediated modulation of T cell function. Interestingly, the result showed that estrogen enhanced the IL-12-induced secretion of IFN-γ by CD4$^+$ T cells; whereas testosterone downregulated IFN-γ production. The similar results were obtained by the measurements of protein secretion and gene expression. Furthermore, sex hormone treatment exhibited identical effects on IL-12-activated CD4$^+$ T cells from either female or male NOD mice. It indicated that in both female and male mice, estrogen orientated CD4$^+$ T cells into Th1 type cells while testosterone might promote Th2 cell differentiation. Therefore, this result partially

explains the gender-related differentiation of cytokine profiles observed in insulitic lesions and *in vitro* activation of T cells isolated from NOD mice.

Since my results indicated that sex hormone treatment directly affected the IL-12-induced IFN-γ expression and production, I further investigated the potential mechanism(s) by which sexual hormones affecting cytokine gene expression. It is possible that signals specific for IFN-γ gene activation may be altered by sex hormones. Therefore, I examined the phosphorylation level of Stat4 in sex hormone-treated $CD4^+$ T cells since Stat4 is a critical transcription factor specific for IFN-γ gene activation. The results showed that estrogen treatment increased the phosphorylation of Stat4 while testosterone decreased the phosphorylation of Stat4. To our knowledge, this is the first evidence showing that individual sex hormone can selectively modify the phosphorylation level of the specific transcription factor and alter gene expression level. This mechanism may be one of the possible mechanisms involved in the sex hormone-related modulation of immune responses.

The sex hormone-mediated modulation of immune responses is a very complicated process because sex hormones not only modulate different cell types, they may also exert different effects on individual genes through specific mechanisms. It is known that cytokine produced by macrophages can be regulated by sex hormones (Chao et al., 1995). Alternatively, several studies reported that sex hormones affected IFN-γ and IL-10 expression by human T cell clones or antigen-specific T cell lines in the EAE model (Gilmore et al., 1997; Correale et al., 1998, Bebo et al., 1999).

Although autoimmune diabetes is a T cell-mediated disease and this study was focused on the sex hormone-mediated modulation of T cell function, the mechanisms of sexual dimorphism in NOD mice and other autoimmune diseases may also involve cell types other than CD4^{-} T cells. Therefore, in the future, I plan to continue to study the roles of sex hormones on the modulation of immune responses. Not only the effects on T cell function but also the function of antigen-presenting cells will be examined. It is important to determine which cell populations are the major targets of sex hormone modulation and what are the driving forces for sexual dimorphism of autoimmune diabetes in NOD mice. The results of the present study may enhance a detailed understanding to the pathogenic mechanisms of other autoimmune diseases. Furthermore, signal transduction pathway modified by sex hormones will be studied in more details. Since in the IL-12-induced signaling pathway, not only Stat4 is phosphorylated upon IL-12 stimulation, other two transcription factors, Jak2 and Tyk2, are also directly involved. Whether transcription factors other than Stat4 can be modified by sex hormones is still unclear. Additionally, whether Th2 cytokines, such as IL-4, are influenced by sex hormones and the involved mechanisms also need to be investigated. Answers to these questions will lead to a further understanding of sexual dimorphism, not only in NOD mice, but also in other autoimmune diseases and their animal models.

Figure 31. Schematic presentation of the impact of sex hormones on the pathogenesis of autoimmune diabetes in female and male NOD mice.

In young NOD mice, T cells infiltrate into pancreatic islets beginning at 3-4-weeks of age. Under the influence of female hormones (●●) , initial infiltrating T cells in female islets produce more IFN-γ and less IL-4 than those in male islets. During the pathogenic progress, more autoantigen-specific T cells are recruited and activated in the islets. In female islets, more recruited autoreactive T cells differentiate into Th1 cells because of a high level of IFN-γ and a low level of IL-4 produced by initial infiltrating T cells. These autoantigen-activated Th1 cells produce more IFN-γ in the islets. IFN-γ is cytotoxic to islet β cells and more importantly it enhances the development and maturation of cytotoxic T cells that will kill insulin-producing β cells via various mechanisms. In contrast, in male islets, male hormones (▲▲) cause the initial infiltrating T cells to produce less IFN-γ but more IL-4 than T cells from female islets. When autoreactive T cells are recruited, they are more likely to differentiate into Th2 cells and the development of Th2-biased autoreactions slows down the process of β cell destruction. Therefore, female NOD mice develop diabetes earlier with higher incidence whereas in males insulitis remains but disease development is delayed. In conclusion, sex hormones may influence cytokine profiles of T cells in young female and male NOD mice, and the different cytokine profiles of early insulitis subsequently affect the pathogenic progress of autoimmune diabetes in adult NOD mice.

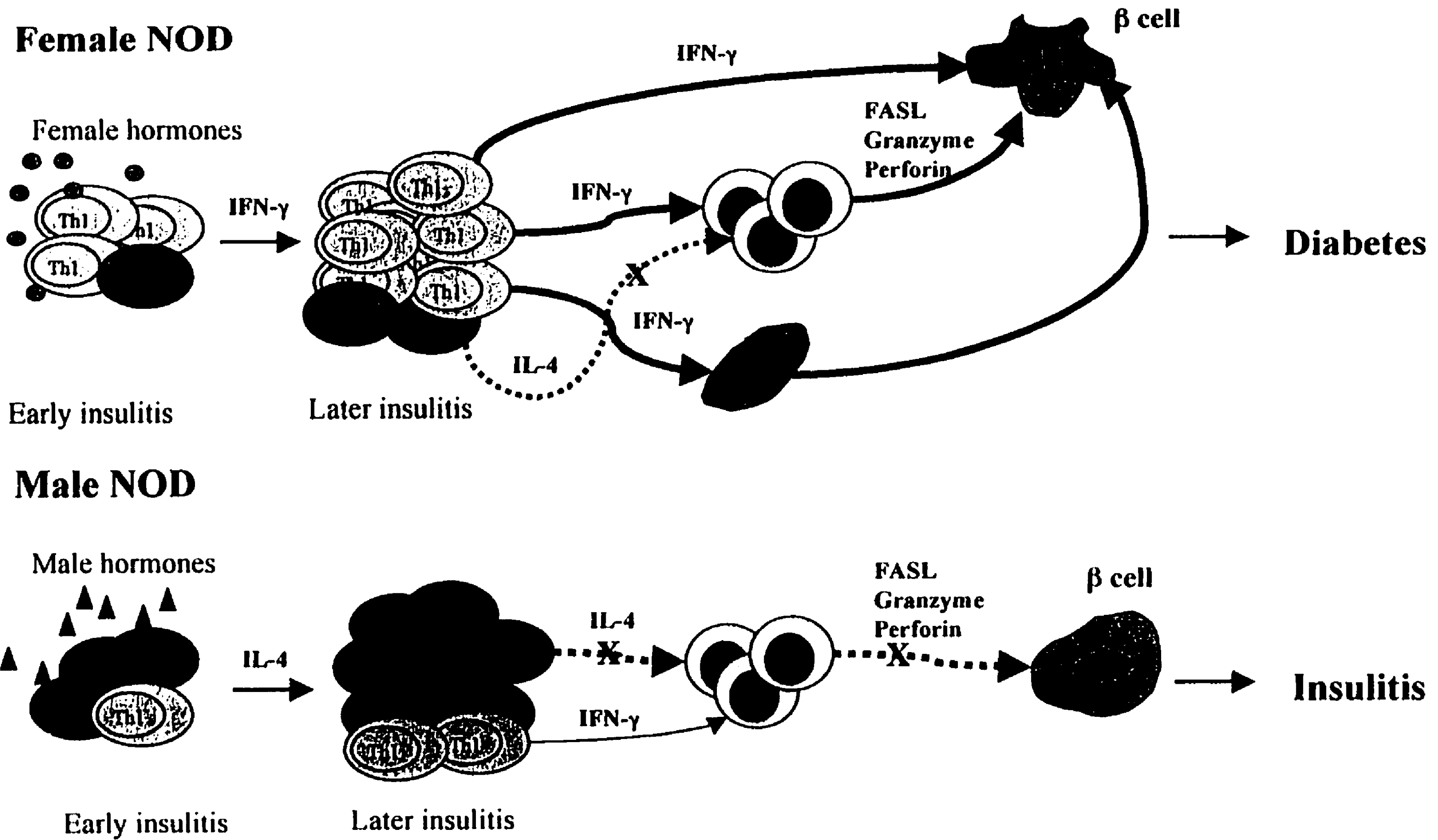

Figure 31. Schematic presentation of the impact of sex hormones on the pathogenesis of autoimmune diabetes in female and male NOD mice

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