Immunoteratological Studies of Diabetic Embryopathy Using Gene Expression Analysis

By

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(ABSTRACT)

Diabetic embryopathy is a major complication of pregnant women with type I diabetes. Immune defects in the pathogenesis of diabetic embryopathy have been suggested. We hypothesized that activated immune system can counteract diabetic effect and result in prevention of diabetic embryopathy. Diabetes was induced in pregnant ICR mice by streptozocin injection. Three different techniques of maternal immune stimulation, complete Freund's adjuvant (CFA), granulocyte-macrophage colony-stimulating factor (GM-CSF), or interferon-γ (IFN-γ), were used to stimulate the maternal immune system. Approximately 50% of fetuses from hyperglycemic (>27 mM/L) dams were malformed, with neural tube defects predominating. Maternal immune stimulation during the time of normoglycemia, i.e. prior to onset of hyperglycemia, was necessary for reducing teratogenic effects associated with hyperglycemia. The immune-stimulated diabetic mice then produced significantly lower numbers of malformed fetuses: CFA 20.9%, GM-CSF 23.3%, IFN-γ 13.9%. A gene microarray was then used to examine a selected panel of placental and splenic genes. We hypothesized that a shared profile of placental or splenic gene expression changes may correlate to the reduced birth defect outcome induced by the different immune stimulation procedures. Diabetes did not cause significant changes in placenta or spleen gene expression profile. In placenta, CFA and GM-CSF changed placental gene expression relative to control or diabetes, but differentially affected such genes relative to each other; further, IFN-γ did not affect gene expression relative to control or diabetes. Thus no common pattern of improved placental cytokine, cell-cycle, apoptotic, transcription factor, or other gene expression was identified in the immune-stimulated mice. In spleen, all 3 immune activators produced a common altered gene expression profile. The overall gene expression profile after all immune stimulation procedures suggested increased splenocyte activity and cytokine production. The cytokine GM-CSF, in particular, was up-regulated in splenic leukocytes.

This cytokine has previously been associated with reduced cleft palate in urethane-exposed mice after immune stimulation, and with reduced limb malformations in cyclophosphamide-treated mice after intra-uterine administration. In contrast, the TGF-β3 gene was down-regulated in immune-stimulated diabetic mice. This gene was up-regulated in urethane-exposed mice, an effect that may be associated with reduced cleft palate. Thus unlike urethane, TGF-β3 gene expression did not show a relationship with reduced diabetes-induced birth defects. Taken together, these data prove our hypotheses and suggest that mechanistically diverse forms of immune activation result in protection against diabetes-related teratogenesis, but only if given prior to onset of hyperglycemia. Such immune stimulation in mice may act through systemic immune organs, i.e. spleen, over-riding adverse effects of diabetes on development.

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LIST OF ABBREVIATIONS

ASTIV	aryl sulfotransferase IV
BCG	bacillus Calmette Guerin
BDNF	brain-derived neurotrophic factor
BG	blood glucose
CFA	complete Freund's adjuvant
CHC	α-cyano-4-hydroxycinnamic acid
CP	cyclophosphamide
CRH	corticotrophin-releasing hormone
CSF-1	colony-stimulating factor-1
COX-2	cyclooxygenase-2
DEPC	diethyl pyrocarbonate
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
γ-GCS	γ-glutamylcysteine synthetase
GD	gestation day
GLUT	glucose transporter
GM-CSF	granulocyte-macrophage colony-stimulating factor
GSH	glutathione
ICM	inner cell mass
IDDM	insulin-dependent diabetes mellitus
IFN-γ	interferon-γ
IGF-I	insulin-like growth factor-I
IGFBP-I	Insulin-like growth factor binding protein-I
IL	interleukin
iNOS	inducible nitric oxide synthetase
LGLs	large granulated lymphocytes
LPS	lipopolysaccharide
MI	myo-inositol
NAC	*N*-acetylcysteine

NGF	nerve growth factor
NK	natural killer
NOD	non-obese diabetic
nNOS	neuronal nitric oxide synthase
NTDs	neural tube defects
PAH	polycyclic aromatic hydrocarbons
PCA	principal components analysis
PCR	polymerase chain reaction
PGs	prostaglandins
PGI_2	prostacyclin
PKC	protein kinase C
RAG 2	recombinase activating gene 2
ROS	reactive oxygen species
RT-PCR	Reverse-transcriptase PCR
SOD	superoxide dismutase
STZ	streptozocin or streptozotocin
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TE	trophectoderm
TGF	transforming growth factor
TNF-α	tumor necrosis factor-α
TUNEL	terminal dUTP nick end labeling
VEGF	vascular endothelial growth factor

CHAPTER 1: HYPOTHESES AND SPECIFIC OBJECTIVES

1.1: Hypotheses

Diabetic embryopathy and other diabetic complications as conditions threatening to human health have been subjected to extensive studies for decades. Information gathered so far indicates that insulin deficiency and hyperglycemia in diabetic patients results in metabolic and physiologic disturbances involving several factors or pathways, so-called multi-factorial basis. These pathways include myo-inositol deficiency, prostaglandin deficiency, and the generation of reactive oxygen species (ROS) (Reece, 1999). These biochemical changes have been linked to abnormally expressed genes that regulate cell growth and apoptotic processes. Several specific genes have been identified in diabetic animal models that were increased or decreased significantly, changes that appeared to correlate with occurrence of embryonic malformations (i.e., PAX-3, NGF, TNF-α) (Chang and Loeken, 1999). Recent studies have also demonstrated effects of diabetes on the immune system that again may relate to the development of fetal malformations (Fein et al., 2001).

The immune system has been shown to play multiple important roles in the success of pregnancy. Immune cells and their cytokine products are active in the utero-placental unit, and their deficiencies could compromise the outcome of pregnancy. An influencing role of immune system in fetal development was supported by the success of immune stimulation techniques to lower birth defects caused by various teratogens (Holladay et al., 2002). The protective effect was associated with improved patterns of cytokine expression, i.e. TNF-α and TGF-β2.

In this study we hypothesized that diabetes alters immune responses during pregnancy, which is associated with development of fetal defects; and immune stimulation can prevent or improve this effect, therefore reducing occurrence of diabetic embryopathy. In addition we also hypothesized that immune stimulation may cause changes in gene expression profiles in systemic immune organs (i.e. spleen) or local immune cells (i.e. placenta), which then relate to fetal defect reduction.

1.2: Specific Objectives

These studies were designed to further elucidate operating mechanisms by which diabetes causes embryonic malformations, as well as the mechanisms by which maternal immune stimulation can reduce these malformations. Study 1 examined teratogenic outcome in diabetic animals with or without immune stimulations. Study 2 examined gene expression of placenta of diabetic mice with or without immune stimulations. Study 3 examined gene expression of spleen of diabetic mice with or without immune stimulations.

1.2.1: Study 1 - Effects of immune stimulations on diabetic embryopathy

A diabetic mouse model was produced that yielded significant numbers of malformed fetuses, characteristic of diabetic embryopathy. The mouse model then was used for assessing the effects of 3 different immune-stimulation methods, CFA, IFN-γ, and GM-CSF with 2 different regimens; after or before diabetes was induced. Theses agents were selected based on previous knowledge of their ability to stimulate the immune system, success in prevention of spontaneous abortion and chemical-induced teratogenesis, and difference in mechanism of actions. Fetal morphology was evaluated among normal, diabetic, and immune-stimulated diabetic mice.

Diabetic induction

Day		Day 1	Day 7	Day 10	Day 14	Day 15		Day 31
Action			- STZ		- mating	- GD 0		- GD 17 - sacrifice

Immune stimulations after diabetes

Day		Day 1	Day 7	Day 10	Day 14	Day 15		Day 31
Action			- STZ	- CFA - GM-CSF - IFN-γ	- mating	- GD 0		- GD 17 - sacrifice

Immune stimulations before diabetes

Day		Day 1	Day 7	Day 10	Day 14	Day 15		Day 31
Action		- CFA - GM-CSF - IFN-γ	- STZ		- mating	- GD 0		- GD 17 - sacrifice

1.2.2: Study 2 – Gene expression profile of placenta in mice with or without immune stimulations

These studies examined gene expression profiles of placentas collected from experimental mice, including control, diabetic, and immune-stimulated diabetic mice. A gene array was used to evaluate expression level of selected genes, including groups of gene that regulate cell growth and differentiation, apoptosis, growth factors and cytokines, transcription factors, and intracellular signal transduction modulators and effectors. Attempts were made to correlate gene expression profile across treatment groups with the level of embryonic defects. These data were used to construct a possible mechanism by which immune stimulations could reduce embryonic malformations caused by diabetes.

1.2.3: Study 3 – Gene expression profile of spleen in mice with or without immune stimulations

These studies examined gene expression profiles of spleens collected from experimental mice including control, diabetic, and immune-stimulated diabetic mice. A gene array was used to evaluate expression of selected genes that regulate cell growth and differentiation, apoptosis, growth factors and cytokines, transcription factors, and intracellular signal transduction modulators and effectors. Attempts were made to correlate gene expression profile across treatment groups with the level of embryonic defects. These data were used to construct a possible mechanism by which immune stimulations could protect embryonic malformations caused by diabetes.

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CHAPTER 2: LITERATURE REVIEW

2.1: Diabetic Embryopathy

Poorly controlled diabetes during the first trimester of pregnancy, and in particular during the first six weeks of intrauterine development, has been associated with a spectrum of developmental abnormalities ranging from growth retardation to discrete congenital anomalies of the nervous, cardiovascular, renal, and skeletal systems to demise of the conceptus and spontaneous abortion (Buchanan and Kitzmiller, 1994). Major congenital anomalies have affected 4 to 12% of infants, a twofold to fivefold increase over that in the general population, and spontaneous abortion rates range from 9 to 45% (Jovanovic-Peterson and Peterson, 1993). In fact, 40% of all perinatal deaths in diabetic mothers are related to congenital malformations.

In the second or third trimester, macrosomia, defined as a birthweight percentile greater than 90, and the foundation for neonatal hypoglycemia are the major concerns (Ryan, 1998). In type I diabetes, the risk of macrosomia is 25%, and the risk of neonatal hypoglycemia is 8% (Weintrob et al., 1996). A particular concern is that the excess fat distribution may be truncal giving rise to the increased risk of shoulder dystocia and fetal trauma.

The mediation of these effects in human remains unclear. However, laboratory studies from *in vivo* diabetic animal models and *in vitro* embryo culture have revealed several important characteristics of diabetic embryopathy. First, Sadler (1980) showed that embryos from normal rats develop abnormally when grown in diabetic serum, indicating that some factor(s) in diabetic serum is teratogenic. Second, studies in which individual components of diabetic serum have been added to normal serum during embryo culture suggest a multifactorial etiology for diabetic embryopathy. Glucose, ketones, and inhibitors of somatomedin activity have been implicated as teratogens in such experiments (Buchanan et al., 1994; Freinkel et al., 1986; Horton and Sadler, 1983; Hunter et al., 1987; Sadler et al., 1986) and are likely synergistic in their toxic effects on rodent embryos. Thus, diabetic teratogenesis must be viewed as resulting from factors that are more complex than hyperglycemia alone. Third, in vitro studies of embryogenesis reveal that a deficiency of myo-inositol, abnormalities of arachidonic acid and prostaglandin metabolism, and the over-

production of oxygen radicals may mediate the embryotoxic effects of hyperglycemia (Baker et al., 1990; Eriksson and Borg, 1991; Pinter et al., 1986; Reece, 1999). Forth, maternal diabetes could affect the embryonic development in different stages and result in embryonic malformations. These stages are preimplantation (Moley et al., 1991; Pampfer et al., 1997), early development, and organogenesis (Reece, 1999). Finally, the susceptibility of individual embryos to the toxic effect of diabetic serum appears to vary. An example of this phenomenon is the finding that only a fraction of embryos from a single litter exposed to the same diabetic serum in vitro will develop abnormally. In addition, Eriksson et al (1988) have shown variability among strains of rats in the frequency of diabetes-related birth defects.

2.1.1: Biochemical pathogenesis

The precise mechanism responsible for abnormal fetal organogenesis in pregnancy complicated by diabetes is unclear. However, studies up to date have shown that the mechanisms may include deficiency states of membrane lipids (myo-inositol, arachidonic acid, etc.), alteration in the prostaglandin cascade, and the generation of excess free oxygen radicals (Reece, 1999). These biochemical alterations result in characteristic morphological and molecular changes, which are considered to be the basis of diabetic embryopathy.

A deficiency of myo-inositol (MI) is a cause for the pathogenesis of other diabetic complications, such as neuropathy, retinopathy, and nephropathy (Winegrad, 1987). Abnormalities in intracellular MI and phosphoinositide metabolism have also been implicated in the pathogenesis of diabetic embryopathy (Baker and Piddington, 1993). This model postulates that elevated glucose levels result in tissue myo-inositol depletion. In turn, this affects phosphoinositide metabolism, a key intracellular signaling system that provides such important second messengers as the inositol phosphates, diacylglycerol and arachidonic acid. Several investigators demonstrated in vitro that increasing concentrations of glucose result in a parallel decrease in the MI concentration in embryos (Hod et al., 1990; Hashimoto et al., 1990; Baker et al., 1990). Conversely, supplementation with MI restores the concentrations to normal values and results in a significant decrease in malformations. The protective effect of myo-inositol supplementation has also been shown in vivo. Baker et al. (1986) demonstrated that the incidence of neural tube fusion defects was reduced from 9.7% in the embryos born to diabetic rats that received the control rat chow versus 3.7% in the embryos born to pregnant diabetic mothers that had received the 1% myo-inositol supplemented chow. While these data support the significant protection afforded by myo-inositol against glucose-induced failure of neural tube fusion, the protection was only partial,

in that the incidence of neural tube defects was still above that seen in the embryos born to nondiabetic control animals.

In vitro and in vivo experimental studies have shown the role of arachidonic acid deficiency in diabetes-induced embryopathy. Goldman et al. (1985) and Pinter et al. (1986) showed that arachidonic acid supplementation of a high glucose culture medium reduced malformations induced by hyperglycemia. Goldman et al. (1985) also demonstrated the protective effects of arachidonic acid in vivo. Arachidonic acid given by subcutaneous injection to pregnant diabetic rats provided a significant reduction of embryonic malformations comparing with diabetic controls. Another group of investigators (Reece et al., 1996) fed diabetic rats with safflower and primrose oil, essential fatty acids that are known to increase serum arachidonic acid levels, and demonstrated a significant reduction of the rate of neural tube defects from 23.6% to 7.6%. A link between the myo-inositol and arachidonic acid pathways to the pathogenesis of embryopathy has been demonstrated by Baker et al. (1990). They showed that the protective effects of myo-inositol supplementation against the teratogenic effects of a high glucose concentration on neural tube fusion could be reversed by indomethacin, an inhibitor of arachidonic acid metabolism.

The disruption in the arachidonic acid cascade eventually results in a deficiency in prostaglandins (PGs). Baker et al. (1990) used the mouse embryo culture system to test $PGE_{2\alpha}$, $PGF_{2\alpha}$, and PGI_2 (prostacylclin), prostaglandins known to be present in embryonic rat and mice. They found prostaglandin E_2 to be the most effective of the prostaglandins in reversing the inhibition of neural tube fusion induced by high levels of glucose. Piddington et al. (1996) measured PGE_2 directly in embryos from normal and diabetic mice. In normal mice a clear developmental pattern was seen. Prostaglandin E_2 levels were high during early formation of the cranial neural folds (day 8), there was a decline during fusion of the cranial neural folds to form the neural tube (day 9), and levels were low after neurulation was completed (day 10-11). The evidence of this study indicates that embryos have cyclooxygenase activity capable of generating PGE_2 during a brief developmental period preceding neural tube closure. In embryos of diabetic mice, PGE_2 levels were significantly lower than normal during the early development of the cranial neural fold (day 8) but similar to normal after the cranial neural tube had closed (day 9-10). The authors suggested that diabetes mellitus promotes cranial neural tube malformations by causing a functional deficiency of PGE_2 during early neurulation.

Free radicals have been implicated in the pathogenesis of a wide variety of human disorders (Halliwell and Gutteridge, 1990). In diabetes, free oxygen radicals have been associated with the pathogenesis of retinopathy, nephropathy, and angiopathy (Morrow et al., 1994). Eriksson and Borg (1991, 1993) have demonstrated that free radicals are involved in the mechanism of diabetic embryopathy. These investigators postulated that increased free oxygen radical formation in embryonic tissues is causally related to malformations. The evidence for this model comes largely from the ability of free oxygen radical scavenging enzymes to protect against glucose-induced malformations. Rat embryos were cultured in medium containing 10 mM glucose to serve as controls, 50 mM glucose (a concentration capable of producing a major malformation rate of 81%), or 50 mM glucose plus either citiolone (an inducer of free oxygen radical scavenging enzymes) or the oxygen radical scavenging enzymes superoxide dismutase (SOD), catalase, or glutathione peroxidase (Eriksson and Borg, 1991). Addition of SOD was completely protective in the presence of 50 mM glucose, returning the malformation rate to levels seen in the presence of 10 mM glucose. The addition of citiolone, catalase, or glutathione peroxidase to the medium containing 50 mM glucose resulted in significant protection, but the rate of major malformations was still elevated above that seen in the control situation. Eriksson and Borg (1993) followed up these observations, and demonstrated the protective effect of free oxygen radical scavenging enzymes in regard to malformations produced by hyperglycemia, β-hydroxybutyrate, and α-ketoisocaproate. The addition of SOD to the hyperglycemic medium protected against the teratogenic effects of all three agents. The addition of pyruvate transport inhibitor α-cyano-4-hydroxycinnamic acid (CHC), however, provided significant protection only against the malformations induced by glucose and pyruvate, suggesting that free oxygen radicals, considered to be responsible for teratogenesis, are produced in the mitochondria because these hexoses are oxidized in the mitochondria. The authors offered the hypothesis that embryos exposed to a diabetic milieu experience too much oxidative substrate and too little mitochondrial capacity to handle the increased load of free oxygen radical.

Several investigators have demonstrated the significance of the embryonic antioxidant system in the pathogenesis of diabetic embryopathy (Hagay et al., 1995; Sivan et al. 1996; Sakamaki et al. 1999). Hagay et al. (1995) used embryos from a mouse strain with increased endogenous superoxide dismutase activity caused by incorporation of a transgene (hCuZnSOD) into the genome. They found that the transgenic embryos were more resistant

to the teratogenic effect of diabetes and had a lower rate of malformation than diabetic nontransgenic animals. Sakamaki et al. (1999) have shown that the concentration of intracellular glutathione (GSH) in embryonic tissues of diabetic pregnant rats on day 11 was significantly lower than that of normal rats. The activity of γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting GSH synthesizing enzyme, in embryos of diabetic rats was also significantly low, associated with reduced expression of γ-GCS mRNA. Moreover, in vivo studies using supplementation of vitamin E (Sivan et al., 1996), vitamin C (Siman and Eriksson, 1997), or lipoic acid (Wiznizer et al., 1999) have demonstrated these factors to be protective against diabetic embryopathy.

The primary effect of increased activity of oxygen free radicals in diabetic pregnancy is believed to be enhanced lipid peroxidation, particularly within plasma and organellar membranes. The hydroperoxides, which are the major products of lipid peroxidation, have been shown to alter prostaglandin biosynthesis by inhibiting the production of prostacyclin (PGI_2) and enhancing the production of thromboxane A_2 (Eriksson and Borg, 1993). Wentzel et al. (1999) followed up these observations in order to assess embryonic oxidative stress by measuring the concentration of the F_2-isoprostane (8-epi-$PGF_{2\alpha}$), a prostaglandin-like compound generated by ROS-derived oxidation of arachidonic acid in cellular membranes. They showed that both hyperglycemia in vitro and maternal diabetes in vivo caused embryonic dysmorphogenesis and increased embryonic levels of 8-epi-$PGF_{2\alpha}$, indicating increased lipid peroxidation. Addition of *N*-acetylcysteine (NAC), a free radical scavenger, to the culture medium normalized the morphology and 8-epi-$PGF_{2\alpha}$ concentration of the embryos exposed to high glucose.

Although these pathways appear to be different, they are interconnected. For example, prostaglandins are synthesized from arachidonic acid by cyclooxygenases (DuBois et al., 1998), in which an inducible cyclooxygenase, COX-2, has been reported to be expressed at lower levels in embryos of diabetic mothers (Wentzel et al., 1999). Likewise, reactive oxygen species (ROS) can be generated from arachidonic acid by lipid peroxidases (Natarajan et al., 1996). Myo-inositol and arachidonic acid abnormalities result in a functional deficiency of prostaglandins, which are able to adversely affect membranogenesis and membrane function (Pinter et al., 1986). The changes in membrane function and fluidity result in an influx of a high level of glucose into the cells, inducing the generation of free oxygen radicals that cause morphologic damage in the mitochondria of the embryos (Yang et

al., 1995). The excess levels of free oxygen radicals and the diminished antioxidant defense status allow enhanced peroxidation of embryonic lipids (Wentzel et al., 1999). In addition, increased lipid peroxidation can cause a reduction in prostacyclin levels and imbalance in prostaglandin synthesis (Warso and Lands, 1983). The functional deficiency of prostaglandins at a critical time of fetal development can cause embryonic malformations.

Studies have shown that the essential fatty acid contents is greatly reduced in the yolk sac membranes of the conceptuses of diabetic animals and arachidonic acid supplementation restores the fatty acid content, along with the reduction in congenital malformations (Pinter et al., 1986; Pinter et al., 1988). On the basis of these studies it has been proposed that hyperglycemia induced "yolk sac failure". Whereas yolk sac failure during the early hemotrophic phase of nutrition results in embryonic death, failure at a later phase leads to several developmental malformations. Thus the yolk sac failure can be considered as the "primary embryopathy" that leads to the "secondary embryopathy" in the conceptuses (Reece et al., 1994).

2.1.2: Effects of diabetes on preimplantation

The majority of studies examining the effect of maternal diabetes or hyperglycemia on embryo development come from postimplantation models. Far less attention has been paid to the preimplantation, preorganogenic states despite the evidence from human and animal studies that poor metabolic control during this period results in increases in malformations and miscarriage (Jovonovic-Peterson and Peterson, 1993).

A study by Diamond et al. (1989) has shown that alloxon- or streptozocin (STZ)-induced diabetes leads to an in vivo embryonic developmental delay as early as 48 h after fertilization. Recovery of this time point revealed that only 77% or 71% of embryos from the STZ-treated or alloxan-treated mice, respectively, had reached a two-cell stage as compared with 93% or 89% in the control mice. When the same two-cell embryos from diabetic mice were cultured in control media for 72 h, they experienced marked impairment of development as assessed by the distribution of developmental stages at 24-h time points and by the rates of progression to a blastocyst, which increasingly diverged at each time point. Insulin administration to lower the blood glucose levels in the mothers prior to mating reversed the developmental delays seen in the embryos. These findings suggest that hyperglycemia or some metabolic derangement characteristic of the diabetic state is responsible for the delay. Vercheval et al. (1990) and Lea et al. (1996) have discovered a

similar delay at a later, peri-implantation stage in the diabetic rat. Both groups reported a smaller number of blastocysts and a larger number of molurae among all embryos recovered on days 5-8 of pregnancy from rats made diabetic with STZ as compared with control rats.

Pampfer et al. (1994a) have reported that blastocysts recovered from STZ-induced diabetic rats and continued in culture exhibit marked impairment of trophoblast outgrowth. Approximately 70% of blastocysts from control rats implant in vitro, compared with 49% in the diabetic group. Moreover, among the control implants, 33% maintain a compact ICM cluster as compared with 15% in the diabetic group. Most diabetic implants display a disorganized outgrowth with only a few scattered ICM cells. It has also been shown that blastocysts recovered from diabetic NOD mice and transferred into control recipient females produce decreased rates of implantation and of viable implants, and increased rates of congenital malformations (Otani et al., 1991).

In vitro studies have also demonstrated deleterious effects of diabetes on preimplantation embryos. Two-cell embryos from control mice were cultured for 72 h in elevated concentrations of substrates and intermediates known to be abnormal in diabetics (Diamond et al., 1990; Moley et al., 1994). Concentrations of D-glucose greater than or equal to 440 mg/dl, 10 mM acetoacetate, or a racemic mixture of DL-β-hydroxybutyrate (16 or 32 mM) markedly retarded development of embryos in the progression to a blastocyst. Ornoy and Zusman (1991) demonstrated similar in vitro findings. They reported not only a delay in development but also a direct degenerative effect of the diabetic sera on the cells of the ICM in the embryos reaching this stage. The severity of this degeneration and the delay were directly related to the concentration of glucose, β-hydroxybutyrate, and acetoacetate in the sera.

Other factors such as cytokines have been demonstrated to associate with the early developmental problems in embryos from diabetic mothers. Pampfer et al. (1994b) have shown that unlike colony stimulating factor-1 (CSF-1), transforming growth factor-β (TGF-β), and epidermal growth factor (EGF), which are all released into the uterine milieu and promote embryo growth, tumor necrosis factor-α (TNF-α) does the opposite. This cytokine binds to both rat and mouse blastocysts via the TNF-α p60 receptor and causes not only a decrease in the rate of development to a blastocyst but also a decrease in cell number. Similarly to high glucose exposure, TNF-α induces an increased incidence of nuclear

chromatin degradation (karyolysis), decreased ability to maintain a structured ICM cluster with trophectoderm (TE) spreading, and increased rate of fetal resorption after transfer to pseudopregnant mice (Wuu et al., 1999). Furthermore, Pampfer et al. (1995a) have shown by both histologic studies and mRNA analysis that maternal diabetes up-regulates the synthesis of TNF-α by uterine cells. In addition, rat blastocytes were cultured in diabetic uterine cells alone or with added anti-TNF-α antibodies (Pampfer et al., 1995a) or antisense oligonucleotides to the p60 TNF-α receptor (Pampfer et al., 1995b). Both techniques resulted in partially reversing the total cell deficit experienced by the blastocysts, suggesting that overproduction of this cytokine in diabetes may be in part responsible for the deleterious effects seen.

Maternal hyperglycemia has been shown to alter glucose utilization in preimplantation embryos, which may result in increased cell death and growth retardation described previously. Moley et al. (1998a) have compared glucose utilization in preimplantation embryos from control versus STZ-induced diabetic mice. Using ultra-microfluorometric enzyme assays, they found that free glucose in embryos from diabetic mice dropped to barely detectable levels at 48 and 96 h after fertilization, corresponding to two-cell and blastocyst stages. They also measured glucose transport using non-radioactive 2-deoxyglucose uptake, and showed that it was significantly lower at both 48 and 96 h, corresponding to the decrease in free glucose levels. They then measured the expression of glucose transporter (GLUT) 1, 2, and 3 at both the protein and mRNA levels. It was found that GLUT1 mRNA levels are 44 and 68% lower at 48 and 96 h in diabetic embryos. GLUT2 and GLUT3 mRNA levels, although not different at 72 h, were reduced 63 and 77% at 96 h. Furthermore, GLUT1 protein was 46 and 66% less at 48 and 96 h, and GLUT2 and GLUT3 protein were 90 and 84% less at 96 h. This study suggested that the hyperglycemic state down-regulates these glucose transporters, and decreases glucose transport of the embryos at two-cell and blastocyst stages.

Several studies have linked a decrease in glucose transport to the initiation of apoptosis. Pampfer et al. (1990) reported a decrease in the number of ICM and TE cells in blastocysts from diabetic rats. Similarly, Lea et al. (1996) found a 20% reduction in the number of cells in the ICM of blastocysts recovered from the BB/E rats. These embryos are morphologically characterized as having cellular blebbing and nuclear condensation, suggestive apoptosis. Also, Pampfer et al. (1997) demonstrated that blastocysts from diabetic

rats exhibit increased DNA fragmentation, predominantly in the ICM. The same diabetic conditions result in an increase in the expression of clusterin, a protein involved in the clearance of dead cell fragments. In addition, Moley et al. (1998b) measured the expression of *Bax*, a death-promoting member of the Bcl-2 family of proteins, in mouse preimplantation embryos at a blastocyst stage. *Bax* was chosen as an indicator of apoptosis because it is expressed in early embryos. Expression of *Bax* mRNA was 2.5-fold higher in blastocysts from STZ-induced diabetic mice than in embryos from control mice by competitive PCR. Sixfold higher Bax protein levels were also detected in blastocysts from diabetic mice than control mice. High-glucose conditions in vitro cause the same overexpression of *Bax* and confirm that hyperglycemia is responsible for the phenomenon. Also, *Bax* overexpression correlates with apoptotic morphologic changes as detected by dual nuclear DNA staining with propidium iodide and terminal dUTP nick end labeling (TUNEL).

Moley et al. (1998b) hypothesized that the decrease in glucose transport is upstream of *Bax* overexpression, rather than the program cell death signal leading to decreased glucose uptake. Decreased free glucose levels then trigger the cell death signal, causing loss of key progenitor cells in the ICM or TE. If the cell death is extensive enough, this event results in fetal loss. If it is less extensive, this event may be manifest later in development as a malformation.

2.1.3: Effects of diabetes on the placenta

The placenta is a fetal organ carrying out an impressive array of different functions, such as transfer of nutrients and waste products, synthesis and secretion of hormones, and immunoprotection of the fetus, which all are essential for fetal growth and development. The placenta is also a rich source of growth factors and cytokines that can affect the growth and differentiation of fetus (Petraglia et al., 1996).

In diabetic pregnancies a wide range of reports on morphologic and ultrastructural placental abnormalities have been reported. Placentomegaly appears to be the most significant effect of severely complicated diabetes in pregnant women (Teasdale, 1985), as well as in experimental diabetic pregnancy (Eriksson and Jansson, 1984; Giavini et al., 1986). The increased placental mass found in these studies may be interpreted as an insufficient compensatory mechanism for the reduced uteroplacental blood flow in diabetic pregnancies (Eriksson and Jansson, 1984). Other morphologic disturbances include thickening of the trophoblastic membranes together with higher degrees of vesiculation and

vacuolization. Increased content of glycogen and an increased number of glycogen cells have also been reported, in accordance with the finding of large aggregates of possibly glycogen-containing cells in the placentas of diabetic rats (Gewolb et al., 1986).

Moreover, augmented cystic degeneration of rat placental spongiosa cells has been detected (Giavini et al., 1986). In diabetic patients with poor blood glucose control an increased number of syncytial knots and vasculosyncytial membranes (which reduces diffusion distance over the interhemal membrane) and greater villous surface areas were found (Reece and Eriksson, 1996). Focal thickening of human trophoblastic basement membrane, larger placental capillary bed, longer placental villi, but unchanged villous diameter were also found in diabetic human placentas (Mayhew et al., 1994). In addition, Gewolb et al. (1983) described an increased deoxyribonucleic acid content in late pregnancy placentas from diabetic rats, indicating prolonged cell division and thus a more immature state of the trophoblastic tissue.

Placentas of diabetic animals have been shown to express abnormal levels of functional proteins, such as extracellular matrix proteins (Forsberg et al., 1998), and epidermal growth factor receptor (EGF-R) (Sissom et al., 1987; Fujita et al., 1991). Basement membranes constitute extracellular matrix networks in which laminin, fibronectin, and collagen-IV are important constituents, providing an epithelial-mesenchymal interface and surrounding vasculature, nerve fibers, and muscles. In addition to serving as structural proteins laminin, fibronectin, and collagen-IV are potent regulators of cellular proliferation and differentiation, providing appropriate signals that are transduced to the cell nucleus and thereby evoking changes in gene expression (Panayotou et al., 1989). Extracellular matrix receptors are expressed in trophoblast cells, and it was speculated that the extracellular matrix is facilitating trophoblast adhesion and invasion of maternal tissues (Isemura et al., 1993). It is thus reasonable to conclude that normal placental morphogenesis depends on a specific extracellular matrix composition. Forsberg et al. (1998) have reported that laminin deposition was reduced whereas fibronectin levels were increased in placentas from diabetic rats. The authors suggested that diabetes-induced alterations of laminin and fibronectin protein levels in the fetal-maternal interface might affect placental development and alter gas exchange and nutrient transfer to the offspring. Interaction between EGF and EGF-R has been shown in many cell types to stimulate an array of biochemical and morphologic changes including cell proliferation, organ differentiation, and transport of small molecules (Zwick et

al., 1999). Sissom et al. (1987) have investigated the binding of EGF to plasma membrane prepared from placentas of control and diabetic rats, and reported that placental membranes from diabetic fetuses showed decreased specific binding (30%) on gestational days 17 and 21 and the absence of a second class of binding sites on day 21 of gestation. These investigators suggested that the failure of second-class receptors to develop in the diabetic condition might be important for the control of maturation and growth of placental tissues. Fujita et al. (1991) have also reported the lower expression of EGF-R in placentas of diabetic humans at both protein and mRNA levels.

Transfer of glucose from the maternal circulation to the fetus is fundamental in the utero-placental-fetal transfer system. Fetal plasma glucose concentrations have been shown to correlate with maternal glucose concentrations (Economides and Nicolaides, 1989). The glucose transfer across the placenta is facilitated, and is independent of the Na+ gradient (Johnson and Smith, 1980). The expression of GLUTs shows a tissue-specific distribution. For example, GLUT1 has been found in erythrocytes, kidney and blood-tissue barriers, including those in the brain and placenta (Asano et al., 1988). GLUT3 has been shown to be abundant in organs with high glucose requirements, such as brain, and to be expressed in the placenta in rodents (Haber et al., 1993). Because of the low level of GLUT3 in human placenta, Shepherd et al. (1992) suggested that GLUT1, not GLUT3, is the major GLUT isoform in human placenta.

Two reports in humans (Jansson et al., 1993, Sakata et al., 1995), a report in rats (Zhou and Bondy, 1993), and a report in mice (Yamaguchi et al., 1996) described the changes in placental GLUT gene expression during pregnancy. In the human placenta, GLUT1 is a dominantly expressed glucose transporter and its level increases during pregnancy. The data in rats showed that placental GLUT1 mRNA decreased throughout pregnancy, whereas placental GLUT3 mRNA showed little change after mid-pregnancy (Zhou and Bondy, 1993). However, in mice, the level of GLUT1 mRNA increased after mid-pregnancy, not only in the placenta, but also in the deciduas (Yamaguchi et al., 1995). An increase in the level of GLUT1 in the utero-placental unit during pregnancy may be helpful in the increased requirement of glucose in the fetus, especially after mid-pregnancy.

Several investigators have demonstrated the effect of diabetes on placental glucose transporters in both human and experimental animals. Hahn et al. (1998) have used cultured human term placental trophoblast to study the effect of sustained hyperglycemia on the glucose transport system. This study has demonstrated for the first time a diminished

capacity of the GLUT1 glucose transport system of highly purified human placental trophoblast primary cultures in the presence of 25 mmol/l glucose in the medium. Decrease of GLUT1 along with kinetic analysis of glucose uptake led the authors to hypothesize that the down-regulation of GLUT1 in the wake of long-standing hyperglycemia might be a mechanism to protect the fetus from maternal peak glucose levels in poorly or uncontrolled diabetic pregnancies, while maintaining glucose supply to the fetus at moderate levels and facilitating adequate build up of the placenta itself.

In a sequential study, Hahn et al. (2000) have demonstrated that in addition to down-regulating human GLUT1 protein concentrations, glucose exerts its autoregulatory effect on hexose transport in term placental trophoblast by altering GLUT1 partitioning between the plasma membrane and intracellular sites in favor of the latter.

A study by Jansson et al. (1999), however, produced results that contradict those of Hahn et al. (1998, 2000). By using syncytiotrophoblast microvillous membrane vesicles and basal membrane vesicles isolated from pregnant women with type-1 diabetes and having low level hyperglycemia, GLUT1 protein expression was found to increase significantly in the basal membrane vesicles and mediated higher uptake of D-glucose. The authors suggested that these alterations might explain the occurrence of macrosomia despite well-controlled diabetes.

Human placental GLUT1 expression in response to hyperglycemia seems to be dependent upon the level of glucose concentration. Low level hyperglycemia up-regulates GLUT1 expression, and pronounced hyperglycemia appears to be required to decrease GLUT1 expression and activity in placental cells.

Studies of animal models of diabetes in pregnancy have produced inconsistent results with respect to the effect on placental glucose transport characteristics. For example, in STZ-treated rats, severe diabetes is often found to be associated with a decreased placental glucose transfer capacity (Thomas and Lowy, 1992), which suggests a down-regulation of placental glucose transporters. In contrast, by means of the same animal model, expression of the facilitative glucose transport GLUT3 and the uptake of 2-deoxyglucose have been shown to be up-regulated fivefold in diabetes (Boileau et al., 1995), while GLUT1 mRNA and protein levels remained unmodified. These results also led the authors to suggest that GLUT3 protein plays a major role in placental glucose uptake and metabolism.

In nonobese diabetic mice with severe hyperglycemia, GLUT1 expression was decreased and GLUT3 expression was increased, but the protein expression of both placental

glucose transporters remained unaltered (Devaskar et al., 1994). However, in vitro and in vivo studies by Ogura et al. (1999) have shown that both GLUT1 mRNA and protein were significantly decreased in response to severe hyperglycemia.

These discrepancies suggest that the effect of diabetes on placental glucose transporters might depend on which species and experimental model are used, as well as on the severity of diabetes achieved.

2.1.4: Gene expression studies on diabetic embryopathy

The effects of maternal diabetes on embryonic gene expression have been investigated using a mouse model of diabetic pregnancy (Phelan et al., 1997). In these studies, insulin-dependent diabetes was induced with STZ approximately 4 weeks prior to pregnancy. Because the half life of STZ is only 15 minutes, genotoxic effects of this procedure would not be due to direct teratogenic effects of the drug. A moderate dose of STZ (75 mg/kg, 3 consecutive days) induced hyperglycemia (200 to > 500 mg/dl) within a week of the first administration. In order to retain pregnancies at least until the end of the embryonic period, it was essential to establish euglycemia in STZ-diabetic mice. This was accomplished with subcutaneously implanted pellets that constitutively release insulin. However, on day 4.5 of pregnancy, STZ-diabetic mice again became hyperglycemia (270 ± 155 mg/dl); these mice remained hyperglycemia for the duration of pregnancy.

Embryos that were recovered near the end of the embryonic period of gestation (day 11.5) from diabetic mice were malformed, displaying anomalies affecting the neural tube that can also be observed in human offspring of diabetic mothers. The neural tube defects (NTDs) were observed at three-fold increased frequency compared with embryos of nondiabetic mice (Phelan et al., 1997). Other externally detectable malformations, affecting craniofacial, limb, and thoracic structures, were observed less frequently. The authors suggested that most such malformed embryos arising during either diabetic or nondiabetic pregnancy appear to be reabsorbed during fetal development, as none of the pups that were delivered at term were detectably malformed, and the reduction in conceptus number between the end of the embryonic period and term delivery (day 19.5) was correlated with the number of detectably malformed embryos observed at the end of the embryonic period.

Most of the NTDs were localized to the rostral neuropore, which, along with the caudal neuropore, is the last portion of the neural tube to fuse. These defects were either open NTDs (exencephaly) or defects affecting the development of structures overlying the fourth ventricle. Defects affecting the caudal neuropore (spina bifida) were also observed but less

frequently than those affecting the rostral neuropore. Because closure of the rostral neuropore is affected in embryos cultured in elevated glucose (Eriksson et al., 1991; Buchanan et al., 1994), a common mechanism may be involved. Neural tube fusion is regulated by several identified genes that are expressed in stereotypical fashion along the longitudinal axis of the embryo (Kessel and Gruss, 1990). A candidate gene that could be involved in the NTDs is *Pax-3*. The *Pax* family of developmental control genes includes eight members that encode a DNA-binding paired domain. Some *Pax* members, including *Pax-3*, also encode a paired-type homeodomain that participates in DNA binding (Gruss and Walther, 1992; Wilson et al., 1993). Pax-3 is expressed in neuroectoderm, neural crest, and somitic mesoderm and is required for closure of the neural tube at the rostral and caudal neuropores, development of neural crest, and limb musculature (Daston et al., 1996; Marato et al., 1997). Defects in structures that are Pax-3 dependent occur in human and animal offspring of diabetic mothers (Buchanan and Kitzmiller, 1994). Moreover, loss-of-function mutations in *Pax-3* cause defective development in all Pax-3-dependent structures in 100% of homozygous mutant embryos, demonstrating that it is an essential gene for which no redundant pathway exists (Machado et al., 2001). Therefore, reduction of *Pax-3* expression below a critical threshold would certainly cause a malformation.

To examine whether expression of *Pax-3* was affected in embryos of diabetic mice, RNA was prepared on the day that *Pax-3* expression and neural tube fusion begin (day 8.5) (Phelan et al., 1997). Pax-3 RNA was found to be reduced approximately threefold by quantitative reverse transcription-polymerase chain reaction (RT-PCR) compared with that in embryos of nondiabetic mice. Similarly, in situ hybridization of individual embryos showed that Pax-3 mRNA was significantly reduced or below the level of detection in embryos of diabetic mice. This was not due to a general inhibition of gene expression as a consequence of diabetic pregnancy, as constitutively expressed genes such as *36B4* and *fibronectin* were unaffected.

Another gene encoding a component of DNA licensing factor, *cdc46*, was found to be overexpressed in embryos of diabetic mice and in Splotch (*Sp/Sp*; *Pax-3* mutation) embryos (Hill et al., 1998). Licensing factor is a complex that binds to un-replicated chromatin and is removed upon passage of a replication fork through it (Romanowski and Madin, 1996). By marking chromatin as “unreplicated”, licensing factor appears to be important for replication of DNA once and only once per cell cycle. *Cdc46*, whose expression is increased during the G1/S phase transition, is highly conserved throughout the plant and animal kingdom (Chong

et al., 1996). In embryos of diabetic mice and *Sp/Sp* embryos, in situ hybridization demonstrated that *cdc46* overexpression was localized to the portion of the neural tube where *Pax-3* was underexpressed and which was prone to apoptosis and malformation in Pax-3-deficient embryos (Hill et al., 1998). Other cell cycle-regulated genes, such as *cyclin A* and *mcm3* (another component of licensing factor), were not overexpressed, suggesting that there was not a general activation of expression of all genes that regulate DNA synthesis. Thus, inhibition of *Pax-3* expression in embryos of diabetic mice would, directly or indirectly, increase the expression of *cdc46*. Because selectively increased expression of some genes that regulate DNA synthesis causes apoptosis (Kowalik et al., 1995), increased expression of *cdc46* could play an important role in the apoptosis that occurs in *Pax-3*-underexpressing cells.

The sensory nervous system is among the major targets of diabetes-mediated damage. One possible explanation is the reduction of the expression of regulatory target-derived neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and insulin-like growth factor-I (IGF-I) (Brewster et al., 1994; Vergani et al., 1998). In situ hybridization studies for assessing mRNA abundance of BDNF, NGF, neurotrophin receptor p75, and IGF-I were performed at embryonic stage E-17, and postnatal days 0, and 14 in controls and diabetic rats (Germani et al., 1999). NGF mRNA expression was clearly detected in several areas; the highest values were observed in the tongue and intestine, which were also the areas most affected by diabetes. In these organs, the reduction of NGF mRNA expression was of the order of 90 and 56%, respectively. There was also a loss on the order of 50% in the eyes and heart. Conversely, there was a marked increase in nerve roots and spinal cord. Treatment with silybin, a drug with antioxidant activity, of diabetic mothers prevented the reduction of NGF mRNA expression in the entire embryo; only the higher level observed in nerve roots remained unchanged. In the diabetic embryos, there was a significant increase of p75 mRNA, that was not observed when the mothers were treated with silybin. The changes in NGF and p75 were prenatal; the assessment performed at birth and thereafter did not reveal significant differences from controls. At embryonic stage, the levels of BDNF mRNA were rather low and the differences among the groups were difficult to determine, whereas at birth the value is higher in retina, nerve roots, tongue, heart, and intestine. Pups from diabetic mothers showed a reduction of BDNF mRNA abundance that was counteracted by silybin treatment. Muscle IGF-I mRNA and protein were reduced in

neonates from diabetic mothers at 14 days of age. Such a reduction was not observed when diabetic mothers were treated with silybin (Germani et al., 1999).

In addition to the genes mentioned above, maternal diabetes was reported to alter expression of several other genes that may be involved in embryonic development. Expression of *Hoxb-5* in the fetal rat lung that normally declines after 16 days of gestation was delayed by diabetes (Jacobs et al., 1998). This high level of *Hoxb-5* expression was suspected to be involved in delayed lung maturation of fetuses in diabetic mothers. Insulin-like growth factor binding protein-I (IGFBP-I), a protein that specifically binds IGF-I and determines its bioavailability to tissues and modulate its actions in target tissues, has been shown to be upregulated in fetal liver in response to diabetes (Rajaratnam et al., 1997). The increased expression of IGFBP-I would result in the reduction of availability of IGF-I and consequently affect fetal growth.

2.2: Immunoteratology : Effects of Immunomodulation on Teratogenesis

2.2.1: Reproductive immunology

The uterus immune axis holds the key to solving major problems in female reproductive health, including infertility, many pathologies of pregnancy, and sexually transmitted disease. The molecular determinants of tolerance and immunity in the reproductive tract are now being identified, and the governing principles are similar to those in other mucosal tissues. Cytokines are implicated as pivotal regulators at important 'decision-making' points in each phase of the induction and elicitation of a response (Robertson, 2000).

The uterus is part of the common mucosal immune system, sharing structural and functional similarities, and common lymphocyte trafficking networks, with the intestinal, bronchial, nasal-associated and ocular tissues, and salivary and mammary glands. Immune response in the uterus is exceptional among mucosal tissues in that ovarian steroid hormones also have considerable effects on both afferent and efferent immune events (Wira et al., 1999).

Decidual tissue contains a large population of bone marrow-derived cells including macrophages and an unusual population of large granulated lymphocytes (LGLs; NK-like cells). There are a small number of T cells but almost no B cells. Dissociation experiments

indicate that human decidua contains about 60% resident cells and 40% bone marrow-derived cells (Aplin, 2000).

The role of immune cell populations in pregnancy outcome can be addressed in mouse strains defective in specific lineages, with the obvious reservation that the results obtained reflect behavior under laboratory conditions and not in the wild. *Scid* mice lack both T and B cells and reproduce essentially normally (Croy and Chapeau, 1990). Mice of the *op/op* strain lack colony-stimulating factor-1 (CSF-1) and, in consequence, have a greatly reduced number of uterine macrophages (Hunt et al., 2000). The phenotype is complex with impairment of fertility at several levels: fewer pregnancies after mating, fewer implants and greater losses during gestation (Pollard et al., 1991). The major site of CSF-1 production is the uterine epithelium. The CSF-1 receptor is present in cells of the monocyte-macrophage lineage, but also in other decidual cells and trophoblast so that it is not possible to ascribe definitely the cause of reproductive abnormalities to the absence of macrophages.

Mice of the tgε strain lack LGLs; here reproductive efficiency is also compromised (Guimond et al., 1997). Ovulation and implantation occur as normal, but litter size is reduced, with an average of 6 as compared with 9 in the wild type. Fetal loss is observed beginning at day 10 of pregnancy. The offspring are smaller by about 30% and about 40% die before weaning. The placentas are about 45% smaller in cross-sectional area than controls. Furthermore, beginning on day 10, changes are seen in the decidual arterioles, with thickening of the walls followed by appearance of lipid deposit and foam cells, endothelial discontinuities and leakage of blood into surrounding tissues. Rescue can be effected by transplanting bone marrow from *scid* mice, confirming that the deficiency is specific to non-B, non-T bone marrow-derived cells (Guimond et al., 1998). A double null strain produced by crossing mice deficient in recombinase activating gene 2 (RAG2) with those deficient in the cytokine receptor common γ chain (GC), also lacks LGLs; these mice breed well and the placenta is normally grown. However, the midgestation deciduas contain fewer cells. Data from this animal, and from the IFNγRα knock out, show that although LGLs play no role in the initiation of decidualisation, they are important for maintaining decidual tissue and vascular architecture from midgestation to term, by virtue of the production of interferon-γ (IFN-γ) (Ashkar and Croy, 1999).

T lymphocytes are sparse in the uteri of non-pregnant mice (Croy et al., 1993), although repeated exposure to semen and multiple parity may increase the number of resident

T cells as exposure to antigen increases. Pregnancy is associated with a substantial increase in the number of αβ and γδ TCR^+ T cells, which comprise up to 20% of the total lymphocytes in midgestation deciduas (Arck et al., 1997). The functional significance of T cells in the uterine immune response is most clearly demonstrated by their associations with pathologies of pregnancy. An excess of type 1 activity in the implantation site is emerging as a key feature of disorders of pregnancy believed to have an immunological etiology, including spontaneous abortion and pre-eclampsia (Raghupathy, 1997). The detrimental effect of type 1 immunity is exerted on the growth and development of the placenta, through eliciting inflammatory and pro-thrombotic cascades that interfere with blood supply (Clark et al., 1998). Cell depletion experiments *in vivo* indicate that $CD8^+$ αβ T cells are absolutely necessary for immunological tolerance of the conceptus, and identify γδ T cells with a type 1 cytokine secretion profile as predisposing to pregnancy loss (Arck et al., 1997). The fact that pregnancy can proceed in T-cell-deficient *scid* mice indicates that the relative balance between these apparently beneficial and detrimental cell subsets is likely to be of paramount importance, rather than any trophic effect of T cells on placental development.

2.2.2: Effects of immunomodulation on pregnancy loss and teratogenesis

In has been abundantly clear for some time now that pregnancy can be compromised by a variety of factors, endocrinologic, genetic, anatomic, and infections. What has become apparent recently is that pregnancy can be affected both in positive and negative ways by immunologic factors. The maternal immune system can promote or inhibit the growth and survival of the feto-placental unit, but the precise mechanisms involved, the key mediators, their mode of action and the extent of the influence of the immune system on gestation are far from clear (Raghupathy and Tangri, 1996).

The positive growth-enhancing influences of the maternal immune system on the fetoplacental unit became evident from Wegmann's observations on maternal lymphoid cells and cytokines in pregnancy (Wegmann, 1984). The groundwork for this was laid by the studies of Beer et al. (1975, 1977), who showed that (1) mean litter sizes and mean placental weights are higher in allogeneic pregnancies than in syngeneic pregnancies, (2) litter sizes are higher in multiparous than in primiparous pregnancies, and (3) litter sizes and placental weights are higher in pregnant mice preimmunized with paternal strain lymphocytes. These lines of evidence and the idea that maternal immune responses may actually promote fetal survival coalesced into the Immunotrophism Hypothesis, which envisages important roles for

maternal immune reactivity, primarily mediated by maternal T cells, macrophages and cytokines (Wegmann, 1984). Wegmann (1986, 1988) proposed that these effectors of immune reactivity may promote fetal survival and well-being by enhancing placental growth and function. According to this hypothesis, allorecognition of the fetomaternal interface by maternal T cells results in the production of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3), which induce improved growth of the placental trophoblast and may thus promote fetal viability. In support of this contention is the demonstration of reduced fetal size and increased abortion rates upon injection of anti-T cell antibodies into mice prone to fetal resorption (Athanassakis et al., 1990). Athanassakis et al. (1987) also demonstrated increased proliferation and enhanced phagocytic activity of placental macrophages upon exposure to cytokine-rich culture supernatants; the cytokines responsible were subsequently shown to be GM-CSF, IL-3 and CSF-1 (Mogil and Wegmann, 1988). There is therefore little doubt that the maternal immune system performs important roles in protecting and nurturing the conceptus.

The role of the immune system in protecting the conceptus is further confirmed by a series of experiments using mouse models for pregnancy loss and chemical-induced teratogenesis.

The CBAxDBA/2 is a mouse model of natural spontaneous fetal resorptions. CBA/J females mated with DBA/2 males experience fetal resorption rates of up to 44%, while CBA/J female mated with males of other strains, including those of the same haplotype such as BALB/c, have low resorption rates of about 5-7% (Raghupathy and Tangri, 1996).

The evidence for an immunologic etiology for pregnancy loss in the CBAxDBA/2 model comes from the demonstration that fetal resorptions can be prevented by immunization of prospective CBA/J mothers with BALB/c cells prior to mating (Chaouat et al., 1985; Clark et al., 1987). Another study also showed evidence supporting the important role of immune cells in that protection against increased abortion brought about by alloimmunization can be adoptively transferred to virgin CBA/J females by sensitized T cells (Kiger et el., 1985).

Results published by Toder et al. (1990) showed that immunostimulation with complete Freund's adjuvant (CFA) might have a role in decreasing the frequency of spontaneous resorption in the CBAxDBA/2 model. It was speculated that CFA stimulation could enhance embryo survival by either increasing the production of maternal cytokines,

which may promote embryo or placental growth and development, or enhance the activation of maternal suppressor cells, which could regulate the maternal immune response to the embryo.

Observations by Baines et al. (1996) confirm the beneficial effects of immuno-stimulation in the CBAxDBA/2 model, and also in lipopolysaccharide (LPS)-induced abortion CFWxCFW mice. These investigators observed that prior immunization with both paternal specific (MHC) and non-specific antigens (LPS and CFA) decreased the frequency of spontaneous early embryo resorption in murine pregnancy. While the protective effect of vaccination with LPS to protect against LPS induced abortion was agent-specific, spontaneous abortion was reduced by both allospecific and non-specific immunostimulation. Furthermore, the abortion inhibiting effects of all these treatments were temporary, which seemed to be inconsistent with the expected duration of specific immune response or the anticipated half-life of specific immune mediators. The beneficial effects seen from vaccination to prevent pregnancy losses could therefore have been due to the activation of embryo enhancing factors or functions unrelated to paternal specific mediators of maternal immune response.

Immunostimulation has also proved to be helpful in case of chemical-induced teratogenesis. Intra-uterine immunization of mice with paternal allogeneic (MHC-associated) or xenogeneic (rat; MHC-non-associated) splenocytes was found to increase embryo tolerance to cyclophosphamide (CP)-induced teratogenesis (Torchinsky et al., 1994; 1995). The protective effect of these immunizations was manifested as a dramatic decrease of the proportion of malformed fetuses and the resorption rate. The embryotoxic effect of CP was shown to be accompanied by very prominent DNA degradation (apoptosis) in cells collected from whole embryos. Increasing fetal survival and fetal weight caused by immunostimulation were found to be accompanied by a clear decrease in apoptosis level in embryo cell populations (Toder et al., 1996).

Further investigations demonstrated changes in cytokine expression as a possible mechanism underlying CP-induced teratogenesis and the protective effect of immunostimulation. CP-treated embryos showed a significant increase in the intensity of TNF-α and its receptor mRNA transcripts, and TNF-α protein expression in cells of the malformed regions of the head and the brain (Ivnitsky et al., 1998). At the uteroplacental unit, CP caused a significant reduction of TGF-β2 and CSF-1 expression compared to control

animals (Gorivodsky et al., 1999a; 1999b). All of these changes were reversed or attenuated by maternal immunostimulation.

In addition to intra-uterine immunization with splenocytes, other means of immunostimulation were explored in order to prevent teratogenesis. Savion et al. (1999) injected GM-CSF by an intra-uterine route 3 weeks prior to mating and teratogenic induction by CP. GM-CSF treatment resulted in a significant decrease in the percentage of CP-treated embryos exhibiting limb malformations. CFA, bacillus Calmette Guerin (BCG), pyran copolymer, or interferon-γ were also used to stimulate immune system of female mice, and produced significant protection against fetal malformations caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), urethane, methynitrosourea, and valproic acid (Holladay et al., 2000).

Diabetic embryopathy is another teratogenic model that can be influenced by the use of maternal immunostimulation. Torchinsky et al. (1997) immunized ICR female mice with splenocytes of male rats 3 weeks before the beginning of mating and induced diabetes by streptozotocin (STZ) injection 10 days after immunization. Immunostimulated female mice with blood glucose over teratogenic levels produced significantly lower rates of malformed fetuses and resorptions. Other changes were also observed in maternal spleens, including increased spleen cellularity, increased mature T-cells, and increased macrophage surface markers. Study of TNF-α expression demonstrated a clear increase in both mRNA and protein expression in the uterus of these diabetic mice (Fein et al., 2001). However, maternal immunostimulation, while improving reproductive performance, was found to be accompanied by a reduced expression of TNF-α, both at the mRNA and protein level.

2.3: Summary

Diabetes caused changes in biochemical parameters of an affected pregnant animal including deficiency of myo-inositol, arachidonic acid, prostaglandins, and increased free radicals that may lead to changes in gene expression and organ functions of both maternal and embryonic tissues. These changes disturbed several processes of developing embryos including preimplantation, yolk sac development, placental damage and neural tube formation, which then ultimately caused fetal malformations. Because of this serious consequence, several preventive strategies have been studied including supplementation of

myo-inositol, arachidonic acid, prostaglandins, and antioxidants. Each of these methods produced partial protection on diabetic embryopathy. The reproduction immune system was also suspected to be disturbed by diabetes and may play a role in pathogenesis of diabetic embryopathy. Because the immune system plays an important role during pregnancy, technique of immune stimulation by various stimulies have been used to strengthen non-specific immune cells, which were believed to be major regulators, and produced a protective effect against spontaneous abortion and chemical-induced tertatogenesis. The immune-stimulation method was also effective for prevention of diabetic embryopathy as well.

2.4: References

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CHAPTER 3: IMMUNOSTIMULATION BY COMPLETE FREUND'S ADJUVANT, GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR, OR INTERFERON-γ REDUCES SEVERITY OF DIABETIC EMBRYOPATHY IN ICR MICE

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3.1: ABSTRACT

Increased risk of fetal malformation is a complication occurring in pregnant women with type-1 diabetes. Immune stimulation has been shown to prevent or reduce similar diabetes-induced teratogenesis in mice. Limited information is available regarding ability of diverse methods of maternal immune stimulation to cause this effect, or timing requirements of the immune stimulation. Diabetes was induced in pregnant ICR mice by streptozocin (STZ) injection. Three different techniques of maternal immune stimulation, complete Freund's adjuvant (CFA), granulocyte-macrophage colony-stimulating factor (GM-CSF), or interferon-γ (IFN-γ), were then used to stimulate the immune system of the mice. Approximately 50% of fetuses from hyperglycemic (>27 mM/L blood glucose) dams were malformed, with neural tube defects predominating. Maternal immune stimulation during the time of normoglycemia, i.e. prior to onset of hyperglycemia, was necessary with each of the immune stimulants for reducing teratogenic effects associated with hyperglycemia. The immune stimulated diabetic mice then produced significant lower and approximately equal numbers of malformed fetuses: CFA 20.9%, GM-CSF 23.3%, IFN-γ 13.9%. These results suggest that mechanistically diverse forms of non-specific immune activation result in protection against diabetes-related teratogenesis, but only if given prior to onset of hyperglycemia.

Keywords: Diabetes; Diabetic embryopathy; Immune stimulation; Teratogenesis; Birth defects; CFA; GM-CSF; IFN-γ

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3.2: INTRODUCTION

Diabetic embryopathy is a complication of diabetes, mostly type I, in which the offspring of a diabetic woman develops congenital anomalies. Insulin deficiency and hyperglycemia lead to abnormal embryonic development through multiple pathways, including myo-inositol depletion, arachidonic acid and prostaglandin deficiencies, oxygen free radical generation (Reece, 1999), and dysregulation of the uterine cytokine network (Pampfer, 2001). Based on these factors, strategic preventive studies have been conducted in animal model and have demonstrated varying levels of success in lowering birth defect incidence. These studies included supplementation with myo-inositol (Baker et al., 1986), arachidonic acid (Goldman et al., 1985; Pinter et al., 1986), and antioxidants such as vitamin E (Sivan et al., 1996), vitamin C (Siman and Eriksson, 1997), or lipoic acid (Wiznizer et al., 1999). Additional techniques including modulation of the uterine immune system have been investigated in an attempt to further decrease the risk of diabetic embryopathy (Torchinsky et al., 1997).

Proper responses of local immune cells in uterus such as uterine macrophages, large granular lymphocytes (LGLs) or uterine natural killer (NK) cells are vital for embryonic development during pregnancy (Head, 1996-97; Hunt and Robertson, 1996; Hunt et al., 2000). Uterine immune cells are under the influence of sex steroids to establish dominant status of non-specific immunity over specific immunity, for protection of the allogeneic embryo. A local cytokine network is also established in which arrays of cytokines and growth factors are secreted to communicate between maternal epithelial, decidual, and immune cells and embryonic cells (Robertson et al., 1994). Abnormalities in these processes could be deleterious to the developing embryo. For instance, in diabetic embryopathy, the cytokine tumor necrosis factor-α (TNF-α) increased significantly in uterine and embryonic tissues, an effect that correlated with increased level of fetal malformations (Pampfer et al., 1997; Fein et al., 2001). The latter results suggest the possibility that immune modulations

that reduce elevated TNF-α levels associated with diabetes may have beneficial effects to developing embryo.

Non-specific immune stimulation has been demonstrated to protect embryonic development against a variety of chemical teratogens, including cyclophosphamide (Torchinsky et al., 1995), urethane, methylnitrosourea, and valproic acid (Holladay et al., 2000, 2002). Such protection was associated with normalized cytokine expression including transforming growth factor-β2 (TGF-β2) (Gorivodsky et al., 1999) and TNF-α (Ivnitsky et al., 1998). These data raised questions regarding the possibility that immune stimulation might also help prevent diabetic embryopathy. Protective results observed in diabetic mice after a single intra-uterine vaccination by xenogeneic splenocytes suggested that such might be the case (Torchinsky et al., 1997).

The above collective reports led us to hypothesize that a strong non-specific immune response is beneficial in nurturing embryonic development and could counteract adverse morphogenic effects of diabetes. If such were the case, then diverse forms of non-specific immune activation should offer protection against diabetic embryopathy. To test this hypothesis, we investigated 3 non-specific immunostimulators, and several timings of immune stimulation in pregnant diabetic ICR mice.

3.3: MATERIALS AND METHODS

3.3.1: Animal Model

Male and female ICR mice, 6-8 weeks of age, were purchased (Harlan Sprague-Dawley, Indianapolis, IN) and housed individually (males) or at 5 per cage (females) for a 2-week acclimation period. For breeding, males were housed overnight with females, and females checked for vaginal plugs the next morning, which was designated day 0 of gestation. Mice were given food (NIH 31 open formula) and distilled water ad libitum, and were maintained under controlled conditions of temperature (72°F), humidity (40-60%) and lighting (14/10 light/dark cycle).

Diabetes was chemically induced in female mice by intraperitoneal injection with 200 mg/kg streptozocin (STZ) (Sigma, St.Louis, Mo., USA) 7 days before mating, or at different times during pregnancy (gestation day 0, 3, 6, 7, and 8). Blood glucose (BG) levels in tail vein blood were determined every 3-5 days post-STZ injection using glucose kits designed for this purpose (Sigma), and were used to categorize diabetic mice. Mice with average blood

glucose of 10-18 mM/L were designated as low BG group. Mice with average blood glucose of 19-26 mM/L were designated as moderate BG group. Mice with average blood glucose of 27 mM/L and over were designated as high BG group. Previous reports indicate significant hyperglycemia-associated teratogenesis occurs in mice when maternal BG levels exceed approximately 26 mM/L (Goldman et al., 1985).

3.3.2: Immune stimulations

To modulate maternal immune responses, female mice received one of the following: Complete Freund's adjuvant (CFA) (Sigma) 20-30 μl, by footpad injection; Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sigma) 8000 units, IP injection; or Interferon-γ (IFN-γ) (Gibco BRL) 1000 units, IP injection. These immune stimulation techniques had previously been found by us to reduce fetal defects in mice caused by chemical teratogens (Holladay et al., 2000; Sharova et al., 2000).

Two timings of immunostimulation regimens were employed. In the first regimen, mice were immune stimulated after diabetes was developed; CFA and GM-CSF were injected twice, at 4 days after STZ injection and at gestation day (GD) 6; IFN-γ was injected once at GD 10. In the second regimen, mice were immune stimulated before diabetes was developed; all of the immune stimulating agents were injected twice, at 1 week and then at 1 day before STZ injection. This pattern of immune stimulation that employed two injections of stimulants separated by approximately one week, followed that used by Sharova et al. (2000) to reduce morphologic defects in chemical-exposed mice.

3.3.3: Teratology

Pregnant mice were killed by cervical dislocation at GD 17. Fetuses were examined freshly for external defects, and then preserved in absolute ethanol for later detailed examination under a dissecting microscope. General information was recorded, including maternal spleen weight, total placental weight, number of total fetuses, number of live fetuses, number of resorptions or dead fetuses, and fetal weight. Collected live fetuses were examined externally for the major neural tube defect abnormalities including exencephaly, microcephaly, and spina bifida. Other abnormalities present were recorded as well. The percentage of abnormal live fetuses and abnormal plus dead per total fetuses were calculated for each litter. The pregnancy rate (proportion of plug positive females that became pregnant) was also calculated.

3.3.4: Statistical analysis

All data are presented as arithmetical mean ± SEM. Analysis of variance (ANOVA) was used with Dunnett's t test to establish significant differences between control group and the treatment groups. The Chi-square test was also used to test for differences across groups in pregnancy rate. Results described as different in this paper indicate significantly different at $p < 0.05$.

3.4: RESULTS

3.4.1: Induction of diabetes

The outbred ICR mice responded variably to the diabetes-inducing effect of STZ. The majority of mice treated 7 days before mating (47 of 68) had blood glucose levels over 26 mM/L, a level previously associated with increased teratogenesis (Goldman et al., 1985). Clinically these diabetic mice demonstrated signs of diabetes including polydipsia and polyuria. In all experiments, some STZ-treated mice developed lower level of blood glucose and were categorized into low (10-18 mM/L) and moderate (19-26 mM/L) BG groups. These mice also demonstrated symptoms of diabetes, primarily observed as polydipsia. STZ injections during pregnancy (at GD 0, 3, 6, 7, or 8) caused similar variable levels of blood glucose and symptoms associated with diabetes to that seen prior to breeding, as well as significant decreases in fetal weight. However, hyperglycemia induced during pregnancy by such STZ injection did not result in increased fetal defects (data not shown).

3.4.2: Relation between diabetic status and abnormalities

Abnormalities in reproductive performance and increased fetal defects were demonstrated following STZ injections 7 days before mating. These disturbances varied depending upon diabetic conditions. Pregnancy rate (% of plug-positive mice that became pregnant) was markedly reduced to about 32% in the high BG group (>27 mM/L) as compared to 100% in control, low and moderate BG groups (**Table 3.1**). However, in high BG mice that became pregnant the number of live fetuses was not reduced compared to control and moderate BG groups. The low BG group produced significantly higher numbers of fetuses than control. The low BG group also had significantly heavier fetuses, while high BG group had significantly lighter fetuses. Neural tube defects were observed in high BG fetuses as exencephaly and spina bifida with variable degree of severity (**Fig. 3.1**). Some fetuses had only one type of defect, while some displayed both. Other external abnormalities

were also observed, including open eyelids and open abdomen with visceral organs exposed to the outside. Diabetic mice in the low BG group produced 0.9±0.9% fetal abnormalities, which was not significant compared to 0.0% in control. Significant levels of fetal abnormalities were generated in the moderate BG and high BG groups: 20.6±7.4% and 50.1±6.95% respectively (**Fig. 3.2**).

3.4.3: Effects of immunostimulation on diabetic embryopathy

High BG mice caused by STZ injection 7 days before mating were selected as a positive group, i.e., mice showing a high percentage of morphologic defects for further immune stimulation experiments. Results of the first immune stimulation regimen (i.e., after hyperglycemia was developed) are shown in **Table 3.2**. Briefly, immune stimulation after diabetes was expressed did not improve the assessed parameters, with the exception that pregnancy rate was significantly improved after maternal GM-CSF administration. A trend toward increased pregnancy rate was present in the CFA and IFN-γ experimental groups. Morphologic defects were present in all groups, and were not affected by the immune stimulation procedures (**Fig. 3.3**).

Results of the second immune stimulation regimen are shown in **Table 3.3**. Both reproductive performance and fetal development were improved significantly by all methods of immune stimulation. Pregnancy rate was significantly higher after maternal injection with CFA (43%), GM-CSF (48%), or IFN-γ (64%) as compared to control (32%). The number of live fetuses per mouse was significantly increased by GM-CSF, and average fetal weight was significantly increased by IFN-γ. Fetal malformations were reduced significantly by CFA (20.9±4.48%), GM-CSF (23.3±6.33%), and IFN-γ (13.9±3.72%) compared to the positive control (50.1±6.95%) (**Fig. 3.4**).

3.5: DISCUSSION

Type I diabetes, also known as insulin–dependent diabetes mellitus (IDDM), is an autoimmune disease with both humoral and cell-mediated immune components. A tendency for skewed immune responses in diabetic patients persists during pregnancy, and may be exacerbated by pregnancy. Further, pregnancy serves as an inducing factor for expression of Type I diabetes in genetically predisposed individuals. Regardless of time of onset of this immune-related disease, abnormally high circulating TNF-α during early embryogenesis has been reported and suggested as a cause of increased cell death in the blastocyst (Pampfer et

al., 1997). Elevated hyperglycemia-related uterine TNF-α was recently also observed in late pregnancy, with unknown consequences (Fein et al., 2001). Local immune cells including macrophages are functionally abnormal under the influence of hyperglycemia and produce TNF-α (Morohoshi et al., 1996), thus may in part be responsible for an altered uterine cytokine profile (Pampfer, 2001). Modulating the maternal immune response in IDDM-prone mice by therapies aimed at activating macrophages, e.g., injection of CFA (Sadelain et al., 1990) or bacillus Calmette-Guerin (BCG; Lakey et al., 1997), resulted in a lower frequency of diabetes developed in these animals. The same or related techniques of non-specific immune stimulation resulted in reduced incidence of diabetic embryopathy in the present mice.

We initially attempted to induce diabetes in mice by STZ injection during early pregnancy. This procedure caused pregnancy failure, resulting in a 10% pregnancy rate when STZ was injected at GD 0. Such reduced pregnancy rate may be an effect of hyperglycemia, stress of handling or direct toxicity of STZ. Malformed fetuses were not observed in these mice, however only a few pregnant mice resulted and were evaluated. When STZ was injected at GD 3, 6, 7, or 8, no increased fetal abnormalities were observed. Hyperglycemia is not maximal until 2-3 days post-STZ injection, thus the sensitive time of diabetic teratogenesis may be during the first 5 days of embryogenesis in mice. This observation is in accord with literature suggesting diabetic embryopathy in humans occurs before 8 weeks of pregnancy (Buchanan and Kitzmiller, 1994). In the present mice, heavy fetuses or macrosomias were seen with moderate hyperglycemia, and small fetuses or growth retardation was seen with more severe hyperglycemia.

STZ injection one week prior to breeding of mice caused a significant increase in malformed fetuses, to 50.1%, and was therefore selected for use in maternal immune stimulation experiments. The three methods used for maternal immune stimulation in the present mice clearly have different scopes of action, however all three produce highly effective non-specific immune activation. Activation of such cells, specifically macrophages, was suggested as necessary for immune protection against structural defects caused by chemical teratogens (Nomura et al., 1990). CFA is well known for being an adjuvant to enhance antigenicity, an important step in macrophage activation. GM-CSF is a cytokine with a more specific range of activity, targeting granulocytes and macrophages. This cytokine has also been implicated as an important placental regulatory molecule during

pregnancy (Robertson et al., 2000). Although IFN-γ can affect development of Th1 cells, e.g., part of specific immunity, this cytokine has a strong stimulating effect on macrophages and natural killer (NK) cells (Ashkar et al., 2000). IFN-γ is also well known for its effects on early embryonic loss if presented at a high level, however IFN-γ in the present mice was administered seven days before pregnancy and as such would not be expected to directly target developing embryos.

It is important to note that maternal immune stimulation with CFA, GM-CSF or IFN-γ produced a similar result on fetal maldevelopment, in the form of reduction of neural tube defects from 50% in non-stimulated mice to 14-23% in stimulated mice. This result may suggest the importance of macrophage activation in the immune protection mechanism. Immune stimulation also improved the pregnancy rate of diabetic mice for all three stimulants, however slightly less similar results were obtained. CFA, GM-CSF and IFN-γ resulted in 1.34, 1.50, and 2.00 fold increased pregnancy rates, respectively. These differential responses could relate to different effects of the immune stimulants on macrophages or other immune cells, for instance the stimulatory effect of IFN-γ on Th1 cells.

Operating mechanisms to explain the beneficial effects of the present immune stimulation procedures remain to be determined. Innate immune cells and their cytokines play important roles during pregnancy and should logically be evaluated as potential mediators. The specific arm of the maternal immune system is suppressed during pregnancy, an effect that is in part compensated for by activation of the non-specific immune system (Sacks et al., 1990). Lacking of innate immune cells such as uterine NK cells causes reproductive deficiency, which can be rescued by transplanting bone marrow from *scid* mice, confirming a beneficial effect specific to non-B, non-T bone marrow derived cells (Guimond et al., 1998). It has also been suggested that cytokine products of activated immune cells could cross the placenta and interact with embryonic tissues in a supporting manner (Sharova et al., 2000; Holladay et al., 2002). This possibility may be supported by evidence that TGF-β1 can cross the placenta and rescue TGF-β1 null mice from embryolethality (Letterio et al., 1994). Additional cytokines that cross the placenta and may exert regulatory activity in the fetus include IFN-α, CSF, and G-CSF (Holladay et al., 2002).

Placental macrophages are also an important source of interleukin-1 (IL-1) (Flynn et al., 1982). Hyperglycemia inhibits IL-1 release but not production by macrophages (Hill et al., 1998). Hyperglycemia stimulates protein kinase C (PKC) activity, which consequently

inhibits the production of cGMP, whereas IL-1 release by macrophages is mediated by nitric oxide via increased cGMP levels (Hill et al., 1996). Activated macrophages produce high levels of nitric oxide, which could counteract the effect of PKC stimulation by hyperglycemia, thereby increasing IL-1 release. IL-1 in turn stimulates uterine prostaglandin production through induction of cyclooxygenase-2 (COX-2) enzyme expression (Rauk and Chiao, 2000; Guan et al., 1998). COX-2 is important for the initiation of decidualisation (Lim et al., 1997), and again was suppressed by diabetes (Wentzel et al., 1999). These pathways represent additional potential targets by which maternal immune stimulation may affect fetal development.

One might argue that activated macrophages could do more harm than good to pregnancy, thus are not likely effector cells in immune protection against diabetes-induced embryopathy. Spontaneous early embryo loss in CBA/J x DBA/2 mice has been demonstrated to associate with macrophage activation and increasing levels of inducible nitric oxide synthetase (iNOS) and TNF-α (Haddad et al., 1997). However, early embryo loss in the same mouse model was protected by maternal immune stimulation in several reports (Toder et al., 1990; Strassburger et al., 1992; Gorivodsky et al., 1998). The macrophage activation in CBA/J x DBA/2 mice that display spontaneous embryo loss may therefore be different in terms of timing or cytokine production from models in which macrophage activation has been associated with improved pregnancy rate and reduced fetal morphologic defects.

In summary, the present study demonstrates the beneficial effect of non-specific immune stimulation on diabetic embryopathy. The timing of the immune stimulation, as well as induction of hyperglycemia, was important for demonstrating this protective effect on fetal morphogenesis. Specifically, immune stimulation during the time of normoglycemia appeared to be necessary for reducing the teratogenic effects associated with subsequent hyperglycemia. All three methods of maternal immune stimulation produced approximately equal protection against neural tube-related defects, suggesting a similar underlying operating mechanism(s). However, maternal IFN-γ injection resulted in the greatest numeric improvement in both morphologic defect occurrence and diabetes-reduced pregnancy rate, suggesting that Th1 stimulation or other targets of IFN-γ may offer further benefits toward reducing diabetic embryopathy. Additional experiments that use combined methods of immune stimulation, e.g., IFN-γ + GM-CSF, will be needed to determine if additive effects

(i.e., non-overlapping mechanisms) may further improve immune protection against hyperglycemia related fetal defects.

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Table 3.1 Maternal and fetal parameters at GD 17 in STZ-induced diabetic ICR mice

	Control	Low BG	Moderate BG	High BG
# of mice	11	10	11	15
Pregnancy rate (%)	100	100	100	32*
# of total fetuses /mouse (mean ± sem)	11.2±0.57	14.1±0.57*	13.1±0.60	12.0±0.32
# of live fetuses /mouse (mean ± sem)	10.8±0.69	13.6±0.58*	11.7±0.65	9.6±0.38
Fetal weight (g) (mean ± sem)	0.89±0.08	1.10±0.05*	0.90±0.04	0.76±0.02*
Placental weight (g) (mean ± sem)	N.A.	0.123±0.003	0.121±0.004	0.118±0.004
Spleen weight (g) (mean ± sem)	N.A.	0.167±0.013	0.224±0.04	0.147±0.011
Abnormal plus dead /total fetuses (%) (mean ± sem)	3.6±2.45	4.4±1.88	27.0±7.61*	59.9±5.85*

* statistically significant ($P < .05$)

Table 3.2 Maternal and fetal parameters at GD 17 in STZ-induced diabetic ICR mice, with or without immune stimulation after occurrence of hyperglycemia

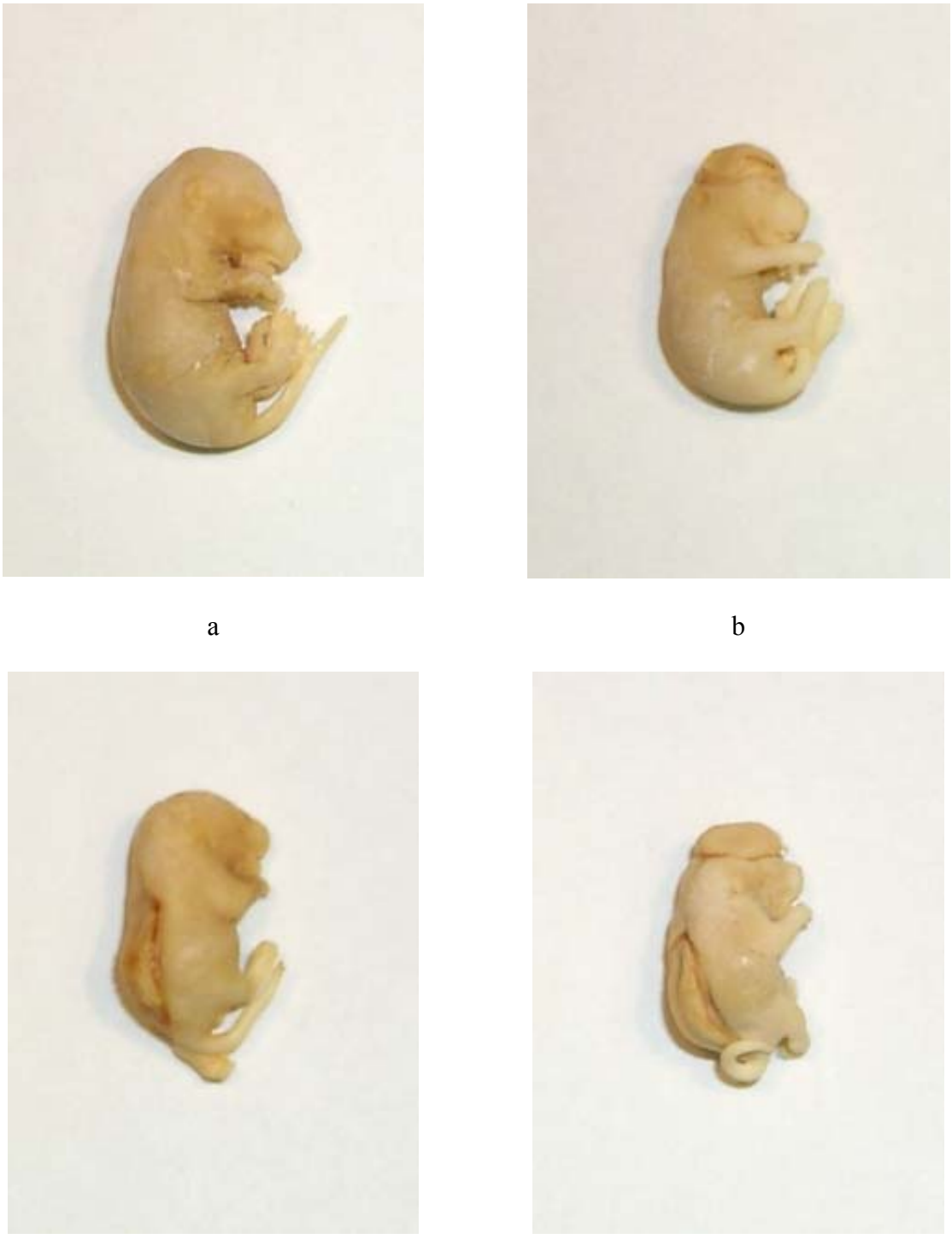
	High BG Positive control	High BG Plus CFA	High BG Plus GM-CSF	High BG Plus IFN-γ
# of mice	15	8	10	8
Pregnancy rate (%)	32	42	47*	42
# of total fetuses /mouse (mean ± sem)	12.0±0.32	10.6±0.57	11.3±0.54	11.8±0.53
# of live fetuses /mouse (mean ± sem)	9.6±0.38	8.3±0.75	7.1±1.01	9.0±1.09
Fetal weight (g) (mean ± sem)	0.76±0.02	0.79±0.06	0.71±0.05	0.69±0.05
Placental weight (g) (mean ± sem)	0.118±0.004	0.128±0.006	0.114±0.005	0.116±0.003
Spleen weight (g) (mean ± sem)	0.147±0.011	0.177±0.021	0.122±0.014	0.155±0.019
Abnormal plus dead /total fetuses (%) (mean ± sem)	59.9±5.85	68.1±8.31	74.1±8.7	54.3±9.31

* statistically significant ($P < .05$)

Table 3.3 Maternal and fetal parameters at GD 17 in STZ-induced diabetic ICR mice, with or without immune stimulation prior to occurrence of hyperglycemia

	High BG Positive control	High BG Plus CFA	High BG Plus GM-CSF	High BG Plus IFN-γ
# of mice	15	10	10	14
Pregnancy rate (%)	32	43*	48*	64*
# of total fetuses /mouse (mean ± sem)	12.0±0.32	11.6±0.37	13.3±0.76	11.6±0.66
# of live fetuses /mouse (mean ± sem)	9.6±0.38	10.2±0.36	12.2±0.76*	10.6±0.67
Fetal weight (g) (mean ± sem)	0.76±0.02	0.82±0.03	0.77±0.03	0.86±0.03*
Placental weight (g) (mean ± sem)	0.118±0.004	0.116±0.003	0.115±0.004	0.133±0.005*
Spleen weight (g) (mean ± sem)	0.147±0.011	0.177±0.015	0.14±0.007	0.188±0.007*
Abnormal plus dead /total fetuses (%) (mean ± sem)	59.9±5.85	29.8±5.6*	30.0±5.56*	21.8±3.81*

* statistically significant ($P < .05$)



a b

c d

Fig. 3.1 Mouse fetuses at gestation day 17 showing neural tube defects caused by diabetes. a) normal; b) exencephaly; c) spina bifida; d) exencephaly and spina bifida

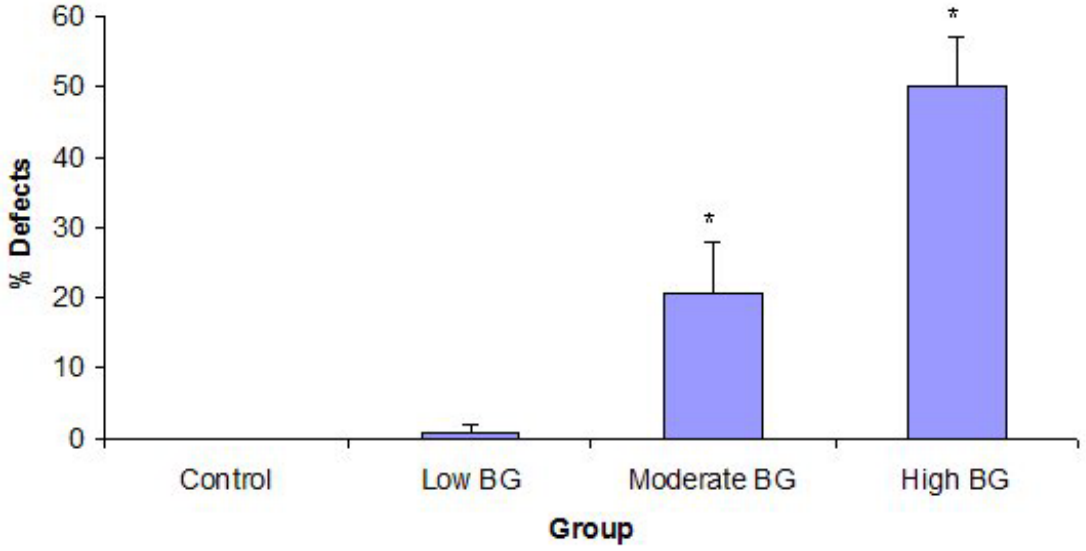


Fig. 3.2 Percentage of abnormal fetuses in STZ-induced diabetic ICR mice. * p < .05, Dunnett's t test. Mice were treated with STZ 7 days before mating and categorized into 3 groups (low BG, moderate BG, and high BG) based on their average blood glucose levels. Pregnant mice were sacrificed at gestation day 17, and their fetuses were examined for neural tube defects and other abnormalities. Moderate and high BG groups produced significant levels of abnormal fetuses, 20.6±7.4% and 50.1±7.0% respectively.

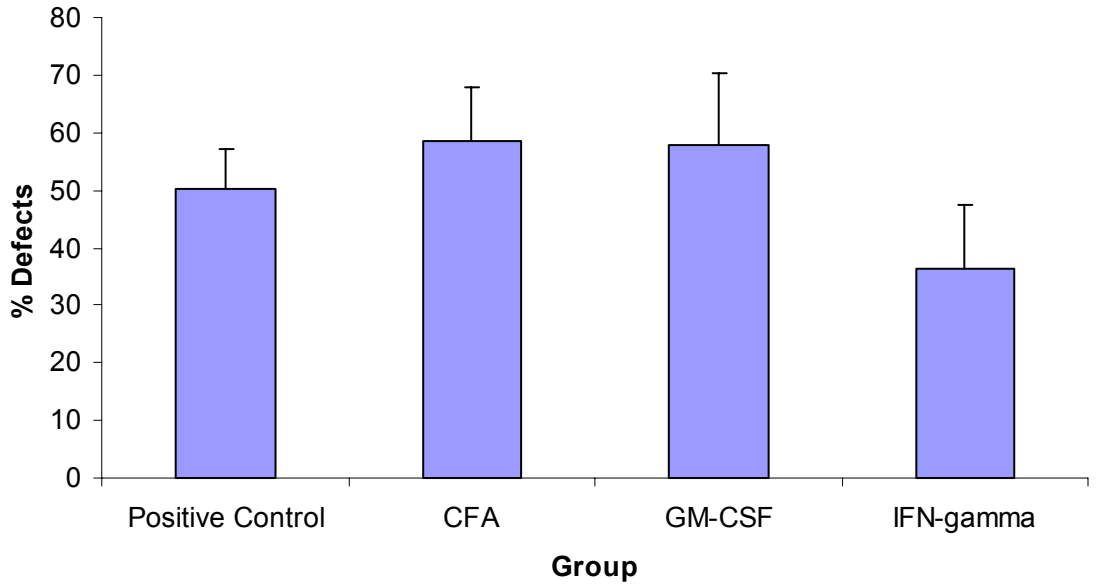


Fig. 3.3 Percentage of fetal abnormalities in STZ-induced diabetic ICR mice with or without maternal immune stimulations after induction of hyperglycemia. Mice were treated with STZ 7 days before mating, and used as a positive control. Three groups of mice that were treated with STZ 7 days before mating and either CFA, GM-CSF or IFN-γ 3 days later were used as treatment groups. Pregnant mice were sacrificed at gestation day 17, and their fetuses were examined for neural tube defects and other abnormalities. All treatments demonstrated no significant effects.

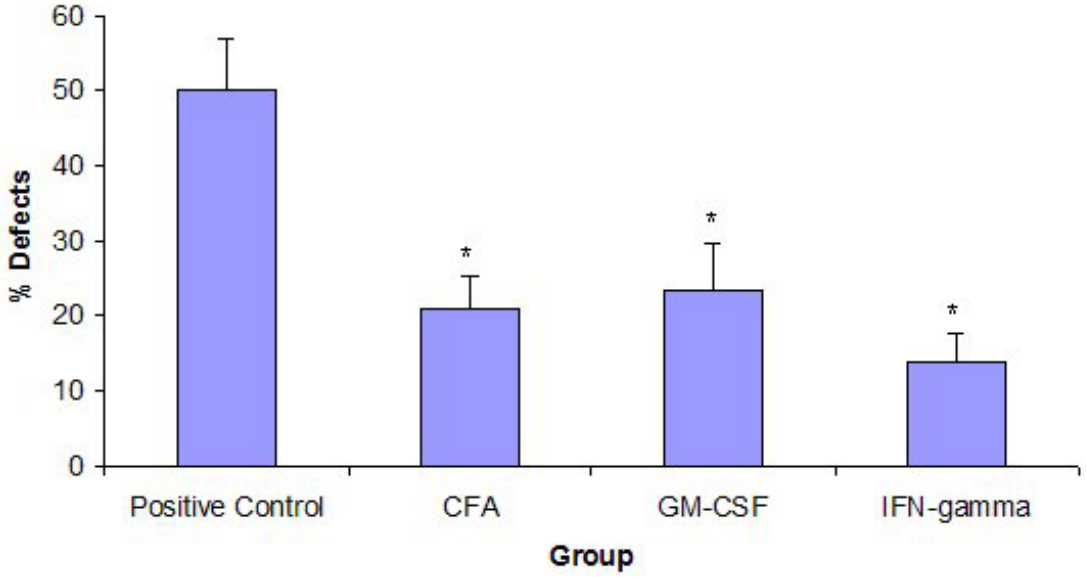


Fig. 3.4 Percentage of fetal abnormalities in STZ-induced diabetic ICR mice with or without maternal immune stimulation prior to induction of hyperglycemia. * $p < .05$, Dunnett's t test. Mice were treated with STZ 7 days before mating, and used as a positive control. Three groups of mice that were treated with CFA, GM-CSF or IFN-γ, and 1 week later with STZ which was 7 days before mating were used as treatment groups. Pregnant mice were sacrificed at gestation day 17, and their fetuses were examined for neural tube defects and other abnormalities. All treatments demonstrated significant lower percentage of fetal abnormalities; positive control (50.1±7.0%), CFA (20.9±4.5%), GM-CSF (23.3±6.3%), and IFN-γ (13.9±3.7%).

CHAPTER 4: REDUCED BIRTH DEFECTS CAUSED BY MATERNAL IMMUNE STIMULATION IN DIABETIC ICR MICE: LACK OF CORRELATION WITH PLACENTAL GENE EXPRESSION

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4.1: ABSTRACT

Maternal immune stimulation in mice decreases expression of morphologic defects caused by diverse chemical teratogens, hyperthermia, x-rays or diabetes mellitus. Diabetes mellitus alters placental structure and function, events that may be related to embryopathy. Three different methods of maternal immune stimulation that result in approximately equal reduction of diabetic embryopathy were used in the present studies: footpad injection with complete Freund's adjuvant, intraperitoneal (IP) injection with granulocyte-macrophage colony stimulating factor (GM-CSF), or IP injection with interferon-gamma (IFN-γ). A gene microarray was then used to examine expression of a selected panel of 151 placental genes. We hypothesized that the maternal immune stimulation may act by overcoming an embryopathy-inducing effect of diabetes on the placenta, and that such might be detected by a shared profile of placental gene expression changes induced by the different immune stimulation procedures. The immune stimulation that caused the greatest reduction in birth defect incidence, IFN-γ, did not significantly change placental gene expression profile as compared to control or diabetes. CFA and GM-CSF significantly changed placental gene expression relative to control or diabetes, but differentially affected such genes relative to each other. No common pattern of improved cytokine, cell-cycle, apoptotic, transcription factor, or other gene expression was identified in placentae of the immune stimulated mice, that might explain the ability of this procedure to reduce fetal birth defects. These data

suggest that maternal immune stimulation reduces birth defects in diabetic mice by a mechanism independent of placenta.

Keywords: Diabetes, diabetic embryopathy, teratogenesis, birth defects, gene expression, CFA, GM-CSF, IFN-γ

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4.2: INTRODUCTION

Diabetic embryopathy is a complication of type I diabetes. Affected newborns display some form of congenital malformation, including neural tube defects, heart defects, and kidney defects. Pregnancy failure and fetal death are also parts of this condition. In humans, these malformations usually occur during early pregnancy, prior to 8 weeks after conception (Buchanan and Kitzmiller, 1994). Investigations of the underlying mechanisms demonstrate that multi-factors are involved, including myo-inositol depletion, arachidonic acid and prostaglandin deficiencies, oxygen free radical generation (Reece, 1999), and dysregulation of the uterine cytokine network (Pampfer, 2001).

Besides assessment of biochemical changes, studies of gene expression have revealed how cells responded to diabetic conditions, and demonstrated roles in mediating teratogenesis. In embryonic tissues of diabetic mice, expression of Pax-3 gene decreased significantly, about threefold, compared with that in embryos of non-diabetic mice (Phelan et al., 1997). Pax-3 is expressed in neuroectoderm, neural crest, and somatic mesoderm and is required for closure of the neural tube at the rostral and caudal neuropores, development of neural crest, and limb musculature (Daston et al., 1996; Marato et al., 1997). Expression of nerve growth factor (NGF) was also decreased in embryos of diabetic mice (Germani et al., 1999). In maternal tissues of diabetic mice such as uterus and placenta, aberrant gene expression has been reported including tumor necrosis factor-α (TNF-α) (Pampfer, 2001; Fein et al., 2001), epidermal growth factor receptor (EGF-R) (Fujita et al., 1991), and glucose transporters (GLUTs) (Jansson et al., 1999; Hahn et al., 2000).

Proper immune responses are associated with the success of pregnancy. Local utero-placental immune cells and cytokine network together establish a condition that promotes

placental and embryonic growth (Robertson, 2000). Besides these local immune responses, systemic immune organs like spleen have a role during pregnancy (Tsoukatos et al., 1994). Interference with such immune responses during pregnancy could therefore produce an adverse effect on the pregnancy outcome. In the case of diabetic embryopathy, there are evidences demonstrating adverse effects of hyperglycemia on immune cells (Hill et al., 1998), and disturbance of cytokine levels at the utero-placental tissues that associated with fetal malformations (Pampfer , 2001; Fein et al., 2001; 2002).

In the present study, we examined the hypothesis that the immune system of pregnant mice may be disturbed by diabetes, and this could play a role in diabetes-related birth defects. We employed immunostimulation in streptozocin-induced diabetic mice, a new strategy that has been shown to be protective against diabetes-induced teratogenesis (Torchinsky et al., 1995; Holladay et al., 2002). The outcome of fetal defects was then compared to gene expression profile of placenta to suggest molecules or pathways that might be responsible for reduced diabetic embryopathy in offspring of immune-stimulated pregnant mice. Placenta was a tissue of choice for examination as it is an interface between the mother and a fetus. Specifically, the placenta provides nutrients, growth factors, hormones, and a variety of secretory proteins (Petraglia et al., 1996), and also contains a large number of immune cells that function as part of the innate immune system during the pregnancy (Guleria and Pollard, 2000).

4.3: MATERIALS AND METHODS

4.3.1: Animal model

Male and female ICR mice, 6-8 weeks of age, were purchased (Harlan Sprague-Dawley, Indianapolis, IN) and housed individually (males) or at 5 per cage (females) for a 2-week acclimation period. For breeding, males were housed overnight with females, and females checked for vaginal plugs the next morning, which was designated day 0 of gestation. Mice were given food (NIH 31 open formula) and distilled water ad libitum, and were maintained under controlled conditions of temperature (72°F), humidity (40-60%) and lighting (14/10 light/dark cycle).

Diabetes was chemically induced in female mice by intraperitoneal (IP) injection with 200 mg/kg streptozocin (STZ) (Sigma, St.Louis, Mo., USA) 7 days before mating. Blood glucose (BG) levels in tail vein blood were determined every 3-5 days using glucose kits

(Sigma), and were used to categorize diabetic mice. Mice with average blood glucose ≥ 20 mmol/L were previously found to produce fetuses with increased birth defects (Punareewattana and Holladay, 2003) and were used in this study.

4.3.2: Immune stimulations

To modulate maternal immune responses, female mice received one of the following: Complete Freund's adjuvant (CFA) (Sigma) 20-30 μl, by footpad injection; Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sigma) 8000 units, IP injection; or Interferon-γ (IFN-γ) (Gibco BRL) 1000 units, IP injection. All of the agents were injected twice, at 1 week and 1 day before STZ injection. These immune stimulation techniques had previously been found by us to reduce fetal defects in mice caused by chemical teratogens (Holladay et al., 2000; Sharova et al., 2000).

4.3.3: Sample collection and teratology

Pregnant mice were killed by cervical dislocation at gestation day (GD) 17. Placentas were collected and snap frozen for later use in gene expression studies. Fetuses were examined freshly and then preserved in absolute ethanol for later detailed examination. Placental weight, number of total fetuses, number of live fetuses, number of resorptions or dead fetuses, and fetal weight were recorded. Collected live fetuses were examined externally for the major neural tube defect abnormalities including exencephaly, microcephaly, and spina bifida. Other abnormalities present were recorded as well.

4.3.4: Microarray preparation

One hundred fifty-one sequence-verified I.M.A.G.E. Consortium clones were purchased from ATCC (Manasas, VA) and Incyte Genomics Inc (Palo Alto, CA). Genes selected for study included growth factors, cytokines, cell cycle genes, apoptotic genes, transcription factors, kinases, oncogenes and tumor suppressors (**Table 4.1**). Equimolar amounts of cDNA generated from the clones by PCR were immobilized on nylon membranes (Hybond N+, Amersham) with VP 384S2 multi-blot replicator and the Vicki Ultra High Density Array & Registration system (V&P Scientific Inc, San Diego, CA) and cross linked by UV irradiation using a TL-2000 UV Translinker (UVP, Upland, CA), as previously described (Sharova et al., 2003).

4.3.5: cDNA synthesis and hybridization

Six or more placental samples from each experimental group were used for gene expression comparison. Total RNA from each placenta was extracted with Tryzol^{TM} (Gibco,

Rockville, MD) according to the manufacturer protocol, and dissolved in diethyl pyrocarbonate (DEPC; Sigma)-treated dH_2O. Two μg of total RNA were combined with 50 ng of random hexamers, and the mix was subjected to 3 cycles of heat denaturation. Double-stranded cDNA synthesis was performed using the AMV RT and Universal Riboclone® cDNA Synthesis System (Promega, Madison, WI) according to the manufacturer protocol. ^{32}P-labeled probe was synthesized from ds-cDNA with the Prime-a-Gene® Labeling System (Promega) according to the manufacturer protocol.

The membranes were pre-hybridized for 15 min at 42°C in: 50% formamide; 0.12 M Na_2HPO_4, pH 7.2; 0.25 M NaCl; 7% SDS, then added with heat denatured ^{32}P-labeled probe, and later incubated overnight at 42°C. After-hybridization membranes were washed in: 2x SSC/0.1% SDS 45 min; 0.5x SSC/0.1% SDS 45 min; 0.1x SSC/0.1% SDS 45 min. The membranes were then exposed and scanned using the phosphoimager system from Molecular Dynamics® (Amersham Biosciences, Piscataway, NJ). Resultant electronic images of membranes were analyzed by ImageQuant® Software (Molecular Dynamics®), after which gene expression data were stored on spreadsheet software for statistical analysis. All data were normalized relative to internal β-actin controls.

4.3.6: Statistical analysis

Gene expression differences across treatment groups were examined by three different data analysis procedures:

a) ANOVA was used to identify individual genes whose expression was different in a treatment group as compared to control, at $p < 0.05$. This comparison was performed using the General Linear Model (GLM; Minitab version 13; Minitab Inc., PA).

b) Principal components analysis (PCA) was used to search for shifts in gene clusters or families, to provide insight into coordinate aspects of gene expression. PCA, a branch of multivariate analysis, is a data reduction technique used to create a small set of uncorrelated variables that account for a large proportion of the total variance in the original variables (Sokal and Rohlf, 1969). The PCA analysis was performed using the SAS system computer program (SAS Institute Inc., Cary 27513, NC). A scree plot was constructed to estimate the number of PCs to attempt to interpret. Attention was focused on those PCs that explained at least 5% of the variation in gene expression. These selected PCs were then subjected to ANOVA to test for treatment effects. PC pairs were also plotted against each other to show co-ordinate space effects on gene expression by treatments.

4.4: RESULTS

4.4.1: Diabetic teratology and effects of immune stimulations

STZ-induced diabetic mice displayed variable hyperglycemia ranging from 10 to 30 mmol/L. Mice with blood glucose level ≥ 20 mmol/L were used as the diabetic group. Fetal malformations in these diabetic mice were significantly increased, predominantly in the form of neural tube defects expressed in fetuses as exencephaly and/or spina bifida with variable degree of severity. Other external abnormalities were also observed, including open eyelids and open abdomen with visceral organs exposed to the outside. We previously reported that abnormalities occurred in 50.1±6.95% of fetuses from these diabetic mice; whereas the control mice displayed no defects; and fetuses from immune stimulated dams displayed significant reductions in defects: CFA: 20.9±4.5%; GM-CSF: 23.3±6.3%; and IFNγ: 13.9±3.7% (Punarrewattana and Holladay, 2003). For the present report, total RNA was extracted from pooled placental tissue collected from each of these control and treatment groups, and reverse transcribed to cDNA. The level of expression of all genes was normalized relative to internal β-actin control and stored in spreadsheets. Measurement of RNA and cDNA concentrations showed significantly higher recoveries for the IFN-γ group over all other groups (data not shown).

4.4.2: Gene expression comparisons and gene relations

4.4.2.1: Gene expression changes relative to control

The comparative gene expression data for all experimental groups were calculated as mean ± sem, along with ANOVA p-values for each gene. Ten of the 151 genes tested were observed with p-values below .05 (**Table 4.2**), and corresponded to differences between non-immune stimulated and immune stimulated diabetic mice. Control and diabetic groups were generally not different in gene expression profile. Genes that changed significantly in the group of diabetes ± CFA included bcr, GAS2, IGF-I, NF-kB-1, NF-kB-2, STAT3, and TGF-β1 (Table 4.2). Genes that changed significantly in the group of diabetes ± GM-CSF included cadherin 3, coagulation factor II, GAS2, Syk, and STAT3. Three genes showed near-significant effects at .10 > p > .05. Expression of GM-CSF (p-value 0.07) tended toward increase by CFA, whereas caspase-6 (p-value 0.08) and TNF-α (p-value 0.08) by GM-CSF.

In addition to ten genes significantly affected by treatment, the ANOVA revealed a relatively large number of genes whose expression was different by > ±50% from control

(**Table 4.3**). This included a small number of genes in the diabetic group (11) and the diabetes plus IFN-γ group (6), and a larger number of genes in the diabetes plus CFA group (33) and the diabetes plus GM-CSF group (48). Most of these genes displayed a numerically higher expression level than control. To visualize this more global effect of each treatment on gene expression, the expression level of Table 4.3 genes was plotted relative to control (**Fig. 4.1**). These graphs again show that expression level for the selected genes was not greatly different in placental tissue of control and diabetic mice (Fig. 4.1 a), and was not changed by the IFNγ immune stimulation (Fig. 4.1 b). In contrast, the maternal CFA or GM-CSF immunizations resulted in a general pattern of up-regulation of placental gene expression (Fig. 4.1 c-d).

4.4.2.2: Gene expression changes that were linked to another changed gene

Changes in expression level of individual genes may be less meaningful than changes in functionally-related gene pairs (Sharova et al., 2000). To search for genes with linked altered expression, raw data for each gene across all groups were used to plot correlation graphs with other all genes. Approximately 22,000 regression graphs were produced and evaluated by visual inspection for evidence of positive or negative correlation. For graphs where a correlation was suggested, regression analysis was used to determine if a significant correlation existed. Representative correlation graphs shown in **Fig. 4.2** demonstrated a positive relationship between p21 and R-ras gene expression (a), and a negative correlation between p21 and bcl2 expression (b). The numbers of such co-regulated genes detected were small and showed no apparent consistent relationship to the immune stimulation treatments and the reduced birth defect outcome of interest (data not shown).

4.4.3: Principal components analysis of gene expression

Given the relatively large number of genes showing non-significant but >50% change in gene expression level, all data from the 5 experiment groups were re-analyzed by principal components analysis. Each principal component had an eigenvalue that represents variability attributed to specific genes considered by the PC. A scree plot showing eigenvalues of these PCs in order is presented (**Fig. 4.3**), and shows three PC with eigenvalue > 10.0, suggesting latent coregulation of gene expression. PCs 1-4 contained genes that accounted for >56% of the total variance detected by the PCA, and were selected on this basis and analyzed for a treatment-related effect using ANOVA (**Table 4.4**).

A significant effect was found in PC 3 by the treatment CFA, and in PC 4 by the treatment GM-CSF. These results agree with and strengthen the visual suggestion of effect of these treatments that was seen in Fig. 4.1 c-d.

The PCA comparisons are shown graphically by box plot in **Fig. 4.4**. These graphs demonstrate that the gene expression eigenvalue of the CFA group in PC 3 is different from the others (middle bar of Fig. 4.4c); a similar but less dramatic effect on gene expression by the treatment GM-CSF was seen in PC 4 (2^{nd} bar from right in Fig. 4.4d). Total shifts in coordinate space gene expression profiles by treatment can be visualized by plotting the PC that contains high variability against each other. **Fig. 4.5** shows such PC pairs for each treatment. In each case, coordinate gene expression for the control, diabetes, and IFN-γ groups share relatively close PC coordinate positions. The CFA and GM-CSF coordinate gene expression is not only shifted away from these former three groups, but the CFA and GM-CSF profiles are not similar to each other.

The PCA analysis suggested the importance of gene variability in PC 3 and 4. The twenty specific genes that most affect PC 3 and 4, both positively and negatively, are shown in Tables 4.6 and 4.7. The genes that most affected PC 3 and were up-regulated included bcl2, AIF, NF-kB-1, Bcr, Proliferin, GAS-2, BID, NF-kB-2, Annexin A6, and Myc. Genes that most affected PC 3 and were down-regulated included p21 (waf), R-ras, Prostaglandin D synthase, Prolactin, Egr-2, PIAS-1, Rxra, IL-2, and IGF-II. The genes with positive PC score in PC 4 were all close to control. The genes with negative PC score in PC 4 represented up-regulated genes, and included GAS-5, E2f1, TIMP-2, MMP-2, DHFR, Mekk, Rxra, c-fos, cytochrome C, Rb1, caspase-6, Syk, IL-16, cadherin 3, and IL-9R.

4.5: DISCUSSION

Altered patterns of gene expression have been related to disease processes associated with insulin dependent diabetes mellitus. Genes that have been found to be up-regulated in different tissues of mature diabetic animals have included protein kinase C (PKC) (Koya et al., 1998), c-jun (Zayzafoon et al., 2000) mitochondrial-encoded genes (Antonetti et al., 1995), vascular endothelial growth factor (VEGF) (Cooper et al., 1999), TGF-β1 (Gilbert et al., 1998), and neuronal nitric oxide synthase (nNOS) (Serino et al., 1998). Down-regulated genes included renal IGF-I (Connors et al., 1997), hepatic hydroxysteroid sulfotransferase-a

(HST-a) and aryl sulfotransferase IV (ASTIV) (Runge Morris and Vento, 1995), and corticotrophin-releasing hormone (CRH) (Schwartz et al., 1997).

Altered regulation of gene expression, in maternal and embryonic tissues, has similarly been associated with diabetic embryopathy. Dysregulation of cytokines, specifically TNF-α and TGF-β2, and increased apoptosis in uterus and placenta have been proposed as causative factors in diabetic embryopathy (Fein et al., 2001; 2002). Changes in placental morphology (Giavini et al., 1986) and glucose tranporter proteins (Boileau et al., 1995) have also been reported, however no evidence indicated that these changes were associated with fetal defects.

For unknown reasons, maternal immune stimulation in mice reduces embryopathy associated with diabetes (Torchinsky et al., 1997; Fein et al., 2001; 2002). It is known that the immune system is active during pregnancy, and lack of specific immune cells or improper expression of cytokines may result in pregnancy failure or contribute to development of fetal defects (Robertson, 2000). In this regard, prior to pregnancy uterine immune cells are scarce, however during pregnancy these are recruited from remote sites including bone marrow by systemic circulation (Robertson, 2000). It has been suggested that maternal immune stimulation may alter immune cell recruitment processes to uterus or placenta, or growth factor / cytokine secretion by these cells, to improve fetal development (Holladay et al., 2002). The later authors had previously reported improved placental ultrastructure and restoration of control-level TGF-β and GM-CSF cytokine gene expression in teratogen-treated pregnant mice as a result of immune stimulation, effects that may have been related to reduced birth defects in these mice (Sharova et al., 2003).

Based on the above collective reports, we hypothesized that improved morphologic outcome in fetuses from diabetic mice, after maternal immune stimulation, may be the result of altered placental function. We selected a panel of genes for preliminary studies in the placenta of diabetic mice. Three different, but approximately equally effective forms of maternal immune stimulation for reducing birth defects, were then employed. Our expectation was that placental gene expression profiles after immune stimulation should show a common effect that correlated with the reduced birth defects, if the placenta was an organ involved in mediating reduced birth defects.

The immune stimulation procedures all caused significantly reduced fetal defects, as previously reported, but differentially altered expression of placental genes relative to control

mice. Interestingly, for the genes we selected, the induction of maternal hyperglycemia did not change placental gene expression level relative to the control mice. This observation included genes for TGF-β2 and TNF-α, i.e., cytokines previously associated with diabetic embryopathy (Fein et al., 2002). These findings suggest that effects of hyperglycemia on the placenta of mice play a limited role in diabetic embryopathy, since dramatic differences in fetal birth defect expression were present between these groups. Alternately, gene expression may not reflect physiologic function, as it has also been shown that diabetes can block the release of cytokines without changes in their production (Hill et al, 1996; 1998). It should further be considered that expression level of 11 placental genes in diabetic mice was 1.5 times or more above the control level, and while not significant differences, a cumulative effect on fetal development may be possible from this up-regulating trend caused by diabetes. For this reason, comparisons of individual gene expression levels between control and treatment groups may be of limited value, and analyses that consider genes as functional clusters may be more meaningful (Sharova et al., 2000).

Principal component analysis was used to detect gene clusters (families) that changed together in placenta by treatment, and that might shed light on mechanisms by which the maternal immune stimulation reduced birth defects associated with hyperglycemia. This data analysis technique has been similarly used to show coordinate changes in fetal head gene expression profile caused by maternal immune stimulation, and linked to reduced teratogen-induced cleft palate (Sharova et al., 2000). For the present mouse placenta, the PCA demonstrated significant changes in gene expression profiles caused by the immune stimulants CFA and GM-CSF, that were suggested by ANOVA for the individual genes. The first apparent observation, however, was that maternal injection with the macrophage activating protein IFN-γ caused the greatest numeric decrease in fetal birth defects associated with hyperglycemia (50% defects reduced to 14%), but did not cause significant effects in coordinate gene expression as detected by PCA. It should be considered that while CFA and GM-CSF act specifically on immune cells, IFN-γ is known to act on several body systems with a wide range of target cells.

Changes in expression level of the anti-apoptotic gene bcl2 had the strongest effect in PC 3 by CFA. This was from an up-regulation of bcl2 activity and was associated strongly with 2 down-regulated apoptotic genes, p21 (waf) and R-ras. Increased level of R-ras induce apoptosis (Wang et al., 1995), and p21 (waf) is a downstream signal of p53 (Zhan et al.,

1999). However, other up-regulated genes included some that may promote apoptosis, e.g., GAS2, Bcr, AIF, and BID. However, increased IGF-I, TGF-β1, and GM-CSF during pregnancy may support bcl2 function toward placental cell growth and survival and embryonic development (Robertson et al., 1994; Rajaratnam et al., 1997; Stewart and Rotwein, 1996).

Genes that contributed to expression differences caused by GM-CSF included transcription factors, e.g., E2f1, Rxra, c-fos, and Rb1; and growth regulators, e.g., GAS5, TIMP-2, MMP-2, and DHFR. For the most part these genes were up-regulated relative to control, and involve both apoptotic and cell survival pathways. As was the case with IFN-γ, no obvious commonality was identified with the effects of CFA on placental gene expression, to suggest a mechanism or pathway by which these interventions reduce birth defects.

In summary, previous reports suggested that the placenta, and placental immune cells, may be targets of maternal hyperglycemia, and related to diabetic embryopathy. We previously found both improved placental architecture and gene expression profile at gd 14 after maternal immune stimulation, changes that associated with reduced cleft palate in urethane-exposed mice (Sharova et al., 2002). Our expectation was that we might similarly show improved placental function in diabetic mice as a result of different forms of maternal immune stimulation, which correlated with the reduction in birth defects. The present experiments showed no such correlations. It should be considered that urethane is a cytotoxic agent, and damages the rapidly proliferating placenta; in contrast, hyperglycemia induces placentomegaly (Eriksson and Jansson, 1984). Thus it is likely that these two teratogens differentially affect placenta. It must also be considered that the present gene expression profiles were from placental tissue obtained late in pregnancy (gd 17), i.e., the time when fetuses were collected to evaluate birth defect incidence. Immune stimulation effects on placenta at earlier points in gestation may have been linked to altered cell death, cytokine production, or other gene expression profiles that could beneficially affect fetal development, but were no longer visible in late gestation. Additional experiments that use placental tissue collected at earlier timepoints in gestation will be required to rule out this possibility.

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Table 4.1 Partial list of genes evaluated in placenta, grouped by function

Growth factors and receptors	Apoptotic genes
Epidermal growth factor (EGF)	Caspase 2, 6, 7, 8, 9, 11, 12, 14
EGF-R	Apoptosis-inducing factor (AIF)
Estrogen receptor (Estr-R)	BAD, BAK, BAX, BID
Fibroblast growth factor (FGF)	Bcl2, bcl2l
Folate binding protein 1 (Fol 1), 2	Bcr, Abl
Insulin-like growth factor-I (IGF-I), II	Granzyme B, F
Platelet derived growth factors A (PDGF-A), B	P53, p21(waf), Rb1
Transforming growth factor-β1 (TGF-β1), 2, 3	**Cell cycle genes**
Growth hormone (GH)	Cyclin B1, D2, G
VEGF	Cell division cycle control protein 2a (Cdc2a)
Cytokines	Growth differentiation factor-1 (GDF-1)
Interferon-γ (IFN-γ)	Growth arrest specific protein (GAS), GAS2, GAS5
Tumor necrosis factor-α (TNF-α)	**Oncogenes and Tumor suppressors**
Granulocyte macrophage-colony stimulating factor (GM-CSF)	Rho A, C
	P53, Rb1
Interleukin-1 (IL-1); 2, 4, 10, 12, 15, 16	A-raf, B-raf
Transcription factors	C-src, R-ras
c-fos	P21/H-ras
c-jun	mSmad3
E2F transcription factor 1 (E2f1)	C-Rel
Early growth response 1 (Egr-1), 2	Myb proto-oncogene
I-kappa B alpha chain (IκBα)	**Kinases**
Msx-1, 2	Protein kinase C (PKC)-α, δ
myc	Jak 1, 2
NF-kB-1, 2	Mekk
Rxra	ERK-1, 2
Wee-1	Syk

Table 4.2 Complete placental gene expression data

No.	Gene name	ANOVA p-value	Control		Diabetes		Diabetes +CFA		Diabetes +GM-CSF		Diabetes +IFN-g	
			Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem
1	c-abl	0.46	109	18.5	113	15.1	158	51.0	155	49.2	91	15.4
2	AIF	0.21	386	55.4	456	32.9	569	46.3	482	100.3	379	56.7
3	Annexin A3	0.88	99	14.8	116	16.6	114	28.4	120	11.9	98	12.2
4	Annexin A4	0.21	140	17.2	132	14.8	179	26.4	98	27.7	164	30.0
5	Annexin V	0.83	243	24.1	231	20.9	252	55.1	290	59.5	253	47.7
6	Annexin A6	0.58	1816	206.7	2162	301.5	2543	295.8	2087	304.4	2017	242.6
7	Annexin A7	0.63	150	14.0	156	18.9	188	44.6	176	43.9	204	29.0
8	Api2	0.72	159	17.3	182	23.9	213	33.5	159	40.7	170	29.1
9	BAD	0.31	149	21.0	165	21.9	202	36.5	224	59.3	124	30.0
10	BAK	0.14	113	19.1	166	17.7	245	82.4	213	57.5	134	34.6
11	BAX	0.68	231	21.4	274	17.6	300	35.9	319	98.4	307	80.8
12	Beta-actin	-	3000	0.0	3000	0.0	3000	0.0	3000	0.0	3000	0.0
13	Bcl2l	0.81	145	21.2	141	16.7	146	37.6	142	50.1	98	19.3
14	bcl2	0.61	1507	246.4	1828	273.0	2068	277.3	1671	149.3	1536	96.9
15	bcr	0.01*	119	18.2	127	20.8	305	109.0	140	25.0	81	10.5
16	BID	0.13	115	20.9	150	25.3	327	153.1	147	46.5	128	27.8
17	BMP-4	0.55	12169	1625.5	13265	1980.2	15198	1566.1	14234	1328.5	10252	2025.7
18	BMP-5	0.67	114	11.9	132	13.3	142	16.5	159	39.1	130	29.3
19	Calmodulin 3	0.99	272	32.7	280	21.1	296	32.0	282	68.9	275	29.5
20	Caspase 2	0.91	201	30.8	207	16.6	235	36.7	241	65.6	218	36.1
21	CASPASE-6	0.08	114	12.4	162	15.0	138	43.6	288	105.5	154	27.3
22	CASPASE-7	0.20	204	33.4	230	20.9	448	196.1	266	91.3	196	46.2
23	CASPASE-8	0.80	2270	388.4	2391	342.5	2382	381.6	1883	299.2	1899	257.5
24	CASPASE-9	0.87	131	15.8	149	19.1	136	28.7	171	37.2	151	35.9
25	CASPASE-11	0.88	139	18.0	149	24.6	124	21.2	173	46.8	153	37.2
26	CASPASE-12	0.33	89	16.1	93	23.1	103	25.0	162	37.6	113	22.0
27	CASPASE-14	0.66	987	103.2	1021	103.9	845	108.7	1239	338.3	998	152.9
28	Cadherin 1	0.71	212	32.0	207	19.0	228	48.1	286	74.5	220	56.0
29	Cadherin 2	0.40	82	11.3	139	15.8	116	33.4	175	67.6	103	63.8
30	Cadherin 3	0.04*	111	39.5	91	19.8	76	20.4	283	103.9	122	41.6
31	Cell division cycle control protein 2a (Cdc2a)	0.89	1653	322.7	1842	345.8	1531	293.9	1406	401.9	1449	232.4
32	Coagulation factor II (protrombin)	0.03*	54	14.5	105	15.4	135	43.4	183	51.3	89	18.4
33	Clathrin (AP19)	0.11	155	21.9	208	28.4	376	145.2	193	60.2	156	42.2
34	*CREB*	0.69	21005	3680.0	24008	4058.2	24043	1961.4	29114	8467.6	18639	3322.0
35	Cyclin B1 (CcnB1)	0.96	87	20.6	93	19.3	97	17.7	112	32.8	93	10.2
36	Cyclin D2 (CcnD2)	0.84	135	15.7	152	16.3	164	29.7	153	46.7	175	30.4
37	Cyclin G (CcnG)	0.66	631	51.0	594	30.9	624	99.9	791	243.7	714	128.0
38	Cytochrome C	0.61	130	18.2	137	14.4	139	29.4	179	28.7	171	48.6
39	DHFR (dihydrofolate reductase)	0.11	100	12.4	122	17.9	123	40.0	220	70.0	106	24.7
40	E2F transcription factor 1 (E2f1)	0.27	116	19.7	134	18.3	80	39.4	211	86.5	120	28.9

Table 4.2 Continued

No.	Gene name	ANOVA p-value	Control		Diabetes		Diabetes +CFA		Diabetes +GM-CSF		Diabetes +IFN-g	
			Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem
41	Egr-1 (Krox-24)	0.39	5769	408.1	6518	501.4	5686	616.6	9881	4300.3	6798	1052.3
42	Egr-2; Krox-20	0.33	5539	441.4	5117	258.2	3721	427.7	6456	2242.1	4583	670.7
43	Endothelin-B receptor	0.37	270	33.9	343	30.0	367	55.0	559	292.7	289	36.0
44	eotaxin	0.76	369	47.1	394	62.4	430	49.8	542	218.0	463	101.9
45	EGF	0.25	196	27.0	194	19.5	316	116.3	235	48.5	152	26.7
46	EGF-R	0.44	249	40.1	221	14.5	197	42.7	243	38.8	170	15.9
47	ERK-1	0.91	197	16.3	205	14.9	237	40.8	201	59.6	208	30.0
48	ERK-2	0.20	109	17.6	108	14.8	156	28.4	99	13.0	77	17.6
49	Estrogen receptor	0.66	174	25.3	156	11.9	167	22.7	211	47.8	188	40.3
50	FADD	0.27	254	25.5	280	15.5	306	38.8	404	116.8	280	50.7
51	Fibroblast growth factor1 (FGF1)	0.44	112	11.2	143	15.3	173	40.9	137	19.9	137	29.5
52	Fibronectin	0.86	223	41.0	183	14.7	171	26.6	186	76.6	213	40.3
53	Folate-binding protein 1	0.56	164	23.1	168	20.1	112	16.8	125	48.8	137	35.1
54	Folate-binding protein 2	0.41	142	18.6	223	31.7	188	36.0	195	58.0	167	33.4
55	c-fos	0.45	205	36.2	187	32.3	152	42.4	281	115.6	128	41.4
56	GAPDH	0.97	2877	237.9	2618	246.0	2565	345.4	2844	814.0	2691	352.5
57	Glutathione synthetase (GST)	0.73	143	20.4	160	16.2	189	18.6	170	35.4	158	24.8
58	GM-CSF	0.07	203	17.5	210	21.0	590	316.7	283	63.9	134	15.5
59	Growth arrest specific protein (GAS)	0.52	117	17.2	123	13.5	164	41.3	121	22.1	144	12.1
60	GAS-2	0.04*	97	13.7	93	15.4	160	31.4	181	52.5	75	33.4
61	GAS-5	0.12	121	18.9	153	9.8	140	30.1	207	40.1	166	22.8
62	Granzyme B (Gzmb)	0.75	92	13.3	120	23.6	142	26.8	109	39.9	119	25.8
63	Granzyme F (Gzmf)	0.71	104	28.1	133	14.4	151	31.8	129	44.5	101	24.6
64	Growth hormone (GH)	0.35	87	21.2	132	16.0	114	30.9	91	23.4	90	10.1
65	Growth differentiation factor 1	0.57	339	26.4	401	38.7	315	65.6	489	173.9	398	69.4
66	HEDGEHOG DESERT	0.35	4469	438.7	4559	328.6	4890	687.4	8262	3935.2	4938	1051.9
67	HEDGEHOG INDIAN	0.57	149	20.3	156	14.9	217	47.6	181	46.1	179	44.6
68	Histone 3a	0.36	391	35.0	398	34.4	401	50.5	522	103.0	465	38.4
69	IAP-3	0.23	322	28.7	330	21.5	398	65.9	471	105.3	406	65.3
70	IGF I	0.05	160	15.5	149	15.5	486	250.0	191	36.6	140	39.6
71	IGF II	0.87	1213	343.1	1160	216.8	1100	222.9	1528	438.4	1499	432.1
72	I-kappa B alpha chain	0.48	140	17.9	186	20.8	200	32.3	182	32.4	184	17.9
73	IL-1beta	0.83	136	17.8	136	14.5	155	36.9	129	29.5	111	24.4
74	IL-2	0.70	2038	188.0	1982	119.9	1765	125.1	2293	530.2	2198	308.0
75	IL-4	0.80	41	9.6	63	13.3	76	25.3	76	46.1	69	31.1
76	*IL-9R*	0.33	291	22.7	320	22.2	275	43.0	636	379.5	289	58.2
77	IL-10	0.72	358	30.8	437	36.0	413	47.0	575	276.9	461	138.5
78	IL-12-35	0.59	87	20.0	101	17.2	118	28.2	131	45.5	70	24.1
79	IL-12-40	0.39	332	26.5	354	27.5	379	38.4	466	103.8	399	51.6
80	IL-15	0.17	142	13.5	183	20.2	220	50.7	220	32.7	147	14.2

Table 4.2 Continued

No.	Gene name	ANOVA p-value	Control		Diabetes		Diabetes +CFA		Diabetes +GM-CSF		Diabetes +IFN-g	
			Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem
81	IL-16	0.42	214	14.6	253	14.5	273	32.7	297	70.3	280	46.5
82	Immediate early response 2	0.60	104	14.7	95	12.5	139	24.0	123	42.5	99	24.0
83	Interferon gamma	0.48	64	11.7	80	11.5	117	27.0	90	42.0	99	15.2
84	Inhibin alpha	0.52	178	20.8	192	17.3	228	44.0	231	73.7	151	22.9
85	Inhibin beta-B subunit	0.88	259	25.2	303	26.8	270	39.7	307	98.1	249	75.9
86	Integrin alpha 1	0.62	331	45.4	383	35.7	360	60.8	475	125.5	362	73.0
87	Integrin 5 alpha	0.47	221	24.4	198	16.1	142	18.2	187	56.9	172	42.4
88	Integrin alpha-6	0.90	2446	335.3	2800	520.4	2947	508.1	2949	1031.8	2196	334.2
89	Integrin beta 1	0.13	148	20.5	151	13.2	192	30.7	538	348.2	133	23.8
90	Integrin beta 2 ; CD18; LFA-1	0.17	100	12.8	119	12.2	181	37.9	115	27.4	122	23.1
91	Janus kinase 2 (Jak2)	0.44	64	9.9	112	19.6	122	22.1	105	38.1	108	36.7
92	Janus kinase 3 (Jak3)	0.39	182	11.8	194	14.4	252	44.5	222	45.7	190	20.6
93	c-jun	0.78	323	25.1	318	25.9	348	34.6	429	154.7	355	99.1
94	MADR-2	0.73	148	19.7	160	17.3	132	41.2	117	18.8	145	23.0
95	MDM-2	0.61	22891	1961.9	23843	2454.2	26510	3702.1	34602	14090	23283	4537.1
96	MEK kinase (Mekk)	0.46	107	15.7	144	17.5	135	54.9	167	51.5	91	16.6
97	*MIP-1alpha*	0.59	365	31.5	408	27.6	407	51.8	518	158.5	380	83.4
98	MMP-2	0.24	166	17.0	178	15.5	159	27.3	267	64.8	211	62.2
99	MMP-9	0.39	320	34.1	348	41.9	525	187.3	342	105.3	288	53.0
100	Msx-1 (Hox7.1)	0.55	109	23.2	118	18.9	134	29.9	170	52.4	103	14.1
101	Msx-2 (homeobox Hox8.1)	0.10	182	20.6	208	23.6	269	66.5	318	75.9	168	28.5
102	Myb proto-oncogene	0.31	86	12.3	117	15.2	128	22.4	139	24.7	93	30.5
103	Myc	0.14	119	15.9	150	21.3	182	22.0	209	58.8	105	26.6
104	NFkB-1(NF-KAPPA-B); p105	0.04*	73	15.1	88	13.1	182	55.2	110	28.2	67	22.2
105	NF-kB-2 P100	0.01*	68	14.4	147	14.5	201	39.8	130	45.2	95	10.3
106	Nos3 "Nitric oxide synthase 3	0.21	95	12.0	145	19.3	140	46.8	174	64.5	69	22.1
107	Oxytocin receptor (human)	0.86	3648	536.2	4461	620.8	3869	530.9	3772	1086.0	3694	592.0
108	P21/H-RAS-1 transforming protein	0.72	87	17.7	122	21.5	122	19.6	116	23.5	109	23.9
109	p21 (waf)	0.68	1781	353.1	1504	252.6	1189	248.1	1895	448.7	1765	353.9
110	p53	0.62	343	57.2	409	39.6	609	299.0	364	177.1	353	72.3
111	PAF acetylhydrolase	0.52	343	27.5	297	26.2	404	53.9	349	74.4	355	70.3
112	PDGF, A	0.30	211	48.6	143	22.0	237	38.8	218	27.8	204	30.7
113	PDGF, B chain	0.77	105	21.2	124	23.3	152	38.8	149	38.0	117	29.1
114	PERFORIN	0.21	1378	102.1	1385	113.3	1657	262.1	2363	787.6	1782	423.2
115	Prolactin	0.64	2299	747.5	1526	309.4	1643	418.2	2494	553.8	2318	764.7
116	Proliferin	0.12	81	19.5	90	14.6	148	21.0	127	43.8	62	23.3
117	Proliferating cell nuclear antigen (Pcna)	0.11	117	31.3	125	13.8	79	33.5	176	59.5	43	15.5
118	Prostaglandin receptpr ep1	0.22	2701	192.2	3034	215.6	2924	404.9	4814	1738.4	3380	566.7

Table 4.2 Continued

No.	Gene name	ANOVA p-value	Control		Diabetes		Diabetes +CFA		Diabetes +GM-CSF		Diabetes +IFN-g	
			Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem
119	Prostaglandin receptor ep2	0.25	107	34.5	137	26.4	75	27.1	666	581.1	98	42.0
120	Prostaglandin D synthase	0.72	11598	2514.1	9371	1841.7	7165	1640.6	11837	3422.3	10190	2431.7
121	PIAS-1	0.53	1592	158.4	1513	94.5	1228	106.0	2021	705.5	1635	351.0
122	PKC alpha	0.52	168	15.2	189	20.4	198	35.3	141	31.0	159	15.8
123	Pkacb	0.52	140	16.9	152	15.5	158	22.8	193	25.2	177	31.2
124	PKC-delta	0.30	92	15.1	127	14.3	148	26.4	177	50.7	128	30.4
125	A-RAF proto-oncogene	0.50	135	12.4	162	19.3	196	50.5	191	53.1	139	16.7
126	B-raf oncogene	0.69	359	48.7	324	25.3	280	29.1	397	117.4	319	43.1
127	RANTES	0.35	81	18.7	124	11.1	107	26.3	71	47.9	64	29.6
128	R-ras	0.53	6221	1233	4816	906.9	3730	688.8	6344	1416.8	5890	1278.9
129	c-Rel	0.71	84	27.0	114	15.2	78	14.8	78	23.3	115	42.1
130	Retinoblastoma 1 (Rb1)	0.30	203	24.5	175	15.7	166	44.0	256	50.4	164	32.2
131	Retinoid X receptor alpha (Rxra)	0.17	245	42.3	168	22.3	177	39.6	352	118.4	258	77.8
132	RhoA	0.89	279	65.9	253	23.7	234	45.6	308	103.4	228	45.2
133	RhoC	0.77	172	15.6	173	15.7	179	17.5	138	32.1	159	36.7
134	SMAD3 (mSmad3)	0.06	118	17.9	151	16.4	198	26.1	206	36.1	181	25.6
135	Syk	0.03*	162	15.6	160	16.0	171	19.8	269	53.6	170	15.0
136	c-Src kinase	0.43	255	17.2	318	26.3	302	54.5	366	73.9	287	54.8
137	STAT1	0.18	95	24.7	138	17.4	111	42.2	173	40.4	75	14.0
138	*STAT3*	0.04*	164	14.2	218	20.8	268	45.8	310	79.6	181	17.2
139	SOCS-1	0.44	156	19.4	182	18.8	152	29.0	248	85.6	176	29.5
140	TIMP-2	0.10	137	20.0	135	10.3	85	19.5	183	34.8	142	28.7
141	TRAF-1	0.49	146	20.1	190	23.8	175	22.8	258	103.4	175	40.6
142	TRAF-2A	0.52	177	40.4	167	21.5	218	44.4	275	95.3	218	47.1
143	TRAF-5	0.90	441	50.0	532	76.8	503	80.4	537	201.8	439	74.6
144	TGFbeta 1	0.04*	451	28.6	438	39.2	767	193.4	507	91.6	415	43.9
145	TGFbeta 2	0.45	330	24.7	329	34.7	346	39.6	462	127.2	317	56.0
146	TGFbeta 3	0.12	182	25.4	170	26.6	185	27.5	311	78.7	224	42.8
147	TNF alpha	0.08	160	13.5	156	15.5	163	29.8	253	64.5	138	10.9
148	Trophoblast specific protein	0.89	128	26.1	129	23.5	124	28.5	157	34.8	158	30.9
149	VEGF	0.33	147	21.5	128	11.9	176	39.4	203	57.0	140	7.0
150	VCAM-1	0.53	151	13.1	163	15.0	218	43.5	217	90.3	142	39.4
151	wee-1	0.31	145	18.8	187	26.0	185	24.3	296	135.8	151	22.8

Table 4.3 Placental gene expression relative to control. Genes shown in the table displayed at least 50% different expression as compared to a different treatment group.

Gene name	Diabetes	Diabetes + CFA	Diabetes + GM-CSF	Diabetes + IFN-γ
AIF	1.2	**1.5**	1.3	1.0
BAD	1.1	1.4	**1.5**	0.8
BAK	**1.5**	**2.2**	**1.9**	1.2
bcr	1.1	**2.6**	1.2	0.7
BID	1.3	**2.9**	1.3	1.1
Caspase-6	1.4	1.2	**2.5**	1.4
Caspase-7	1.1	**2.2**	1.3	1.0
Caspase-12	1.0	1.2	**1.8**	1.3
Cadherin 2	**1.7**	1.4	**2.1**	1.3
Cadherin 3	0.8	0.7	**2.5**	1.1
Coagulation factor II	**1.9**	**2.5**	**3.4**	**1.7**
Clathrin (AP19)	1.3	**2.4**	1.2	1.0
DHFR	1.2	1.2	**2.2**	1.1
E2f1	1.2	0.7	**1.8**	1.0
Egr-1	1.1	1.0	**1.7**	1.2
Endothelin-B receptor	1.3	1.4	**2.1**	1.1
eotaxin	1.1	1.2	**1.5**	1.3
EGF	1.0	**1.6**	1.2	0.8
FADD	1.1	1.2	**1.6**	1.1
FGF 1	1.3	**1.5**	1.2	1.2
Folate binding protein 2	**1.6**	1.3	1.4	1.2
GM-CSF	1.0	**2.9**	1.4	0.7
GAS-2	1.0	**1.7**	**1.9**	0.8
GAS-5	1.3	1.2	**1.7**	1.4
Granzyme B	1.3	**1.5**	1.2	1.3
Growth hormone	**1.5**	1.3	1.0	1.0
Hedgehog Desert	1.0	1.1	**1.8**	1.1
Hedgehog Indian	1.0	**1.5**	1.2	1.2
IAP-3	1.0	1.2	**1.5**	1.3
IGF-I	0.9	**3.0**	1.2	0.9
IL-4	**1.5**	**1.8**	**1.8**	**1.7**
IL-9R	1.1	0.9	**2.2**	1.0
IL-10	1.2	1.2	**1.6**	1.3
IL-12-35	1.2	1.4	**1.5**	0.8
IL-15	1.3	**1.6**	**1.5**	1.0

Table 4.3 Continued

Gene name	Diabetes	Diabetes + CFA	Diabetes + GM-CSF	Diabetes + IFN-γ
Interferon-γ	1.2	**1.8**	1.4	**1.5**
Integrin-β1	1.0	1.3	**3.6**	0.9
Integrin-β2 (LFA-1)	1.2	**1.8**	1.2	1.2
Janus kinase 2 (Jak2)	**1.7**	**1.9**	**1.6**	**1.7**
Mekk	1.3	1.3	**1.6**	0.9
MMP-2	1.1	1.0	**1.6**	1.3
MMP-9	1.1	**1.6**	1.1	0.9
Msx-1 (Hox7.1)	1.1	1.2	**1.6**	0.9
Msx-2 (Hox8.1)	1.1	**1.5**	**1.7**	0.9
Myb proto-oncogene	1.4	**1.5**	**1.6**	1.1
Myc	1.3	**1.5**	**1.7**	0.9
NFkB-1	1.2	**2.5**	**1.5**	0.9
NFkB-2	**2.2**	**3.0**	**1.9**	1.4
Nos3	**1.5**	**1.5**	**1.8**	0.7
p53	1.2	**1.8**	1.1	1.0
Perforin	1.0	1.2	**1.7**	1.3
Proliferin	1.1	**1.8**	**1.6**	0.8
Pcna	1.1	0.7	**1.5**	**0.4**
Prostaglandin receptor ep1	1.1	1.1	**1.8**	1.3
PKC-δ	1.4	**1.6**	**1.9**	1.4
A-raf proto-oncogene	1.2	**1.5**	1.4	1.0
RANTES	**1.5**	1.3	0.9	0.8
SMAD3	1.3	**1.7**	**1.7**	**1.5**
Syk	1.0	1.1	**1.7**	1.0
STAT1	**1.5**	1.2	**1.8**	0.8
STAT3	1.3	**1.6**	**1.9**	1.1
SOCS-1	1.2	1.0	**1.6**	1.1
TRAF-1	1.3	1.2	**1.8**	1.2
TRAF-2A	0.9	1.2	**1.5**	1.2
TGF-β1	1.0	**1.7**	1.1	0.9
TGF-β3	0.9	1.0	**1.7**	1.2
TNF-α	1.0	1.0	**1.6**	0.9
wee-1	1.3	1.3	**2.0**	1.0
Total number	**11 genes**	**33 genes**	**48 genes**	**6 genes**

Table 4.4 Principal components that explain 56% of variability in gene expression between treatments, and ANOVA comparison among the 5 treatments

	PC 1	**PC 2**	**PC 3**	**PC 4**
Eigenvalue	49.976	14.605	10.385	7.478
Proportion of variation explained	0.342	0.100	0.071	0.051
Cumulative variation explained	0.342	0.442	0.514	0.565
ANOVA				
Df	4	4	4	4
p	0.480	0.927	0.046	0.011

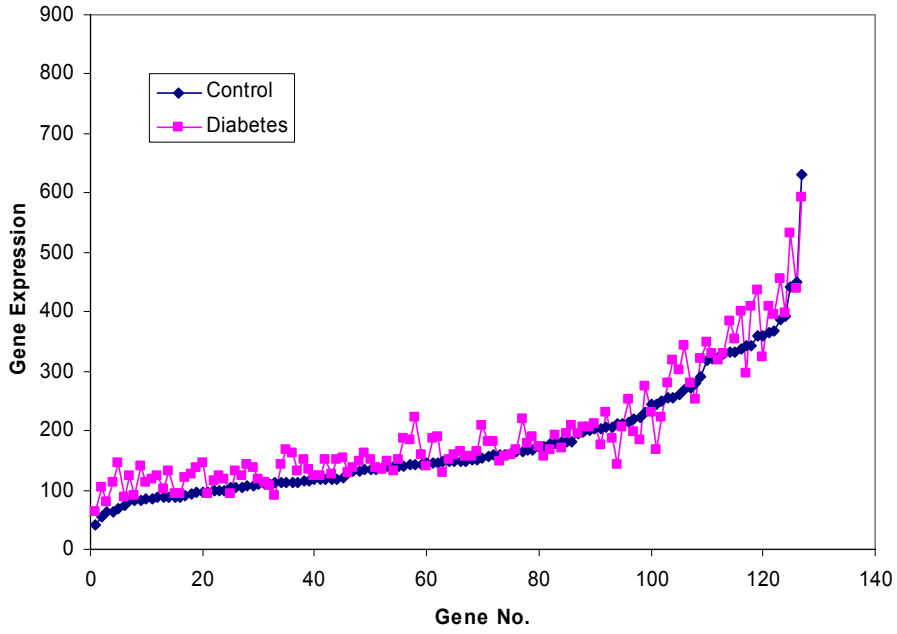
* PC 3 and PC 4 were significant at $p < .05$, and further tested to identify the group that was different from the others.

Table 4.5 Twenty selected genes of both positive and negative PC scores in PC 3. This PC represents the effect of CFA. Genes with positive PC score were up-regulated, while genes with negative PC score were down-regulated.

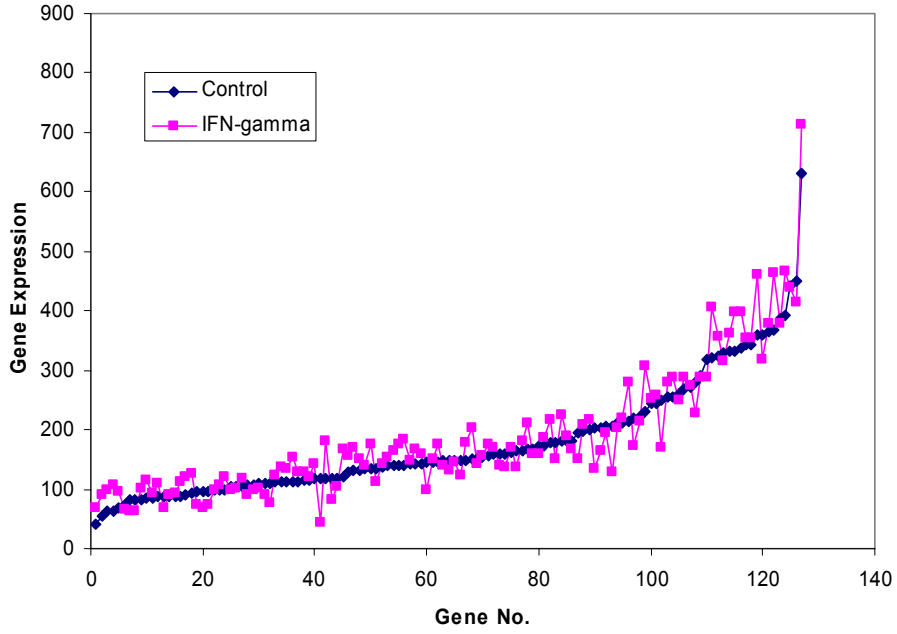
Genes that affect PC 3 positively		Genes that affect PC 3 negatively	
Gene name	PC score	Gene name	PC score
Bcl2	0.20001	P21 (waf)	-0.22413
AIF	0.18777	R-ras	-0.22304
NFkB-1	0.16337	Prostaglandin D synthase	-0.21915
Bcr	0.15915	Prolactin	-0.16241
Proliferin	0.15811	Egr-2	-0.14690
GAS 2	0.15176	PIAS-1	-0.14629
BID	0.14803	Rxra	-0.14428
NFkB-2	0.14668	IL-2	-0.14305
Annexin A6	0.14472	IGF-II	-0.14244
Myc	0.13832	Caspase-6	-0.12472
P21/H-ras-1	0.13783	MADR-2	-0.12405
Msx-1(Hox7.1)	0.12522	Fibronectin	-0.11247
EGF	0.11883	E2f 1	-0.10809
GM-CSF	0.11842	Histone 3a	-0.10245
Clathrin (AP19)	0.11729	RhoA	-0.10190
GAS	0.10805	c-abl	-0.09548
STAT3	0.10783	B-raf	-0.09163
Coagulation factor II	0.10608	TGF-β3	-0.08632
ERK-2	0.10283	Bcl2l	-0.08319
Folate binding protein 2	0.10172	Annexin A4	-0.08267

Table 4.6 Twenty selected genes of both positive and negative PC scores in PC 4. This PC represents the effect of GM-CSF. Genes with positive PC score showed control-leveled expression. Genes with negative PC score showed up-regulated expression.

Genes that affect PC 4 positively		Genes that affect PC 4 negatively	
Gene name	PC score	Gene name	PC score
Cyclin D2	0.28297	GAS 5	-0.17236
Annexin A4	0.25093	E2f 1	-0.17163
c-abl	0.24804	TIMP-2	-0.16277
Bcl2l	0.24156	MMP-2	-0.14714
p53	0.22334	DHFR	-0.14517
RANTES	0.20411	Mekk	-0.13714
PAF acetylhydrolase	0.17578	Rxra	-0.13033
RhoC	0.14568	c-fos	-0.12837
Janus kinase 2	0.13948	Cytochrome C	-0.12189
c-jun	0.13696	Prostaglandin receptor ep2	-0.11741
Hedgehog Indian	0.13414	Retinoblastoma 1 (Rb 1)	-0.11350
VCAM-1	0.12405	Caspase-6	-0.10530
Janus kinase 3	0.12394	Syk	-0.10224
Granzyme B	0.11190	IL-16	-0.09816
ERK-2	0.10377	Cadherin 3	-0.09778
Granzyme F	0.10341	IL-9R	-0.09487
LFA-1	0.09852	TRAF-2A	-0.09065
Intergrin 5α	0.08815	Caspase-9	-0.09026
Cyclin B1	0.08044	GDF-1	-0.08741
Calmodulin 3	0.07789	TRAF-1	-0.08239

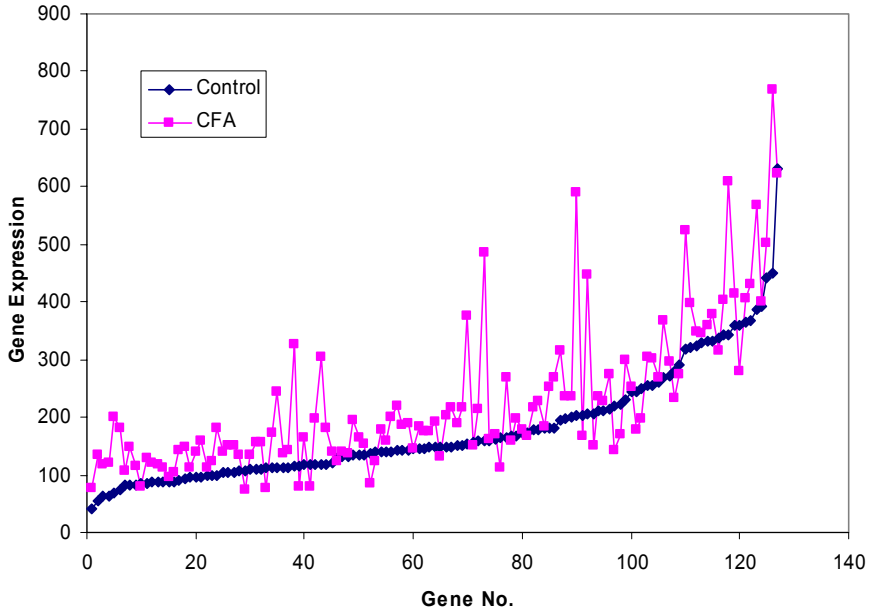


a



b

Fig. 4.1 Scatter plots of placental gene expression levels relative to control with connected line for better visualization. a) Diabetic group; b) IFN-γ group; c) CFA group; d) GM-CSF. Diabetic and IFN-γ groups show unaltered pattern, while CFA and GM-CSF show altered patterns with most genes higher than control.



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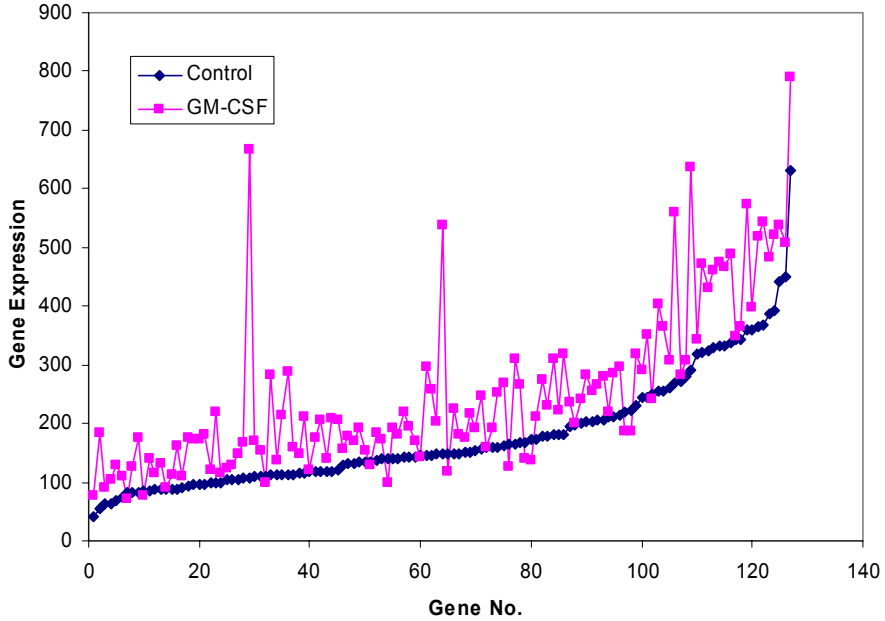
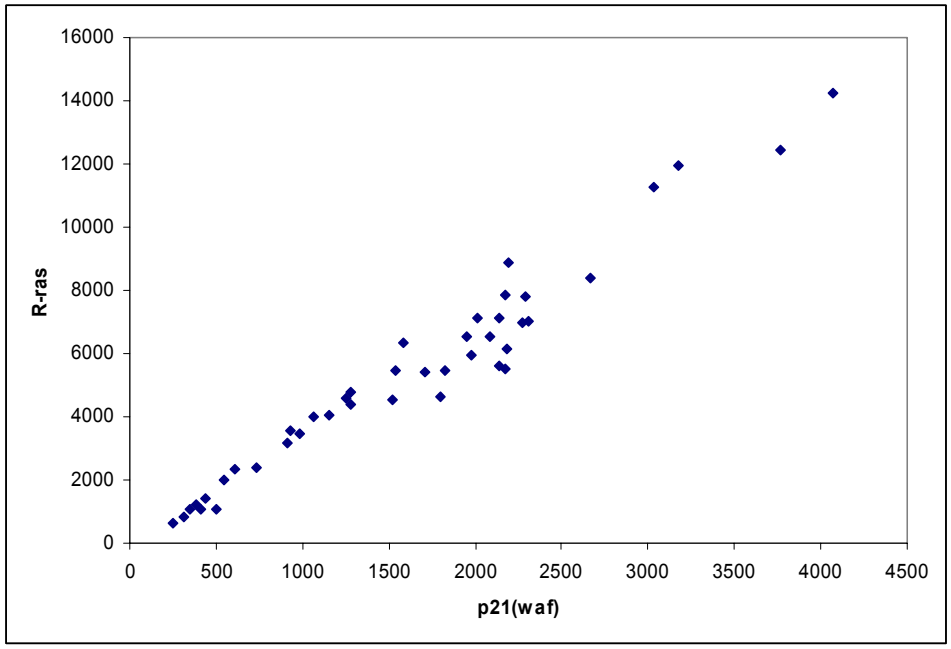
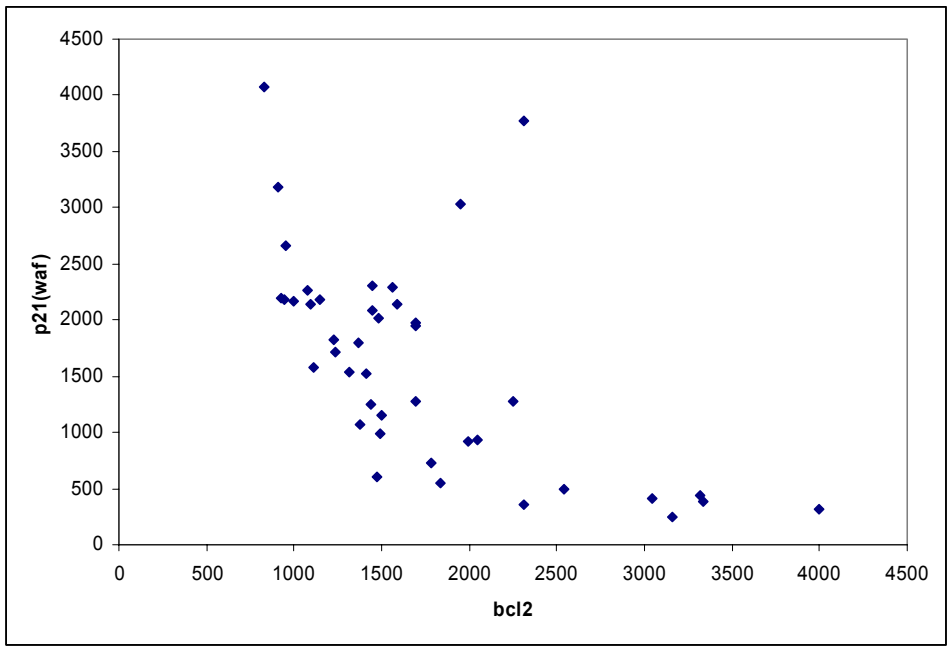


d

Fig. 4.1 Continued



a



b

Fig. 4.2. Scatter plots demonstrating positive and negative relationships between gene pair expression: Graph (a) shows a positive relationship between expression of p21 (waf) and R-ras; while graph (b) shows a negative relationship between expression of p21 (waf) and bcl2.

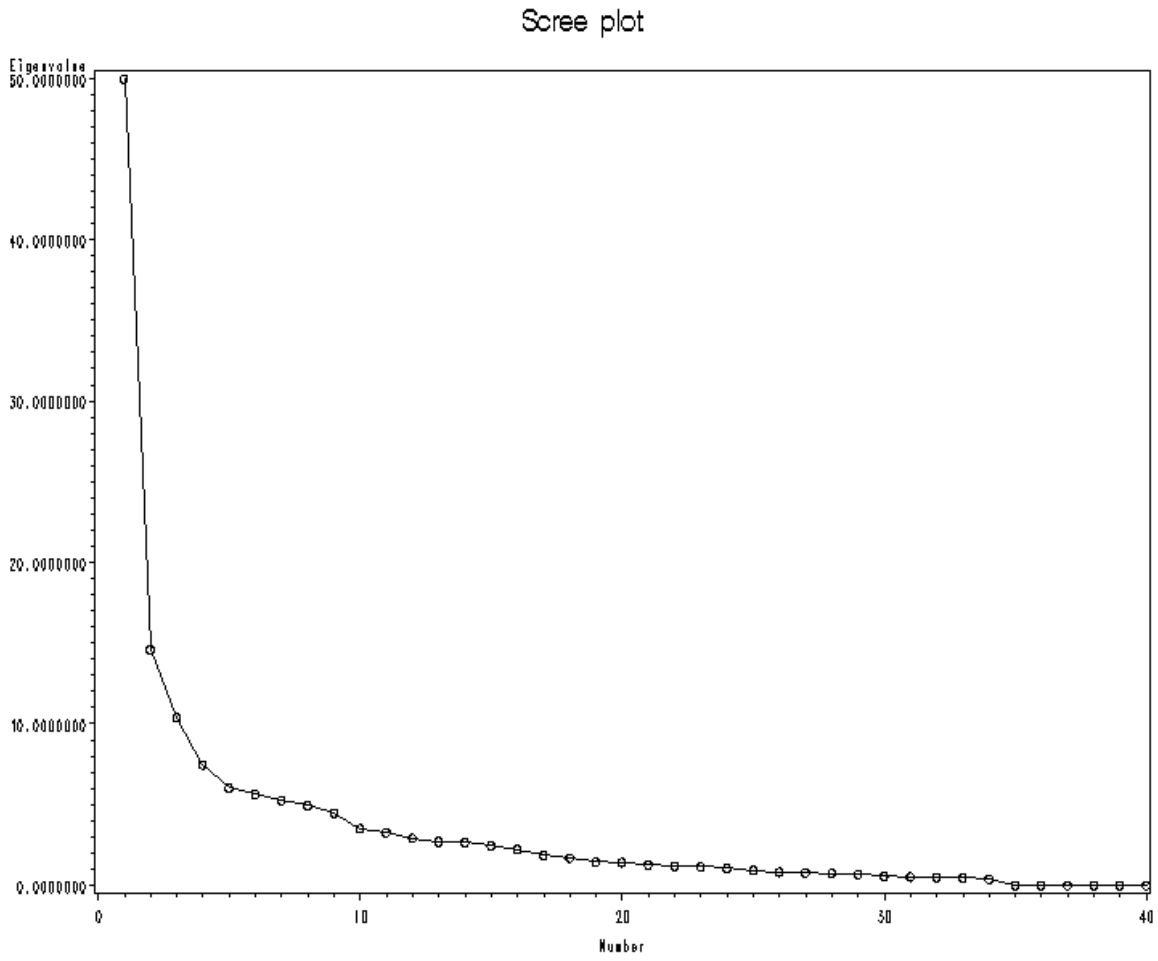
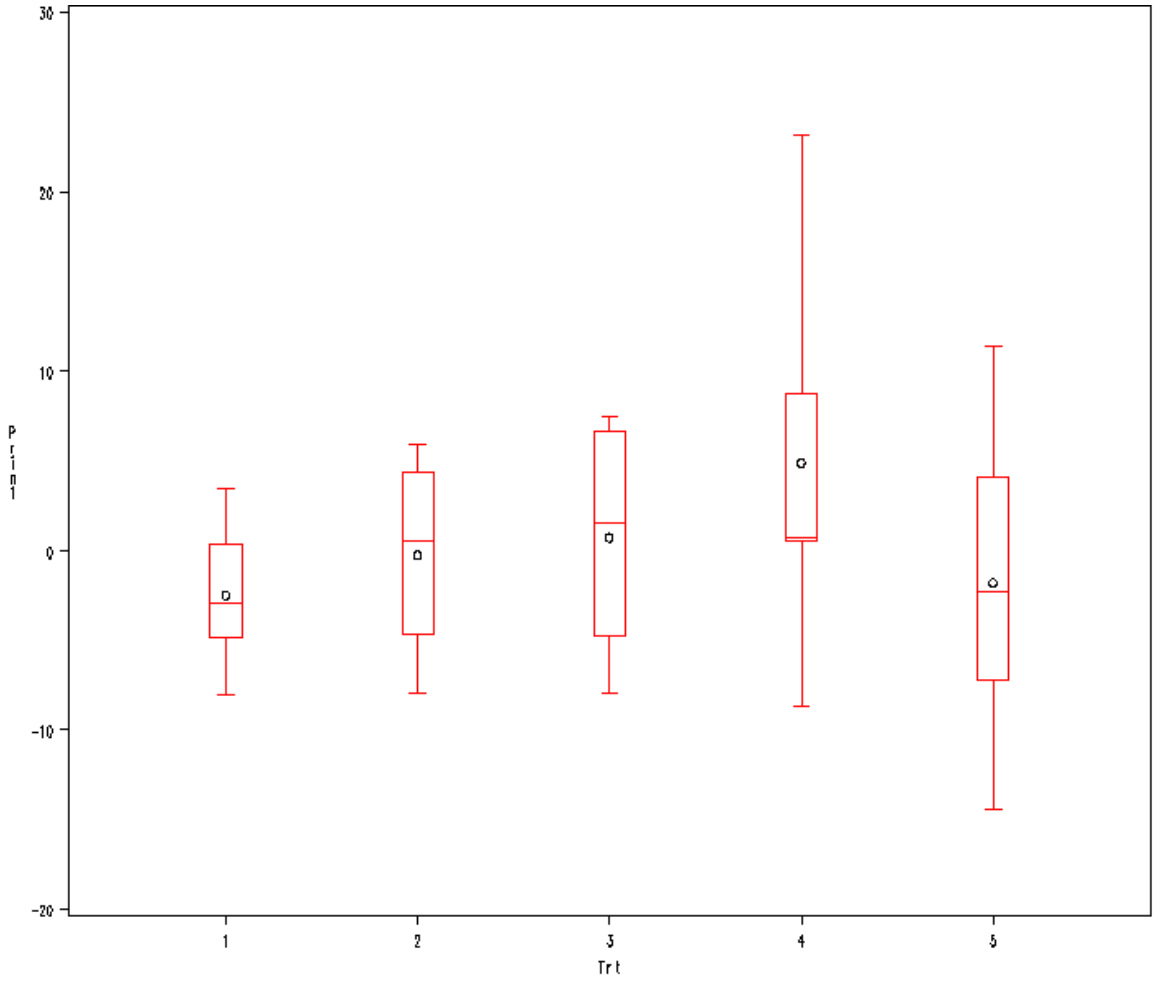
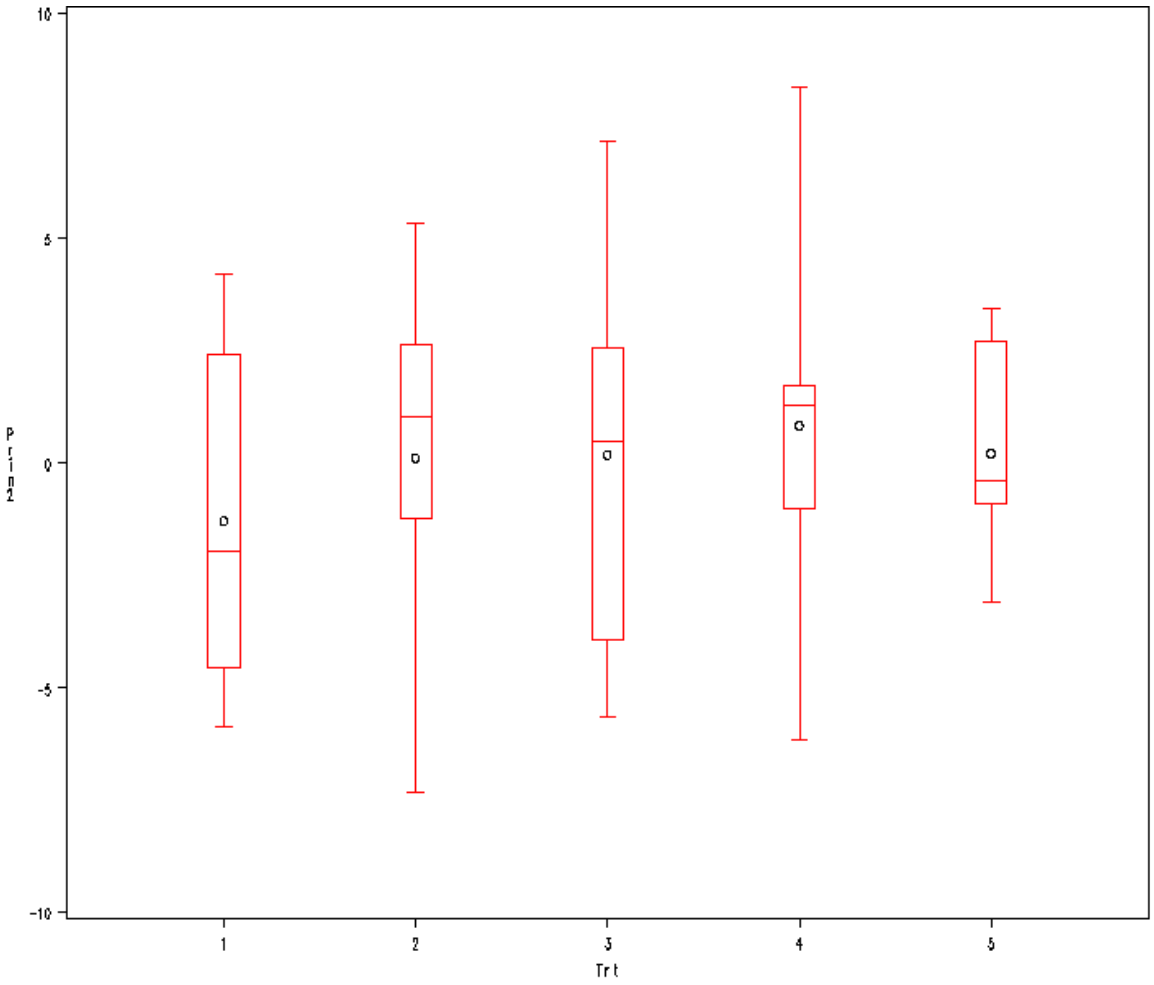


Fig. 4.3. Scree plot of eigenvalues of principal components (PCs) 1-40. PCA analysis creates the number of PCs, and each PC contains eigenvalue or the variance of PC, the proportion and cumulative proportion of the total variance explained. PCs that explained at least 5% of the variation in gene expression were subjected to further analysis.



a

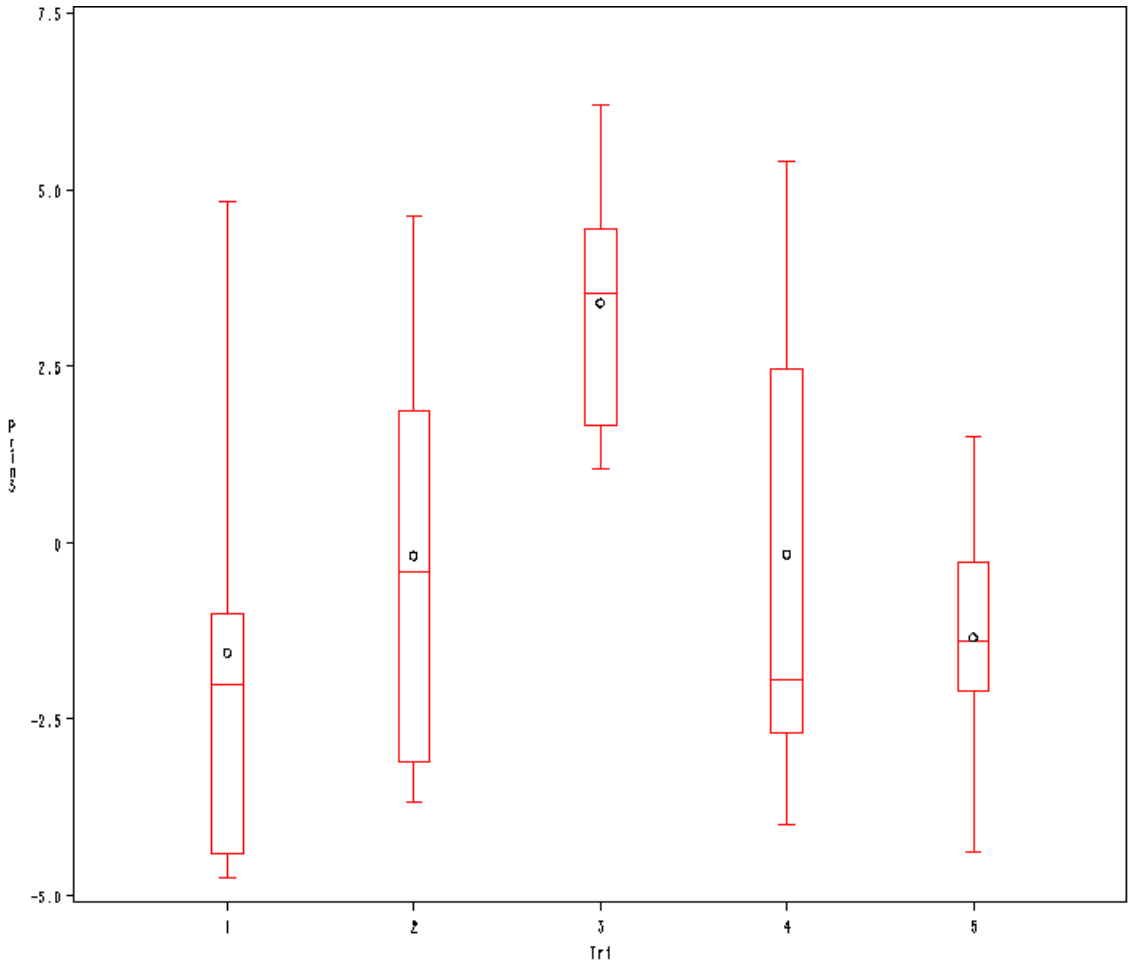
Fig. 4.4 Boxplots of PC 1-4 demonstrating a treatment effect of CFA in PC 3; and of GM-CSF in PC 4. Fig. a (PC 1), b (PC 2), c (PC 3), d (PC 4). PC 1-4 were selected from Scree plot and analysed by ANOVA comparing values among control and treatment groups. PC 3 and 4 were further analysed to identify genes that contribute to the treatment effects.



control diabetes CFA GM-CSF IFN-γ

b

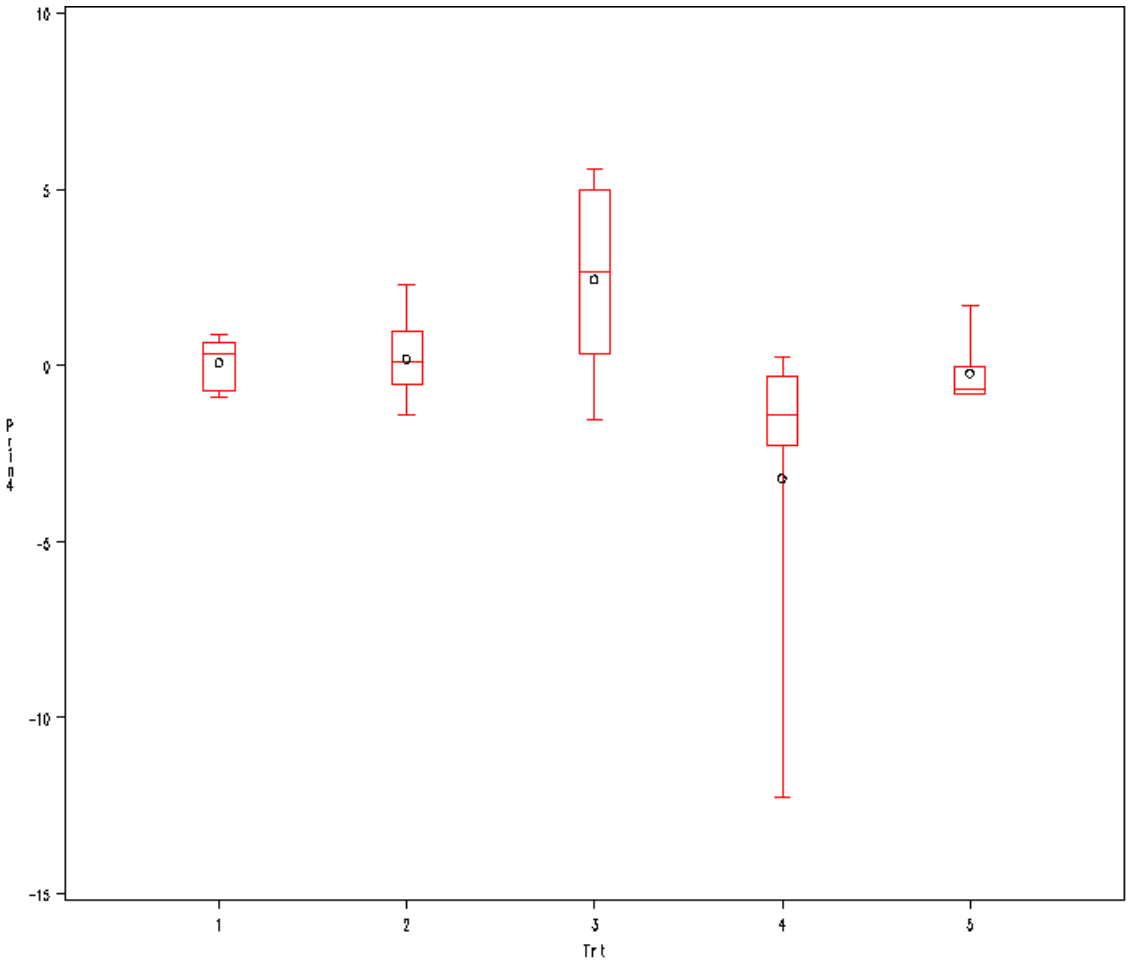
Fig. 4.4 Continued



control diabetes CFA GM-CSF IFN-γ

c

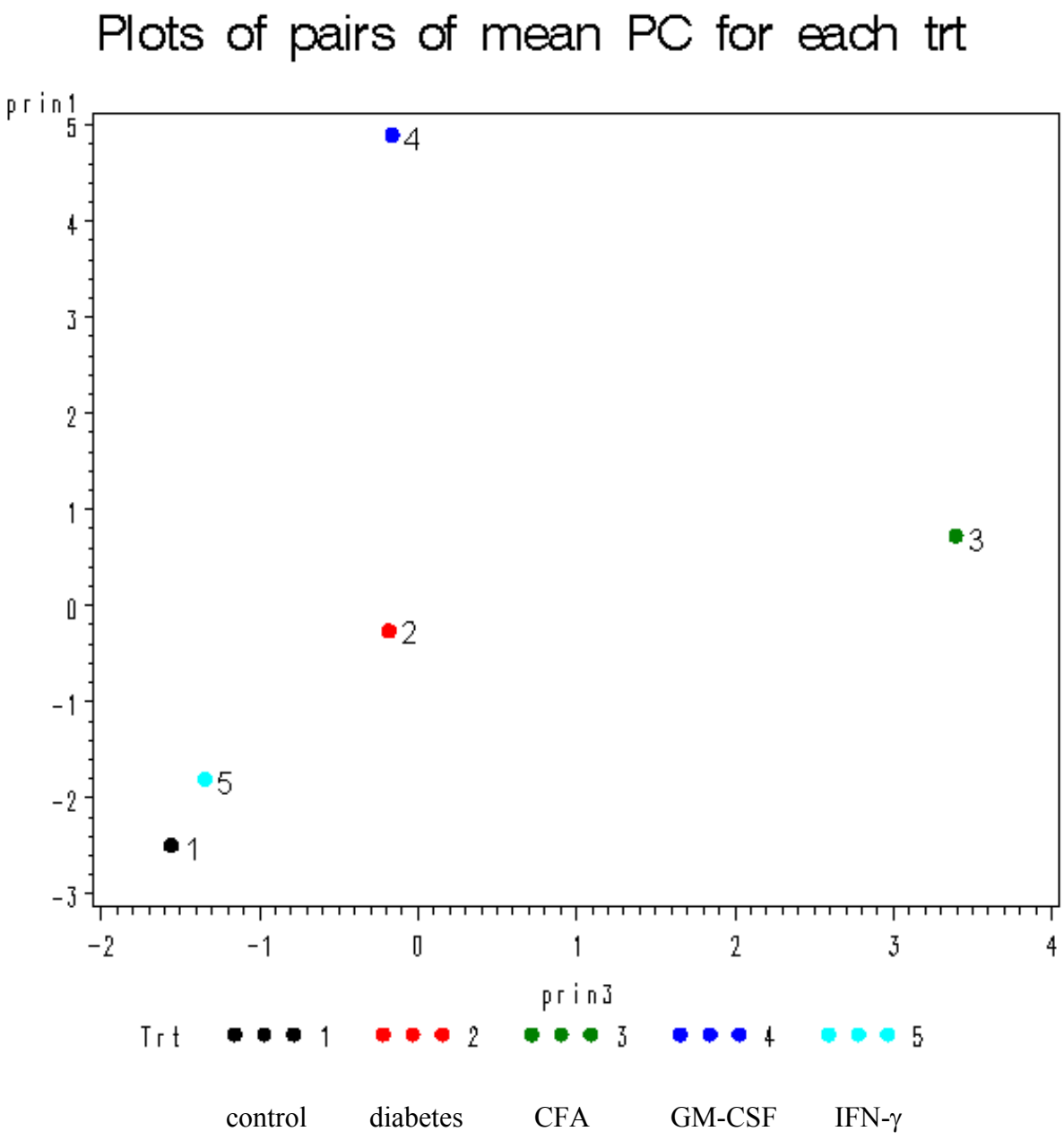
Fig. 4.4 Continued



control diabetes CFA GM-CSF IFN-γ

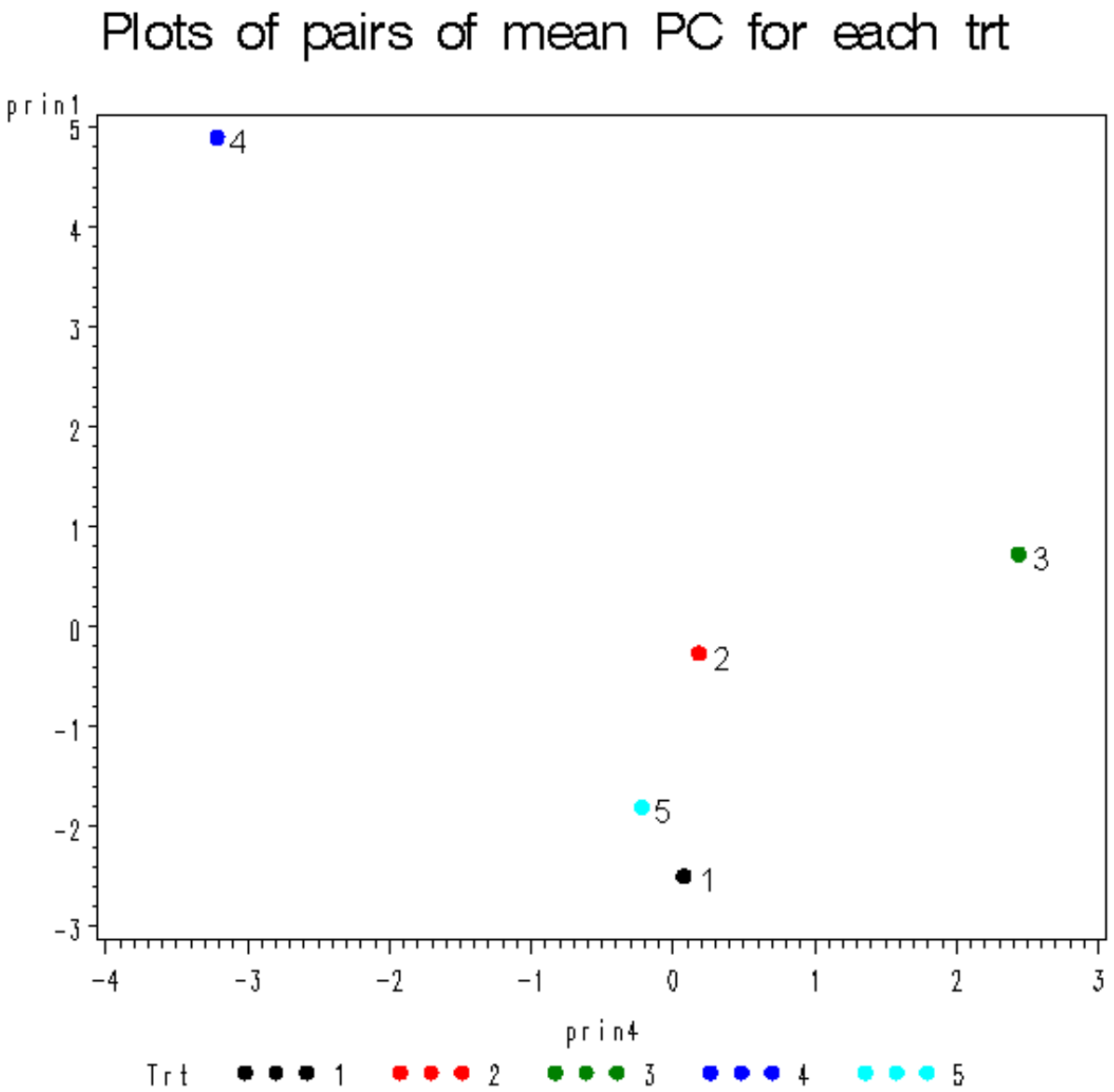
d

Fig. 4.4 Continued



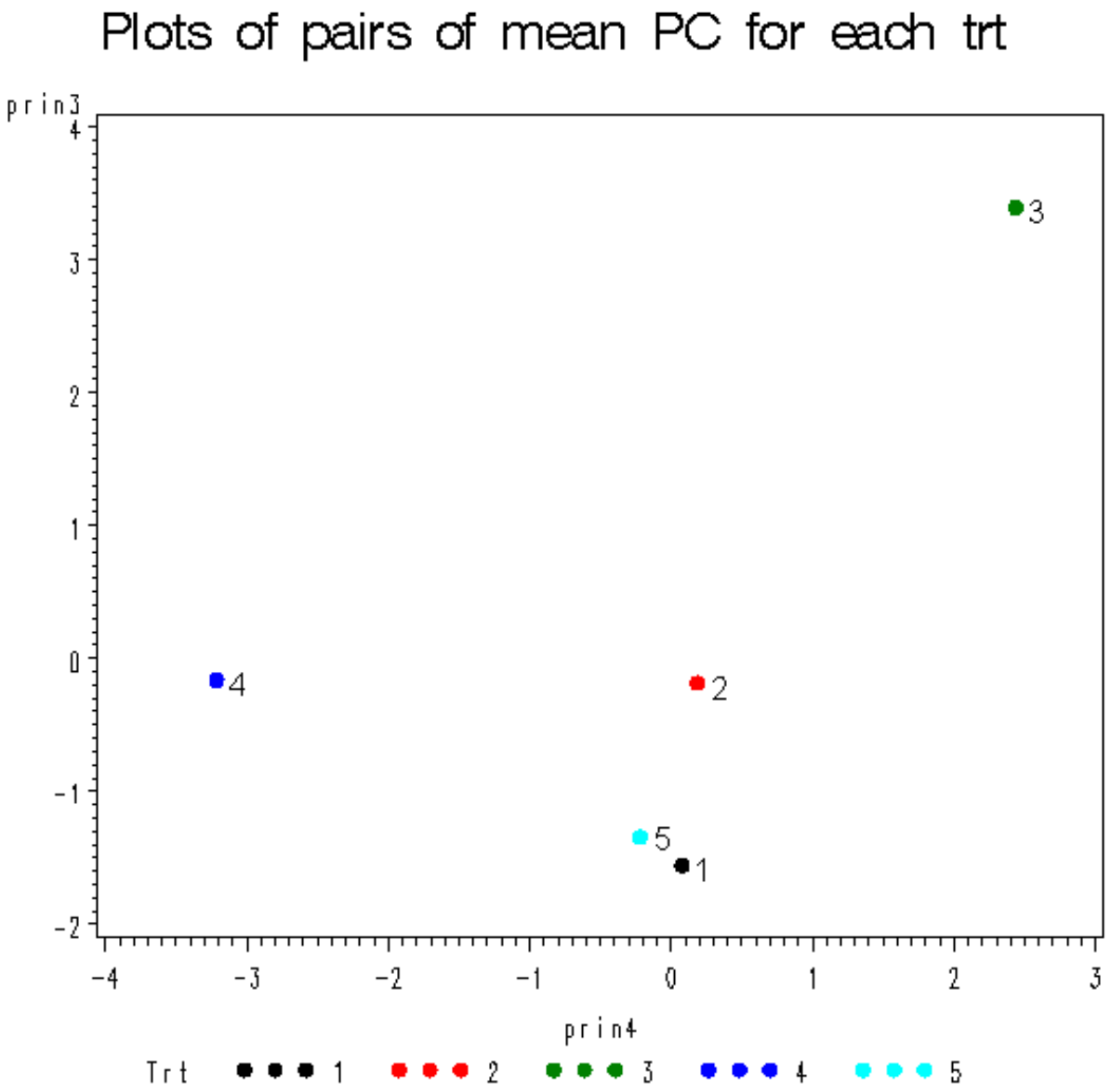
a

Fig. 4.5 Scatter plots of PCA analysis of placental gene expression, showing mean of PCs that were significantly affected by experimental treatments. a) PC 1 / PC 3 coordinate space; b) PC 1 / PC 4 coordinate space; c) PC 3 / PC 4 coordinate space. Control, diabetic, and IFN-γ groups had relatively similar profiles, while CFA and GM-CSF had different profiles from the others and from each other.



b

Fig. 4.5 Continued



c

Fig. 4.5 Continued

CHAPTER 5: REDUCED BIRTH DEFECTS CAUSED BY MATERNAL IMMUNE STIMULATIONS MAY INVOLVE INCREASED EXPRESSION OF GROWTH PROMOTING GENES AND CYTOKINE GM-CSF IN SPLEEN OF DIABETIC ICR MICE

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5.1: ABSTRACT

Maternal immune stimulation in mice decreases incidence of pregnancy failure and fetal abnormalities caused by diverse etiologies including chemical teratogens and diabetes mellitus. Growth factors or cytokines produced by activated immune cells were proposed to be key mediators that may exert their effects on placenta or embryonic development. Diabetes disrupts the secretion of these cytokines, which may associate with diabetic embryopathy. Three different methods of maternal immune stimulation that result in approximately equal reduction of diabetic embryopathy were used in the present studies: footpad injection with complete Freund's adjuvant (CFA), intraperitoneal (IP) injection with granulocyte-macrophage colony stimulating factor (GM-CSF), or IP injection with interferon-gamma (IFN-γ). A gene microarray was then used to examine expression of a selected panel of 151 genes in splenic leukocytes. We hypothesized that maternal immune stimulation may act by overcoming altered gene expression patterns of immune cells in the diabetic mice, which partially mitigates the teratogenic effect of diabetes. It further seemed likely that a shared profile of splenic gene expression changes induced by the different immune stimulation procedures may be identified and related to reduced teratogenesis. The three procedures produced a common altered gene expression profile. Significantly affected genes included apoptotic and anti-apoptotic genes, and genes controlling cellular proliferation, and likely reflect a state of immune activation. GM-CSF gene was also up-regulated. The protein product of this gene regulates placental development, and was recently

associated with reduced cleft palate in immune stimulated pregnant mice after exposure to urethane. In contrast, the TGF-β3 gene was down-regulated in immune-stimulated diabetic mice. This gene was up-regulated in urethane-exposed mice, an effect that may be associated with reduced cleft palate. Thus unlike urethane, TGF-β3 gene expression did not show a relationship with reduced diabetes-induced birth defects. These results suggest that further studies of GM-CSF as mediator of reduced birth defects in teratogen-challenged, immune-stimulated mice are warranted.

Keywords: Diabetes, diabetic embryopathy, immune stimulation, teratogenesis, birth defects, CFA, GM-CSF, IFN-γ

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5.2: *INTRODUCTION*

Diabetic embryopathy is a pathological complication of newborns from women with diabetes. The lesions range from mild effects like macrosomia or growth retardation to severe or lethal conditions that include neural tube defects, heart defects, and kidney defects. Diabetic women are generally placed on a strict glucose control program during pregnancy to maintain steady levels of glucose; however the incidence of birth complications is still higher than in non-diabetic pregnancies. Insulin deficiency and hyperglycemia are the primary causes, and act through generation of other harmful products including ketone bodies, somatomedin inhibitors, and advance glycation end products (AGEs) (Buchanan and Kitzmiller, 1994). At the cellular level, diabetes mediates its toxic effects through multiple pathways. Myo-inositol level is low in diabetic tissues, which may interfere with signal transduction in target tissue cells. Arachidonic acid and prostaglandin levels are also deficient, which again may interrupt cellular functions that require prostaglandins. Oxygen free radicals are generated at higher rates in diabetic tissues, leading to cellular damage and apoptosis (Reece, 1999). Based on these pathological pathways, several strategic preventive studies have been conducted in animal models and have demonstrated success in lowering birth defect incidence. These studies included supplementation with myo-inositol (Baker et al., 1990), arachidonic acid (Goldman et al., 1985; Pinter et al., 1986), and antioxidants such

as vitamin E (Sivan et al., 1996), vitamin C (Siman and Eriksson, 1997), and lipoic acid (Wiznizer et al., 1999).

Another factor that might be affected by diabetes and contribute to fetal defect development is maternal immune system modulation. Immune cells like macrophages displayed abnormal cytokine secretion in diabetes (Hill et al., 1998), and such cytokine aberration was related to diabetic complications (Hoffman et al., 1998; Cooper et al., 2001). Production of prostaglandin E_2 (PGE_2), which can inhibit Th1 cytokine production and enhance Th2 type response (Harris et al., 2002) was inhibited by diabetes (Reece, 1999). Dysregulation of the uterine cytokine network was also reported to be an influencing factor related to diabetic embryopathy (Pampfer, 2001). Immune stimulation has demonstrated protective effects against spontaneous abortion (Baines et al., 1996) and both chemical-induced teratogenesis (Holladay et al., 2002) and diabetic embropathy (Torchinsky et al., 1997). In diabetic mice, abnormally high expression of tumor necrosis factor-α (TNF-α) in utero-placental tissues was decreased after immune stimulation (Fein et al., 2001).

To further examine mechanisms of immunostimulatory protection against diabetic embryopathy, we tested 3 different immune stimulation methods, CFA, GM-CSF, IFN-γ. Even though each of these agents has different targets, each produces a non-spicifically activated state of the immune system. Each of these agents also lowered the incidence of diabetes-related fetal malformations (Punareewattana and Holladay, 2003). The understanding of specific proteins or pathways responsible for such protective results remains unclear. In this regard, several immune proteins have been suggested to be involved in immune protection against chemical-induced teratogenesis including TGF, CSF-1, GM-CSF (Hollady et al., 2002).

In the present study, we examined the hypothesis that the immune system of pregnant mice may be disturbed by diabetes, and this could play a role in diabetes-related birth defects. Spleens collected from diabetic pregnant mice were examined for their gene expression profile and compared with the outcome of fetal defects. The spleen was selected for examination because it is a major immune organ, and during pregnancy splenic cells produce growth factors, i.e. CSF-1, IL-3, GM-CSF that are able to promote placental growth (Tsoukatos et al., 1994). Assessment of splenic gene expression therefore might suggest mechanisms underlying the immune protection phenomenon.

5.3: MATERIALS AND METHODS

4.3.1: Animal model

Male and female ICR mice, 6-8 weeks of age, were purchased (Harlan Sprague-Dawley, Indianapolis, IN) and housed individually (males) or at 5 per cage (females) for a 2-week acclimation period. For breeding, males were housed overnight with females, and females checked for vaginal plugs the next morning, which was designated day 0 of gestation. Mice were given food (NIH 31 open formula) and distilled water ad libitum, and were maintained under controlled conditions of temperature (72°F), humidity (40-60%) and lighting (14/10 light/dark cycle).

Diabetes was chemically induced in female mice by intraperitoneal (IP) injection with 200 mg/kg streptozocin (STZ) (Sigma, St.Louis, Mo., USA) 7 days before mating. Blood glucose (BG) levels in tail vein blood were determined every 3-5 days using glucose kits (Sigma), and were used to categorize diabetic mice. Mice with average blood glucose ≥ 20 mmol/L were previously found to produce fetuses with increased birth defects (Punareewattana and Holladay, 2003) and were used in this study.

4.3.2: Immune stimulations

To modulate maternal immune responses, female mice received one of the following: Complete Freund's adjuvant (CFA) (Sigma) 20-30 μl, by footpad injection; Granulocyte-macrophage colony-stimulating factor (GM-CSF) (Sigma) 8000 units, IP injection; or Interferon-γ (IFN-γ) (Gibco BRL) 1000 units, IP injection. All of the agents were injected twice, with the first injection at 1 week and the second injection at 1 day before STZ injection. These immune stimulation techniques had previously been found by us to reduce fetal defects in mice caused by chemical teratogens (Holladay et al., 2000; Sharova et al., 2000).

4.3.3: Sample collection and teratology

Pregnant mice were killed by cervical dislocation at gestation day (GD) 17. Spleens were collected and snap frozen for later gene expression determination. Fetuses were examined freshly and then preserved in absolute ethanol for later detailed examination. General information was recorded, including spleen weight, number of total fetuses, number of live fetuses, number of resorptions or dead fetuses, and fetal weight. Collected live fetuses were examined externally for the major neural tube defect abnormalities including

exencephaly, microcephaly, and spina bifida. Other abnormalities present were recorded as well.

4.3.4: Microarray preparation

One hundred fifty-one sequence-verified I.M.A.G.E. Consortium clones were purchased from ATCC (Manasas, VA) and Incyte Genomics Inc. (Palo Alto, CA). Genes selected for study included growth factors, cytokines, cell cycle genes, apoptotic genes, transcription factors, kinases, oncogenes and tumor suppressors (**Table 5.1**). Equimolar amounts of cDNA generated from the clones by PCR were immobilized on nylon membranes (Hybond N+, Amersham) with VP 384S2 multi-blot replicator and the Vicki Ultra High Density Array & Registration system (V&P Scientific Inc, San Diego, CA) and cross linked by UV irradiation using a TL-2000 UV Translinker (UVP, Upland, CA), as previously described (Sharova et al., 2002).

4.3.5: cDNA synthesis and hybridization

Six or more spleen samples from each experimental group were used for gene expression comparison. Total RNA from each spleen was extracted with Tryzol^{TM} (Gibco, Rockville, MD) according to the manufacturer protocol, and dissolved in diethyl pyrocarbonate (DEPC; Sigma)-treated dH_2O. Two μg of total RNA were combined with 50 ng of random hexamers, and the mix was subjected to 3 cycles of heat denaturation. Double-stranded cDNA synthesis was performed using the AMV RT and Universal Riboclone® cDNA Synthesis System (Promega, Madison, WI) according to the manufacturer protocol. ^{32}P-labeled probe was synthesized from ds-cDNA with the Prime-a-Gene® Labeling System (Promega) according to the manufacturer protocol.

The membranes were pre-hybridized for 15 min at 42°C in: 50% formamide; 0.12 M Na_2HPO_4, pH 7.2; 0.25 M NaCl; 7% SDS, then added with heat denatured ^{32}P-labeled probe, and later incubated overnight at 42°C. After-hybridization membranes were washed in: 2x SSC/0.1% SDS 45 min; 0.5x SSC/0.1% SDS 45 min; 0.1x SSC/0.1% SDS 45 min. The membranes were then exposed and scanned using the phosphoimager system from Molecular Dynamics® (Amersham Biosciences, Piscataway, NJ). Resultant electronic images of membranes were analyzed by ImageQuant® Software (Molecular Dynamics®), after which gene expression data were stored on spreadsheet software for statistical analysis. All data were normalized relative to internal β-actin controls.

4.3.6: Statistical analysis

Gene expression differences across treatment groups were examined by three different data analysis procedures:

a) ANOVA was used to identify individual genes whose expression was different in a treatment group as compared to control, at $p < 0.05$. This comparison was performed using the General Linear Model (GLM; Minitab version 13; Minitab Inc., PA).

b) Principal components analysis (PCA) was used to search for shifts in gene clusters or families, to provide insight into coordinate aspects of gene expression. PCA, a branch of multivariate analysis, is a data reduction technique used to create a small set of uncorrelated variables that account for a large proportion of the total variance in the original variables (Sokal and Rohlf, 1969). The PCA analysis was performed using the SAS system (SAS Institute Inc., Cary 27513, NC). A scree plot was constructed to estimate the number of principal components (PC) to attempt to interpret. Attention was focused on those PCs that explained at least 5% of the variation in gene expression. These selected PCs were then subjected to ANOVA to test for treatment effects. PC pairs were also plotted against each other to show coordinate space effects on gene expression by treatments. Finally, PC scores for each gene were examined to identify genes that affected the PC both positively and negatively.

5.4: RESULTS

5.4.1: Diabetic teratology and effects of immune stimulations

We previously reported that abnormalities occurred in 50.1±6.95% of fetuses from the present diabetic mice, predominantly in the form of neural tube defects expressed in fetuses as exencephaly and/or spina bifida with variable degree of severity. The control mice displayed no defects; and fetuses from immune stimulated dams displayed significant reductions in defects: CFA: 20.9±4.5%; GM-CSF: 23.3±6.3%; and IFNγ: 13.9±3.7% (Punareewattana and Holladay, 2003). For the present report, total RNA was extracted from pooled maternal spleens collected from each of these control and treatment animals, and reverse transcribed to cDNA.

5.4.2: Gene expression comparisons and gene relations

5.4.2.1: Gene expression changes relative to control

The comparative gene expression data for all experimental groups were first calculated as mean ± SEM, along with ANOVA *p*-values for each gene. Twenty eight of the 151 genes tested were detected with $p < .05$ (**Table 5.2**), and for the most part corresponded to differences between non-immune stimulated and immune stimulated diabetic mice. The control and diabetic groups were generally not different in gene expression profile by the ANOVA, although many genes approached significance. The diabetes + CFA group was also minimally different from control, except for Msx-2 (Hox8.1) and Pcna (proliferating cell nuclear antigen) which were expressed at significant lower levels. Genes that increased significantly in both the groups of diabetes + GM-CSF or diabetes + IFN-γ included annexin A6, bcl2, bcr, BID, BMP-4, caspase-8, Cdc2a, Fol-2, c-fos, GM-CSF, integrin-α6, MDM-2, Myc, Nos3, oxytocin receptor, P21/H-ras-1, and TRAF-5 (**Table 5.2**). Genes that decreased significantly in both groups of diabetes + GM-CSF or diabetes + IFN-γ included histone 3a, Msx-2 (Hox8.1), p21(waf), prostaglandin D synthase, R-ras, and TGF-β3 (**Table 5.2**). Genes that decreased significantly only in the group of diabetes + GM-CSF included cytochrome C, integrin-β, and Rxra. Genes that increased significantly only in the group of diabetes + IFN-γ included IER-2 and integrin-5α (**Table 5.2**). It was noted that these up- or down-regulated genes observed in the GM-CSF or IFN-γ groups showed similar trends toward effect in the CFA group that were not statistically significant. To visualize the shared effects of the immune stimulation procedures, the expression level of selected up- and down-regulated genes from table 5.2 was plotted in separate graphs (**Fig. 5.1**). These graphs show of spleen gene expression effects by the 3 immune stimulators, but with level of effect varying by stimulator.

In addition to twenty seven genes significantly affected by treatment, the ANOVA revealed a relatively large number of genes whose expression was not significantly affected but numerically different by > ±50% from control (**Table 5.3**). This included a small number of genes in the diabetic group (10) and the diabetes + CFA group (14), and a larger number of genes in the diabetes + GM-CSF group (31) and the diabetes + IFN-γ group (35). Most of these genes displayed a numerically higher expression level than control. To visualize this potentially more global effect of each treatment on gene expression, the expression level of Table 5.3 genes was plotted relative to control (**Fig. 5.2**). These graphs once again suggested that expression level for the selected genes was not greatly different in splenic tissue of control and diabetic mice (**Fig. 5.2 a**), and was not greatly changed by the CFA immune

stimulation (**Fig. 5.2 b**). In contrast, the maternal GM-CSF or IFN-γ immunizations resulted in visually-apparent up- and down-regulation of splenic gene expression (**Fig. 5.2 c-d**).

5.4.2.2: Gene expression changes that were linked to another changed gene

Changes in expression level of individual genes may be less meaningful than changes in functionally-related gene pairs (Sharova et al., 2000). To search for genes with linked altered expression, raw data for each gene across all groups were used to plot correlation graphs with other all genes. Approximately 22,000 regression graphs were produced and evaluated by visual inspection for evidence of positive or negative correlation. For graphs where a correlation was suggested, regression analysis was used to determine if a significant correlation existed. Several groups of gene relationships were identified, and examined by ANOVA for a relationship to immune stimulation treatment. Up-regulating genes (annexin a6, bcl2, BID caspase-8, Cdc2a, Fol-2, GST, GM-CSF, Myc, Nos3 Oxytocin receptor, p21/H-ras and TRAF-5) had a strong correlation to each other, as did down-regulating genes (histone 3a, Msx-2, p21(waf), PGD synthase, R-ras, Rxra, and TGF-β3). Significant reverse correlations, i.e. between up-regulated and down-regulated genes, were noted as well.

5.4.3: Principal components analysis of gene expression

Given the relatively large number of genes showing non-significant but >50% change in gene expression level, all data from the 5 experiment groups were re-analyzed by principal components analysis. Each PC has an eigenvalue that represents variability attributed to the PC. A scree plot showing eigenvalues of these PCs in order is presented (**Fig. 5.3**), and shows three PC with eigenvalue > 10.0, suggesting latent coregulation of gene expression. PCs 1-5 contained genes that accounted for the majority of the total variance detected by the PCA, and were selected on this basis and analyzed for a treatment-related effect using ANOVA (**Table 5.4**).

A significant effect was found in PC 2 by the treatment GM-CSF and IFN-γ, and in PC 5 by the difference between the CFA and IFN-γ groups. These results agree with and strengthen the visual suggestion of effect of these treatments that was seen in Fig. 5.1 and 5.2 c-d.

The PCA comparisons are shown graphically by box plot in **Fig. 5.4**. These graphs demonstrate that the gene expression eigenvalues of the GM-CSF and IFN-γ groups in PC 2 that are different from the others (last two bars of Fig. 5.4b); a different and less dramatic effect on gene expression by the treatment CFA vs IFN-γ was seen in PC 5 (middle and last

bar in Fig. 5.4d). Total shifts in coordinate space gene expression profiles by treatment can be visualized by plotting the PC that contain high variability against each other (Sharova et al., 2000; 2002). **Fig. 5.5** shows such PC pairs for each treatment. In each case, coordinate gene expression for the control, diabetes, and CFA groups share relatively close PC coordinate positions, as do the GM-CSF and IFN-γ groups (**Fig. 5.5 a-b**). In Fig. 5.5 d-c, PC 5 was plotted against other PCs, and once again shows that the CFA coordinate gene expression is shifted away from the IFN-γ groups as seen in Fig. 5.4d.

The PCA analysis suggested the importance of gene variability in PC 2 and 5. The twenty specific genes that most affect PC 2 and 5, both positively and negatively, are shown in Tables 5.6 and 5.7. The genes that most affected PC 2 and were up-regulated included caspase-8, Cdc2a, Bcl2, TRAF-5, oxytocin receptor, annexin A6, Fol-2, GM-CSF, P21/H-ras-1, Nos3, EGF, Glutathione synthase (GST), Bcr, and Myc. Genes that most affected PC 2 and were down-regulated included p21(waf), prostaglandin D synthase, R-ras, histone 3a, TGF-β3, Msx-2 (Hox 8.1), Rxra, PIAS-1, and IL-2.

The genes with positive PC score in PC 5 represented down-regulated genes in the group of CFA, and included STAT1, Pcna, annexin A4, and IFN-γ. The genes with negative PC score in PC 4 represented down-regulated genes in the group of IFN-γ, and included Egr-1, E2f1, RANTES, Jak2, Jak3, and growth hormone.

5.5: DISCUSSION

Non-specific maternal immune stimulation has been shown to effectively reduce birth defects in several teratogenic models, including chemical-induced teratogenesis and metabolic disorder-related teratogenesis (e.g., diabetes mellitus) (reviewed by Holladay et al., 2002). Very limited information is available regarding mechanisms mediating these protective results. It is recognized that immune cells, including macrophages and NK cells, and cytokine products of these cells, play critical roles during pregnancy (Robertson, 2000). As such, immune cell or cytokine pathway disturbances by teratogens could adversely affect pregnancy outcome.

Sharova et al. (2002) reported increased TBF-β3 and GM-CSF gene expression in maternal splenic leukocytes after immune stimulation and urethane treatment, which correlated significantly with reduction of urethane-induced cleft palate. Sharova et al. (2003) also demonstrated improved placental morphology and gene expression in urethane-exposed

pregnant mice, events once again suggested as possibly related to protection against cleft palate. It was not known if these placental changes were downstream to the effects on maternal spleen, or if such changes were direct effects of immune stimulation. In this regard, the spleen of pregnant but not virgin mice produces cytokines that stimulate placental-cell proliferation (Athanassakis et al., 1996; Tsoukatos et al., 1994).

We previously reported that 3 diverse immune stimulators, CFA, GM-CSF, and IFN-γ, each significantly reduced the incidence of birth defects caused by diabetes mellitus (Punareewattana et al., 2003). However, we were unable to demonstrate a correlation between improved placental function in these immune stimulated pregnant diabetic mice, and reduced birth defects (Punareewattana et al., unpublished data). The present studies therefore examined effects of immune stimulation on maternal spleen cells in diabetic mice with or without immune stimulation. A microarray that contained selected cytokine and growth factor genes, and additional genes regulating cell cycle-related events, was used as an initial attempt to monitor splenic leukocyte function. A weakness of this approach is the recognition that cytokine secretion by immune cells may be altered independent of cytokine production (Hill et al., 1996; 1998), thus such effects may be missed. However, the array approach offered a relatively rapid method to broadly examine effects of the immune stimulation on spleen leukocytes.

The diabetic spleen showed a general non-significant trend toward up-regulated expression of studied genes. In contrast, the three immune stimulation procedures produced altered patterns of both up- and down-regulated gene expression that appeared similar, but with different magnitudes. GM-CSF or IFN-γ injection produced a largely shared profile of increased and decreased gene expression, while CFA injection resulted in mostly non-significant trends in the same directions. The time-course of immune activation by these immune stimulants is different, which may explain these observations. Specifically, CFA consists of bacterial components in a slow absorption oil vehicle, while GM-CSF and IFN-γ are purified cytokines.

Three data analysis procedures were used to search for treatment-related differences that may show correlation to reduced birth defects. The initial comparison of expression level of individual genes across treatments resulted in the most changed genes. However, such changes in expression level of individual genes may be less meaningful than coordinate changes in functionally-related gene pairs or gene clusters (Sharova et al., 2000). The

regression and principal component analyses were therefore used to extend observations seen for individual genes, with the hope of identifying subsets of affected genes whose coordinate changed expression may relate to reduced birth defects. Of possible importance, each of the three analysis procedures demonstrated altered expression level for a shared group of genes.

An unexpected observation was that apoptosis-related genes were identified as most influenced, including by PCA. These included both up-regulated (caspase-8, bcl2) and down-regulated (p21, R-ras) genes. Caspase-8 is normally associated with Fas-associated death domain (FADD) and death signal from Fas receptor (Kidd, 1998). However this death signal can be blocked by elevated bcl2, which normally acts upstream to all caspases (Korsmeyer, 1992; Kidd, 1998). BID was also up-regulated in spleen leukocytes. This gene is related to caspase-8 as it is cleaved by caspase-8 (Chou et al., 1999), and induces a conformational change of Bax which is then responsible for mitochondrial cytochromc c release during apoptosis (Desagher et al., 1999). R-ras and p21(waf), whose gene products may act against anti-apoptotic activity of bcl2, were down-regulated. Transcription factors c-fos and c-myc that regulate expression of genes important for cell growth, were both up-regulated. Additional up-regulated genes that favor cell growth included Cdc2a, BMP-4, folate binding protein 2, oxytocin receptor, p21/H-ras, intergrin 5α, and intergrin α6. BCR was also up-regulated, which is normally associated with ABL (the latter gene was not significant up-regulated), and has been associated with prolonged hematopoitic cell survival due to anti-apoptotic activity (Cortez et al., 1995). Msx-2 was down-regulated in all groups of immune stimulated mice. This effect could again favor cell growth because Msx-2 regulates BMP-4 expression and suppresses morphogenesis (Ferrari et al., 1998). Apoptosis level in the present maternal spleens was not examined because this endpoint was not expected to be affected. However, it seems likely that the often-opposing apoptotic gene expression patterns detected may simply reflect an activated state of the immune system.

Nitric oxide synthase 3 (Nos3) and Glutathione synthase (GST) were both increased by immune stimulation. Nitric oxide release increases cGMP levels and is a mediator of cytokine release by macrophages (Hill et al., 1996). As such, the inhibitory effect of diabetes on cytokine release by immune cells might in part be counteracted by up-regulated expression of Nos in immune stimulated mice. Increased expression of GST may result in higher levels of glutathione, which are low in diabetic embryonic tissues (Sakamaki et al.,

1999) and have been related to increased free radical toxicity in embryonic and placental tissues.

Expression level of several growth factor genes in maternal spleen was also altered by the immune stimulation procedures. Growth factor gene expression changes that were identified by the PCA as contributing most to variability between diabetic animals with or without immune stimulation treatments included GM-CSF, EGF and TGF-β3. GM-CSF and EGF were increased by immune stimulations, and TGF-β3 was decreased by immune stimulations. These results agree partially with a previous report, where GM-CSF and TGF-β3 were implicated as cytokines related to maternal immune protection against urethane-induced cleft palate (Sharova et al., 2002). These cytokines therefore might be further evaluated for a relationship to the reduced birth defects that occurred in immune stimulated diabetic mice. The involvement of these cytokines in reproductive processes may further support this idea.

Specifically, GM-CSF was originally described as a regulator of proliferation and differentiation of hemopoietic progenitor cells, but now is also recognized as a principal determinant of survival and functional phenotype of mature macrophages and granulocytes (Baldwin, 1992). This cytokine mediates the recruitment and modifies the behavior of uterine leukocytes during the post-mating inflammatory response in mice (Robertson et al., 2000). Its ability to stimulate cytotrophoblast differentiation has also been demonstrated (Garcia-Lloret et al., 1994). GM-CSF also induces uterine-associated macrophages to synthesize immunosuppressive molecules, which act to down-regulate local T-lymphocyte activation through binding to the IL-1 receptor; and increase PGE_2 which inhibits cytotoxic T-lymphocyte generation (Robertson et al., 1994). Another increased-level growth factor, EGF, identified by PCA may be less important than GM-CSF in that its level was only about 1.3 time compare to control in the GM-CSF and IFN-γ groups, and did not change in the CFA group.

TGF-betas have widely demonstrated importance during fetal development. TGF-β2 has been described as an important signal for triggering mesoderm differentiation in digit formation (Merino et al., 1999), and addition of TGF-β to culture medium improved 11-day mouse embryo digit formation (Milaire, 1996). In zebrafish, mutations in the cyc gene, which encodes a signal that activates TGF-β family member Nodal, results in neural tube defects (Muller et al., 2000). Mutations of the TGF-β3 gene have also been associated with cleft

palate in both humans and mice (Tudela et al., 2002). Further, addition of TGF-β3 to TGF-β3 null mutant palatal shelves in culture results in shelf adhesion (Gato et al., 2002). TGF-β1 crosses the mouse placenta (Letterio et al., 1994), suggesting the possibility that increased TGF-β production in immune stimulated pregnant mice could lead to increased TGF-β in fetal tissues. In the current study, down-regulated TGF-β3 but unaltered TGF-β1 and TGF-β2 of splenic cells were observed after the immune stimulations. These results in diabetic mice differ from a previous report, where immune stimulation increased TGF-β3 gene expression in urethane exposed mice (Sharova et al., 2002). TGF-β3 has been demonstrated to be a potent suppressor of hematopoietic cell proliferation which was different from TGF-β1 and TGF-β2 that have bidirectional effects on hematopoiesis (Jacobsen et al., 1991). TGF-β3 may also induce apoptosis by a Fas-independent pathway (Fogli et al., 2000), therefore its down-regulation by immune stimulation in splenic cells correlates very well with the activated state of the immune system, but did not correlate with reduced birth defects.

In summary, non-specific immune stimulation by different procedures produced shared alterations in maternal spleen leukocyte gene expression profile. The expression of several pro- and anti-apoptotic regulatory genes was consistently changed, however some genes were up-regulated and others down-regulated. Concurrent with this observation, we observed increased activity of genes that stimulate proliferation rather than cell death. These summative changes might lead to increased splenocyte proliferation after the immune stimulations rather than increased cell death, however these endpoints were not examined. The increased Nos3 expression would be expected to associate with increased cytokine release from macrophages, and macrophages have repeatedly been implicated as potentially important cells in immune protection against birth defects (Nomura et al., 1990; Sharova et al., 2002). Finally, increased GM-CSF gene expression across immune stimulation treatments may suggest increased production and release of this cytokine. GM-CSF is critically important in placental and fetal development, and was also suggested as a potential mediator of reduced cleft palate in urethane-exposed mice after immune stimulation (Sharova et al., 2002), and of reduced limb malformations in cyclophosphamide-treated mice after intra-uterine administration (Savion et al., 1999). Continued studies will be required to determine if such systemic immune changes in pregnant mice, induced by different immune stimulation procedures, may relate to reduced birth defects associated with these procedures.

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Table 5.1 Partial list of genes used in microarray grouping by function

Growth factors and receptors	Apoptotic genes
Epidermal growth factor (EGF)	Caspase 2, 6, 7, 8, 9, 11, 12, 14
EGF-R	Apoptosis-inducing factor (AIF)
Estrogen receptor (Estr-R)	BAD, BAK, BAX, BID
Fibroblast growth factor (FGF)	Bcl2, bcl2l
Folate binding protein 1 (Fol 1), 2	Bcr, Abl
Insulin-like growth factor-I (IGF-I), II	Granzyme B, F
Platelet derived growth factors A (PDGF-A), B	P53, p21(waf), Rb1
Transforming growth factor-β1 (TGF-β1), 2, 3	**Cell cycle genes**
Growth hormone (GH)	Cyclin B1, D2, G
VEGF	Cell division cycle control protein 2a (Cdc2a)
Cytokines	Growth differentiation factor-1 (GDF-1)
Interferon-γ (IFN-γ)	Growth arrest specific protein (GAS), GAS2, GAS5
Tumor necrosis factor-α (TNF-α)	**Oncogenes and Tumor suppressors**
Granulocyte macrophage-colony stimulating factor (GM-CSF)	Rho A, C
	P53, Rb1
Interleukin-1 (IL-1); 2, 4, 10, 12, 15, 16	A-raf, B-raf
Transcription factors	C-src, R-ras
c-fos	P21/H-ras
c-jun	mSmad3
E2F transcription factor 1 (E2f1)	C-Rel
Early growth response 1 (Egr-1), 2	Myb proto-oncogene
I-kappa B alpha chain (IκBα)	**Kinases**
Msx-1, 2	Protein kinase C (PKC)-α, δ
myc	Jak 1, 2
NF-kB-1, 2	Mekk
Rxra	ERK-1, 2
Wee-1	Syk

Table 5.2 Complete splenic gene expression data

No.	Gene name	ANOVA p-value	Control		Diabetes		Diabetes +CFA		Diabetes +GM-CSF		Diabetes +IFN-g	
			Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem
1	c-abl	0.60	65	10.3	62	7.4	76	11.8	72	12.7	85	18.0
2	AIF	0.82	362	83.6	418	45.1	405	91.8	474	107.8	484	76.4
3	Annexin A3	0.14	99	17.5	95	10.9	71	7.1	67	6.2	113	19.4
4	Annexin A4	0.69	87	9.7	73	11.7	65	16.9	64	7.8	86	17.1
5	Annexin V	0.17	132	13.6	161	13.2	142	9.2	168	22.7	193	21.2
6	Annexin A6	0.008	2261	276.7	2251	150.0	2499	323.6	3391	351.1	3558	533.6
7	Annexin A7	0.07	85	13.1	99	9.1	103	10.1	80	9.3	136	22.8
8	Api2	0.16	151	25.2	120	10.7	134	20.7	89	15.9	106	14.6
9	BAD	0.16	80	14.0	120	9.5	141	34.7	93	13.9	122	11.8
10	BAK	0.89	130	34.0	124	19.3	140	28.9	157	24.7	125	24.2
11	BAX	0.85	244	29.5	221	11.9	216	12.1	218	17.0	218	22.8
12	Beta-actin	-	3000	0.0	3000	0.0	3000	0.0	3000	0.0	3000	0.0
13	Bcl2l	0.14	99	23.1	132	17.3	67	15.9	126	20.5	122	14.3
14	bcl2	0.007	2159	255.3	1967	147.4	2169	286.8	3029	416.7	3495	569.1
15	bcr	0.01	79	11.9	86	10.2	108	7.5	125	12.8	147	23.8
16	BID	0.001	49	13.5	85	12.2	100	7.9	115	21.7	164	18.2
17	BMP-4	0.004	11891	1151.2	11874	1113.9	9700	692.7	19227	2045.0	18415	3702.4
18	BMP-5	0.98	96	16.0	94	13.6	84	16.6	84	12.5	88	23.4
19	Calmodulin 3	0.30	179	8.2	252	44.5	183	35.3	151	12.1	198	14.5
20	Caspase 2	0.21	121	20.4	165	15.0	140	8.5	145	23.9	115	13.6
21	CASPASE-6	0.66	140	53.4	109	8.8	104	20.1	83	18.6	103	20.8
22	CASPASE-7	0.26	190	24.0	186	12.9	215	23.9	208	27.7	252	31.8
23	CASPASE-8	0.001	2334	343.4	2344	224.9	2810	278.8	4423	617.7	4074	579.2
24	CASPASE-9	0.14	67	10.0	102	6.9	98	14.9	128	27.6	141	37.8
25	CASPASE-11	0.41	53	16.4	74	9.0	84	14.9	80	15.4	100	26.5
26	CASPASE-12	0.71	29	10.7	46	6.9	39	6.6	33	11.6	43	14.3
27	CASPASE-14	0.05	1016	81.3	1344	103.0	1031	115.5	1390	105.7	1321	86.9
28	Cadherin 1	0.79	133	20.7	167	13.3	155	14.1	150	23.6	168	38.9
29	Cadherin 2	0.09	81	14.1	62	8.5	122	33.4	64	10.9	97	18.2
30	Cadherin 3	0.20	93	21.2	40	16.2	33	15.8	39	17.7	32	20.7
31	Cell division cycle control protein 2a (Cdc2a)	0.003	1796	287.8	1842	217.8	2191	324.0	3349	489.5	3171	362.2
32	Coagulation factor II (protrombin)	0.18	61	16.4	42	8.8	65	11.4	94	31.7	46	9.4
33	Clathrin (AP19)	0.47	190	28.6	172	14.1	191	13.7	185	27.4	225	22.8
34	*CREB*	0.07	26119	4082.2	25646	2842.1	24377	3937.6	42600	7456.3	37855	8352.9
35	Cyclin B1 (CcnB1)	0.56	41	5.6	46	8.5	50	14.9	65	10.6	55	6.7
36	Cyclin D2 (CcnD2)	0.18	140	17.5	141	6.6	137	9.6	144	12.2	174	11.8
37	Cyclin G (CcnG)	0.40	614	41.4	686	40.2	726	47.5	773	94.4	763	77.5
38	Cytochrome C	0.04	98	10.2	144	18.8	122	16.9	68	11.0	101	15.2
39	DHFR (dihydrofolate reductase)	0.78	91	9.7	97	11.8	97	12.1	96	19.2	75	12.3
40	E2F transcription factor 1 (E2f1)	0.24	72	22.3	136	14.7	120	37.3	98	16.7	97	17.5

Table 5.2 Continued

No.	Gene name	ANOVA p-value	Control		Diabetes		Diabetes +CFA		Diabetes +GM-CSF		Diabetes +IFN-g	
			Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem
41	Egr-1 (Krox-24)	0.37	6755	348.6	7219	679.9	7878	903.5	6619	599.8	5755	503.3
42	Egr-2; Krox-20	0.52	3496	272.3	4116	220.9	3677	307.0	4377	628.3	4077	463.2
43	Endothelin-B receptor	0.86	361	93.6	302	24.2	274	26.3	304	67.1	311	51.2
44	eotaxin	0.79	472	45.1	479	44.6	509	56.7	569	81.8	535	82.7
45	EGF	0.14	160	21.6	157	14.5	159	19.5	212	24.9	202	16.5
46	EGF-R	0.67	243	58.5	219	21.3	191	25.3	194	16.2	183	20.4
47	ERK-1	0.07	213	13.3	240	12.1	197	9.2	189	11.9	203	22.7
48	ERK-2	0.54	85	19.3	77	6.2	92	17.4	65	14.8	63	13.8
49	Estrogen receptor	0.61	123	20.1	140	9.3	121	10.9	115	7.9	128	13.7
50	FADD	0.64	225	24.8	272	20.2	265	36.1	290	41.0	290	39.7
51	Fibroblast growth factor1 (FGF1)	0.25	92	9.8	102	10.0	113	20.0	94	15.0	137	17.6
52	Fibronectin	0.39	128	23.2	130	10.0	147	19.3	95	7.6	121	26.0
53	Folate-binding protein 1	0.97	86	12.2	100	13.8	95	19.9	95	26.6	104	19.7
54	Folate-binding protein 2	0.000	169	20.3	157	16.1	153	10.8	270	36.1	277	22.4
55	c-fos	0.09	174	13.9	186	15.6	171	18.7	249	36.4	241	36.1
56	GAPDH	0.77	2541	243.2	2942	280.1	2889	155.4	2758	355.4	3076	202.1
57	Glutathione synthetase (GST)	0.34	124	14.2	123	11.9	166	50.8	165	14.0	169	12.8
58	GM-CSF	0.02	130	19.0	180	20.3	207	22.6	228	37.0	278	37.7
59	Growth arrest specific protein (GAS)	0.11	110	19.6	89	7.4	98	17.1	60	7.9	90	5.9
60	GAS-2	0.91	106	27.7	85	11.5	92	20.5	89	15.4	99	9.1
61	GAS-5	0.83	126	10.3	139	12.0	132	8.5	116	16.1	135	23.6
62	Granzyme B (Gzmb)	0.31	88	9.8	85	8.3	68	6.4	64	10.6	71	10.9
63	Granzyme F (Gzmf)	0.59	95	27.0	81	11.5	115	14.7	117	40.3	120	19.9
64	Growth hormone (GH)	0.44	68	8.9	97	13.3	86	9.7	79	16.8	67	14.0
65	Growth differentiation factor 1	0.64	360	19.3	364	21.0	355	45.4	396	19.3	410	37.9
66	HEDGEHOG DESERT	0.96	3998	485.1	3960	376.4	3729	503.8	3929	484.8	4291	452.8
67	HEDGEHOG INDIAN	0.37	115	18.7	122	8.1	125	16.6	125	16.2	155	14.0
68	Histone 3a	0.002	393	33.7	395	24.4	362	22.5	260	17.9	287	20.1
69	IAP-3	0.33	255	11.4	336	27.3	316	36.9	345	45.9	367	52.8
70	IGF I	0.68	126	18.1	152	19.9	178	25.7	165	31.3	168	25.7
71	IGF II	0.89	128	13.1	133	8.8	146	20.9	133	10.7	140	11.2
72	I-kappa B alpha chain	0.56	149	17.7	143	11.7	130	20.2	112	10.7	129	21.5
73	IL-1beta	0.45	46	11.9	84	11.0	80	7.9	86	28.8	84	20.3
74	IL-2	0.58	1900	197.5	2055	151.6	1952	108.6	1803	149.6	1722	135.5
75	IL-4	0.74	45	9.6	45	17.6	61	25.5	43	12.0	22	9.2
76	*IL-9R*	0.30	248	21.2	284	18.6	247	26.9	315	40.4	319	38.8
77	IL-10	0.53	339	28.4	399	31.5	341	46.3	417	50.0	411	44.0
78	IL-12-35	0.88	54	20.7	67	5.6	75	19.9	74	20.9	63	9.3
79	IL-12-40	0.48	354	28.3	366	28.6	402	33.1	449	59.7	413	50.0
80	IL-15	0.07	148	18.3	121	5.8	142	11.1	114	12.0	108	7.4

Table 5.2 Continued

No.	Gene name	ANOVA p-value	Control		Diabetes		Diabetes +CFA		Diabetes +GM-CSF		Diabetes +IFN-g	
			Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem
81	IL-16	0.93	270	10.2	251	17.1	271	22.2	265	19.8	256	28.4
82	Immediate early response 2	0.002	77	12.4	71	4.2	94	7.1	97	15.6	142	23.1
83	Interferon gamma	0.51	31	10.2	39	8.3	25	9.0	22	8.4	45	25.2
84	Inhibin alpha	0.58	137	20.0	162	16.9	190	32.8	185	21.3	184	33.3
85	Inhibin beta-B subunit	0.55	174	24.0	197	19.8	179	35.7	179	26.5	237	33.9
86	Integrin alpha 1	0.84	295	26.7	350	28.3	311	67.1	365	69.6	350	49.8
87	Integrin 5 alpha	0.01	136	11.4	153	15.2	144	6.1	201	31.0	233	29.8
88	Integrin alpha-6	0.03	2201	310.6	2474	341.7	1953	164.9	3633	457.1	3492	566.5
89	Integrin beta 1	0.02	113	18.2	130	11.8	99	12.9	63	13.6	105	6.2
90	Integrin beta 2 ; CD18; LFA-1	0.43	75	8.2	68	7.8	68	7.9	54	12.2	82	11.7
91	Janus kinase 2 (Jak2)	0.35	64	13.1	58	8.4	79	16.6	64	12.0	41	9.4
92	Janus kinase 3 (Jak3)	0.31	192	18.0	200	7.9	201	15.1	174	13.1	168	18.6
93	c-jun	0.70	275	18.5	324	23.1	319	49.1	326	53.0	366	61.4
94	MADR-2	0.22	110	11.0	114	11.9	111	13.5	76	13.6	95	9.5
95	MDM-2	0.05	21985	1980.7	23723	1995.0	20542	1642.1	32480	4747.3	32578	5734.5
96	MEK kinase (Mekk)	0.14	154	20.9	115	18.4	134	14.1	142	21.2	182	10.8
97	*MIP-1alpha*	0.63	323	13.9	386	33.6	359	51.3	421	61.3	406	53.5
98	MMP-2	0.64	154	16.5	175	23.8	152	19.5	184	23.0	202	22.8
99	MMP-9	0.80	355	38.4	370	33.4	373	26.9	332	39.6	406	50.5
100	Msx-1 (Hox7.1)	0.18	88	21.1	112	12.4	125	17.1	117	15.5	146	6.7
101	Msx-2 (homeobox Hox8.1)	0.000	574	100.1	417	84.2	200	33.9	83	18.0	93	14.3
102	Myb proto-oncogene	0.61	91	9.0	80	8.2	81	1.1	66	10.1	75	17.3
103	Myc	0.02	116	14.3	141	11.9	163	28.7	200	27.8	215	26.2
104	NFkB-1(NF-KAPPA-B); p105	0.28	83	15.9	84	8.4	87	9.3	62	12.9	103	14.1
105	NF-kB-2 P100	0.87	88	16.9	87	11.0	91	16.1	75	17.3	99	15.6
106	Nitric oxide synthase 3 (NOS3)	0.03	106	13.8	88	14.3	110	19.0	158	24.7	163	26.8
107	Oxytocin receptor (human)	0.000	4297	411.3	4331	341.0	4877	562.7	7759	700.2	7855	868.8
108	P21/H-RAS-1 transforming protein	0.005	72	20.0	93	16.1	112	37.8	188	20.5	176	24.4
109	p21 (waf)	0.000	924	209.3	1083	126.1	867	118.5	246	23.0	246	25.3
110	p53	0.38	351	43.2	379	27.6	354	49.0	385	28.8	460	53.1
111	PAF acetylhydrolase	0.45	255	22.7	279	14.4	268	16.6	297	34.4	318	33.2
112	PDGF, A	0.58	163	41.2	144	20.1	160	17.4	106	26.4	121	27.4
113	PDGF, B chain	0.37	85	11.5	92	15.4	97	14.4	65	12.6	60	12.2
114	PERFORIN	0.99	1492	193.4	1477	144.2	1400	150.3	1478	120.5	1425	161.5
115	Prolactin	0.68	70	13.3	61	6.4	82	7.9	77	23.8	57	19.3
116	Proliferin	0.25	92	16.4	63	8.5	68	7.4	84	12.6	88	11.5
117	Proliferating cell nuclear antigen (Pcna)	0.05	79	12.5	88	18.2	30	10.8	83	22.7	122	18.7
118	Prostaglandin receptpr ep1	0.96	2397	431.8	2597	261.6	2371	294.0	2345	232.3	2540	180.9

Table 5.2 Continued

No.	Gene name	ANOVA p-value	Control		Diabetes		Diabetes +CFA		Diabetes +GM-CSF		Diabetes +IFN-g	
			Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem
119	Prostaglandin receptor ep2	0.55	70	18.8	92	24.1	57	12.4	54	13.8	93	9.3
120	Prostaglandin D synthase	0.000	4626	977.3	6474	828.9	4911	677.5	818	108.4	951	150.9
121	PIAS-1	0.43	1312	121.3	1401	102.4	1268	111.6	1195	100.4	1134	100.0
122	PKC alpha	0.93	148	12.0	143	14.6	128	8.1	134	25.7	140	6.8
123	Pkacb	0.09	110	6.7	140	17.6	141	15.2	82	8.4	132	16.2
124	PKC-delta	0.51	57	13.1	72	11.0	83	15.1	63	12.4	87	11.3
125	A-RAF proto-oncogene	0.30	142	14.2	152	10.5	109	8.9	144	24.5	131	15.7
126	B-raf oncogene	0.74	309	24.8	354	32.7	309	26.4	305	48.0	306	26.5
127	RANTES	0.58	72	7.5	94	26.2	81	18.1	73	10.9	43	11.0
128	R-ras	0.000	2774	486.8	4048	453.5	2923	446.4	589	84.5	540	81.8
129	c-Rel	0.97	52	14.9	51	10.8	46	20.0	48	11.8	60	6.1
130	Retinoblastoma 1 (Rb1)	0.77	132	11.7	143	17.3	151	18.5	117	19.8	125	21.8
131	Retinoid X receptor alpha (Rxra)	0.01	120	17.8	163	16.0	141	16.9	82	10.7	112	13.6
132	RhoA	0.23	164	33.4	165	10.9	116	18.1	126	15.4	157	20.2
133	RhoC	0.88	137	15.3	140	9.0	146	24.8	146	13.9	160	17.5
134	SMAD3 (mSmad3)	0.19	129	3.6	129	11.8	125	19.7	87	15.1	128	9.4
135	Syk	0.15	96	8.5	114	8.3	100	4.2	120	10.4	130	13.5
136	c-Src kinase	0.20	208	23.4	264	18.6	205	19.9	281	41.0	261	29.0
137	STAT1	0.47	64	5.9	82	12.5	51	14.1	80	16.1	86	21.8
138	*STAT3*	0.30	178	15.3	204	18.0	181	20.3	250	35.1	221	31.8
139	SOCS-1	0.47	130	25.3	139	13.4	121	9.1	127	13.6	164	15.8
140	TIMP-2	0.23	115	15.8	105	13.7	100	9.0	66	11.5	99	13.4
141	TRAF-1	0.83	202	33.0	213	27.1	226	32.8	225	28.6	258	42.2
142	TRAF-2A	0.19	106	10.7	99	10.3	124	24.2	66	12.0	103	19.8
143	TRAF-5	0.000	516	33.6	530	33.3	615	73.2	890	93.0	836	82.6
144	TGFbeta 1	0.18	505	56.6	504	27.8	429	26.4	408	37.4	426	33.6
145	TGFbeta 2	0.08	257	18.6	350	20.8	275	23.9	376	62.8	331	22.2
146	TGFbeta 3	0.001	121	13.7	132	13.0	102	13.7	56	4.7	84	7.8
147	TNF alpha	0.38	182	46.4	133	12.9	149	14.6	127	11.1	123	13.3
148	Trophoblast specific protein	0.45	43	10.1	56	8.4	54	15.7	29	9.4	44	12.6
149	VEGF	0.22	67	7.3	100	6.6	113	26.3	100	10.5	93	13.4
150	VCAM-1	0.93	148	34.0	157	15.0	133	16.8	136	26.7	142	28.1
151	wee-1	0.41	104	21.6	106	9.5	114	14.7	96	12.2	141	24.3

Table 5.3 Relative level of gene expression of each experiment groups compared to control. Selected genes that are shown in the table are at least 50% different in a specific group.

Gene name	Diabetes	Diabetes + CFA	Diabetes + GM-CSF	Diabetes + IFN-γ
Annexin V	1.2	1.1	1.3	**1.5**
Annexin A6	1.0	1.1	**1.5**	**1.6**
Annexin A7	1.2	1.2	0.9	**1.6**
BAD	**1.5**	**1.8**	1.2	**1.5**
Bcl2l	1.3	0.7	1.3	1.2
bcl2	0.9	1.0	1.4	**1.6**
bcr	1.1	1.4	**1.6**	**1.9**
BID	**1.7**	**2.0**	**2.3**	**3.3**
BMP-4	1.0	0.8	**1.6**	**1.5**
Caspase-8	1.0	1.2	**1.9**	**1.7**
Caspase-9	**1.5**	**1.5**	**1.9**	**2.1**
Caspase-11	1.4	**1.6**	**1.5**	**1.9**
Caspase-12	**1.6**	1.4	1.2	**1.5**
Cadherin 2	0.8	**1.5**	0.8	1.2
Cadherin 3	**0.4**	**0.4**	**0.4**	**0.3**
Cdc2a	1.0	1.2	**1.9**	**1.8**
Coagulation factor II	0.7	1.1	**1.6**	0.8
CREB	1.0	0.9	**1.6**	1.4
Cyclin B1	1.1	1.2	**1.6**	1.3
Cytochrome C	**1.5**	1.2	0.7	1.0
E2f1	**1.9**	**1.7**	1.3	1.3
FGF1	1.1	1.2	1.0	**1.5**
Folate-binding protein 2	0.9	0.9	**1.6**	**1.6**
GM-CSF	1.4	**1.6**	**1.8**	**2.1**
GAS	0.8	0.9	**0.5**	0.8

Table 5.3 Continued

Gene name	Diabetes	Diabetes + CFA	Diabetes + GM-CSF	Diabetes + IFN-γ
IL-1beta	**1.8**	**1.7**	**1.9**	**1.8**
IL-4	1.0	1.4	1.0	**0.5**
Immediate early response 2	0.9	1.2	1.3	**1.8**
Integrin 5 alpha	1.1	1.1	**1.5**	**1.7**
Integrin alpha-6	1.1	0.9	**1.7**	**1.6**
MDM-2	1.1	0.9	**1.5**	**1.5**
Msx-1 (Hox7.1)	1.3	1.4	1.3	**1.7**
Msx-2 (homeobox Hox8.1)	0.7	**0.3**	**0.1**	**0.2**
Myc	1.2	1.4	**1.7**	**1.8**
Nos3	0.8	1.0	**1.5**	**1.5**
Oxytocin receptor (human)	1.0	1.1	**1.8**	**1.8**
P21/H-RAS-1	1.3	**1.5**	**2.6**	**2.4**
p21 (waf)	1.2	0.9	**0.3**	**0.3**
Proliferating cell nuclear antigen (Pcna)	1.1	**0.4**	1.1	**1.5**
Prostaglandin D synthase	1.4	1.1	**0.2**	**0.2**
PKC-delta	1.3	**1.5**	1.1	**1.5**
R-ras	**1.5**	1.1	**0.2**	**0.2**
TRAF-5	1.0	1.2	**1.7**	**1.6**
TGFbeta 2	1.4	1.1	**1.5**	1.3
TGFbeta 3	1.1	0.8	**0.5**	0.7
VEGF	**1.5**	**1.7**	**1.5**	1.4
Total number	**10 genes**	**14 genes**	**31 genes**	**35 genes**

Table 5.4 Principal components that explain 54% of variability in gene expression between treatments, and ANOVA comparison among 5 treatments

	PC 1	**PC 2**	**PC 3**	**PC 4**	**PC 5**
Eigenvalue	33.416	18.877	10.500	8.799	6.674
Proportion of variation explained	0.229	0.129	0.072	0.060	0.046
Cumulative variation explained	0.229	0.358	0.430	0.490	0.536
ANOVA					
Df	4	4	4	4	4
p	0.586	< 0.0001*	0.335	0.816	0.009*

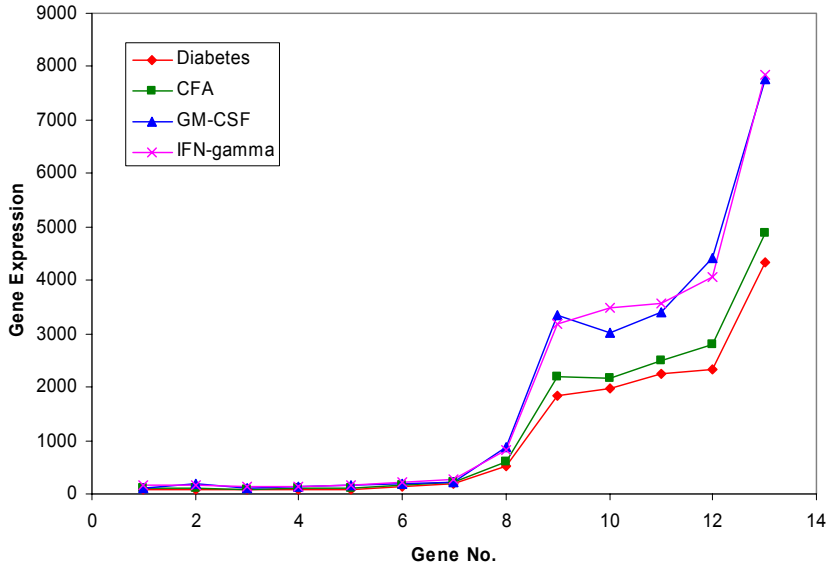
* PC 2 and PC 5 were significant at $p < .05$, and further tested to identify the group that was different from the others.

Table 5.5 Twenty selected genes of both positive and negative PC scores in PC 2. This PC represents the effect of GM-CSF and IFN-γ. Genes with positive PC score were down-regulated, while genes with negative PC score were up-regulated.

Genes that affect PC 2 positively		Genes that affect PC 2 negatively	
Gene name	PC score	Gene name	PC score
P21(waf)	0.21542	Caspase-8	-0.18276
Prostaglandin D synthase	0.20902	Cdc2a	-0.15772
R-ras	0.20401	Bcl2	-0.15100
Histone 3a	0.19612	TRAF-5	-0.14855
TGF-β3	0.18047	Oxytocin Receptor	-0.14586
Msx-2 (Hox 8.1)	0.17971	Annexin A6	-0.14361
Rxra	0.14157	Fol-2	-0.12927
PIAS-1	0.13859	GM-CSF	-0.12851
IL-2	0.13471	P21/H-Ras-1	-0.11305
RhoA	0.13356	Nos3	-0.10843
MADR-2	0.12947	EGF	-0.10721
TIMP-2	0.12799	GST	-0.09059
IL-15	0.12740	Bcr	-0.08927
Integrin-β1	0.12499	Mekk	-0.08704
GAS	0.12496	Myc	-0.08406
ERK-1	0.12183	AIF	-0.06856
Calmodulin 3	0.11732	Msx-1 (Hox 7.1)	-0.06804
I-kappa B alpha chain	0.11708	PAF acytylhydrolase	-0.06268
B-raf	0.11392	BID	-0.06086
Cytochrome C	0.11253	Cyclin D2	-0.05922

Table 5.6 Twenty selected genes of both positive and negative PC scores in PC 5. This PC represents the difference between the effect of CFA and IFN-γ. Genes with positive PC score showed down-regulated expression with the effect of CFA. Genes with negative PC score showed down-regulated expression with the effect of IFN-γ.

Genes that affect PC 5 positively		Genes that affect PC 5 negatively	
Gene name	PC score	Gene name	PC score
STAT1	0.22406	Egr-1	-0.18785
Pcna	0.18892	E2f1	-0.16569
Annexin A4	0.18386	Inhibin-α	-0.16247
IER-2	0.18363	RANTES	-0.15487
Hedgehog Indian	0.17514	Janus kinase 3	-0.14483
IFN-γ	0.16788	IL-16	-0.14018
RhoC	0.16317	Growth hormone	-0.12770
EGF	0.16157	Janus kinase 2	-0.12242
Integrin-β1	0.14406	VEGF	-0.11658
MMP-2	0.14204	BAD	-0.11612
RhoA	0.13812	IGF-II	-0.11269
A-raf	0.13755	R-ras	-0.10302
Calmodulin 3	0.13353	Caspase-12	-0.10278
Bcl2l	0.12245	IL-15	-0.10168
Inhibin-βB	0.12012	TRAF-2A	-0.09735
Annexin A3	0.11806	IGF-I	-0.09646
Cadherin 3	0.10819	BAK	-0.09329
Coagulation factor II	0.09846	IL-4	-0.09281
MADR-2	0.09650	PDGF-B	-0.09005
Integrin-5α	0.09505	Prolactin	-0.08892



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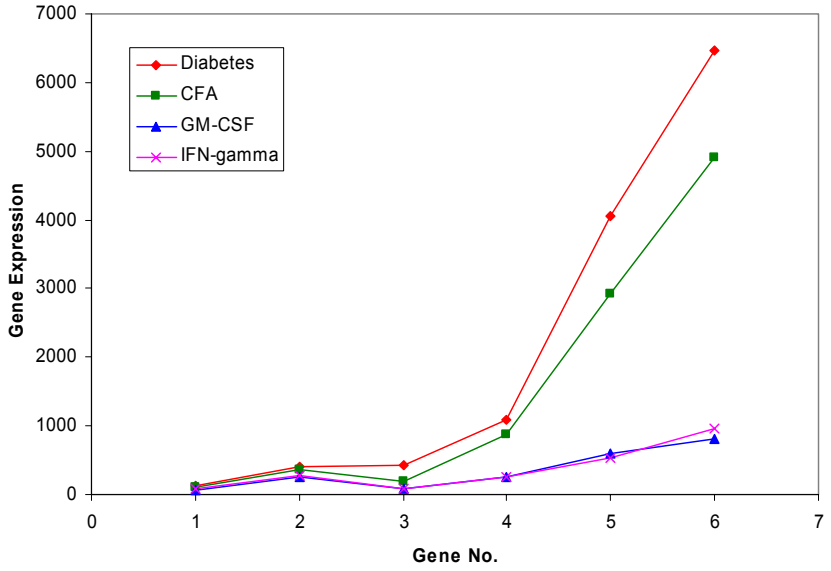
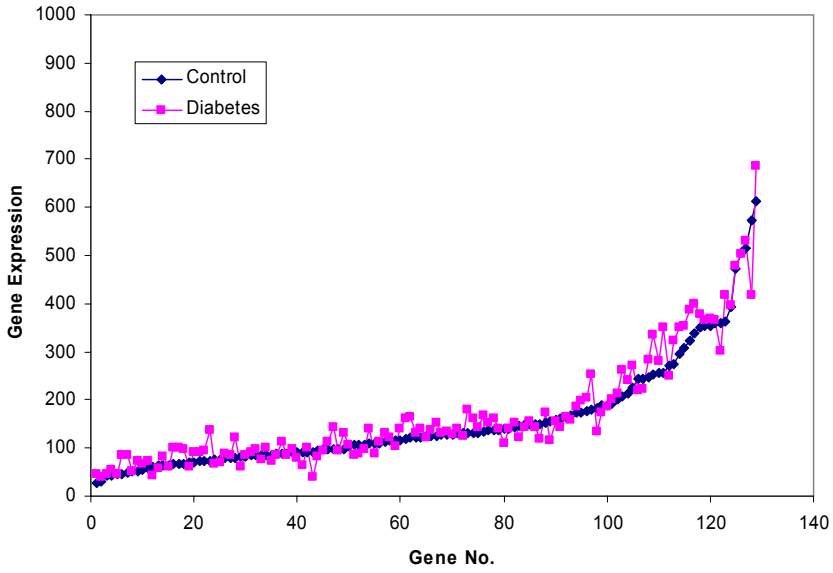


b

Fig. 5.1 Plots of gene expression levels of selected significant altered genes with connected line for better visualization of each groups, showing extreme changes in GM-CSF and IFN-γ groups, and moderate change in CFA group with the same pattern. a) up-regulated genes; b) down-regulated genes



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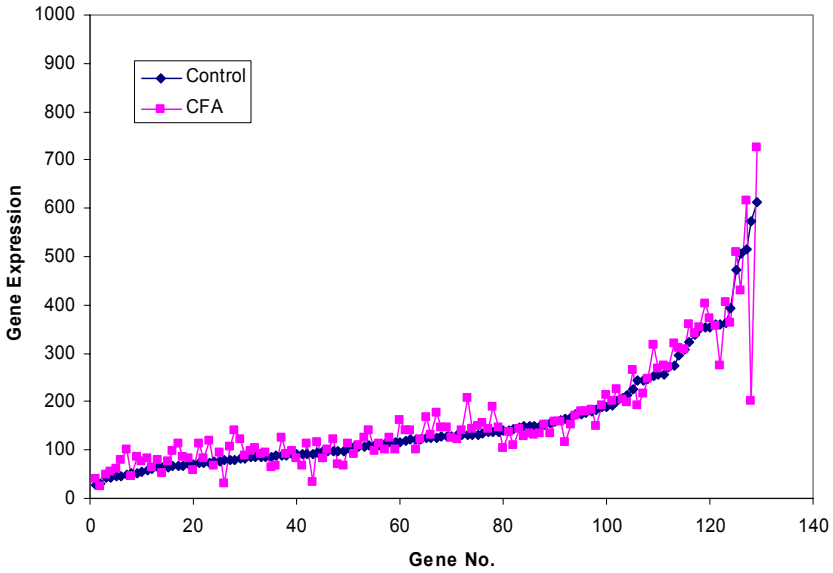
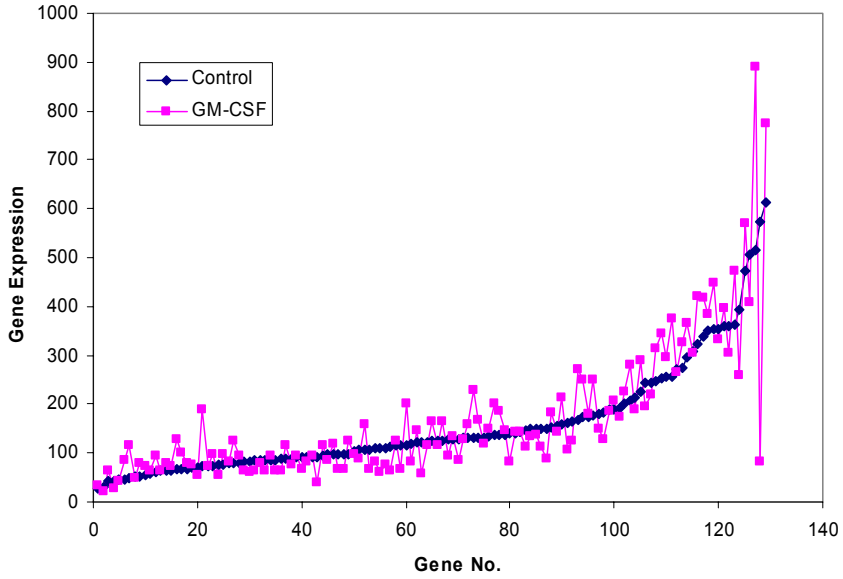


b

Fig. 5.2 Scatter plots showing comparison of splenic gene expression relative to control with connected line for better visualization. a) Diabetic group; b) CFA group; c) GM-CSF group; d) IFN-γ group. Diabetic and CFA groups show unaltered pattern, while IFN-γ and GM-CSF show altered patterns with genes both higher and lower than control.



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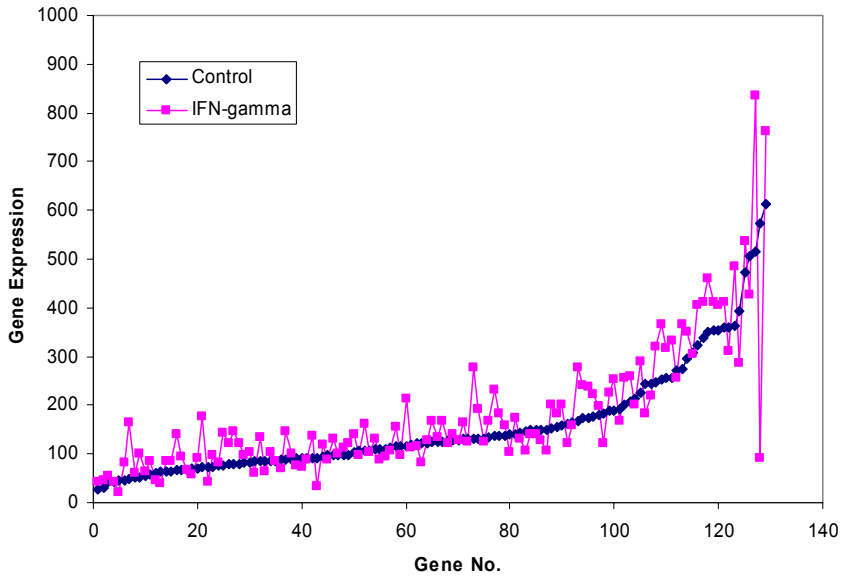


d

Fig. 5.2 Continued

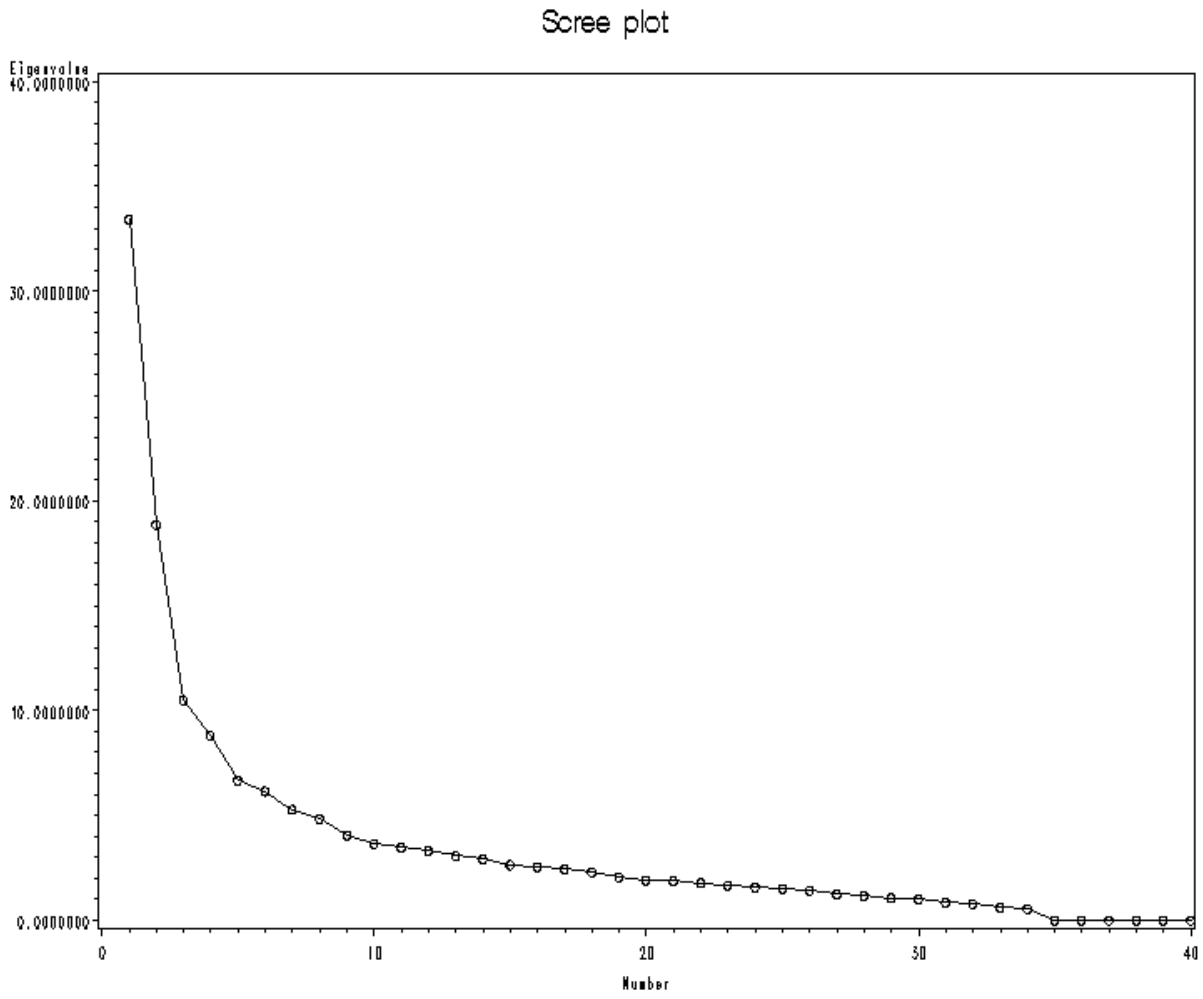
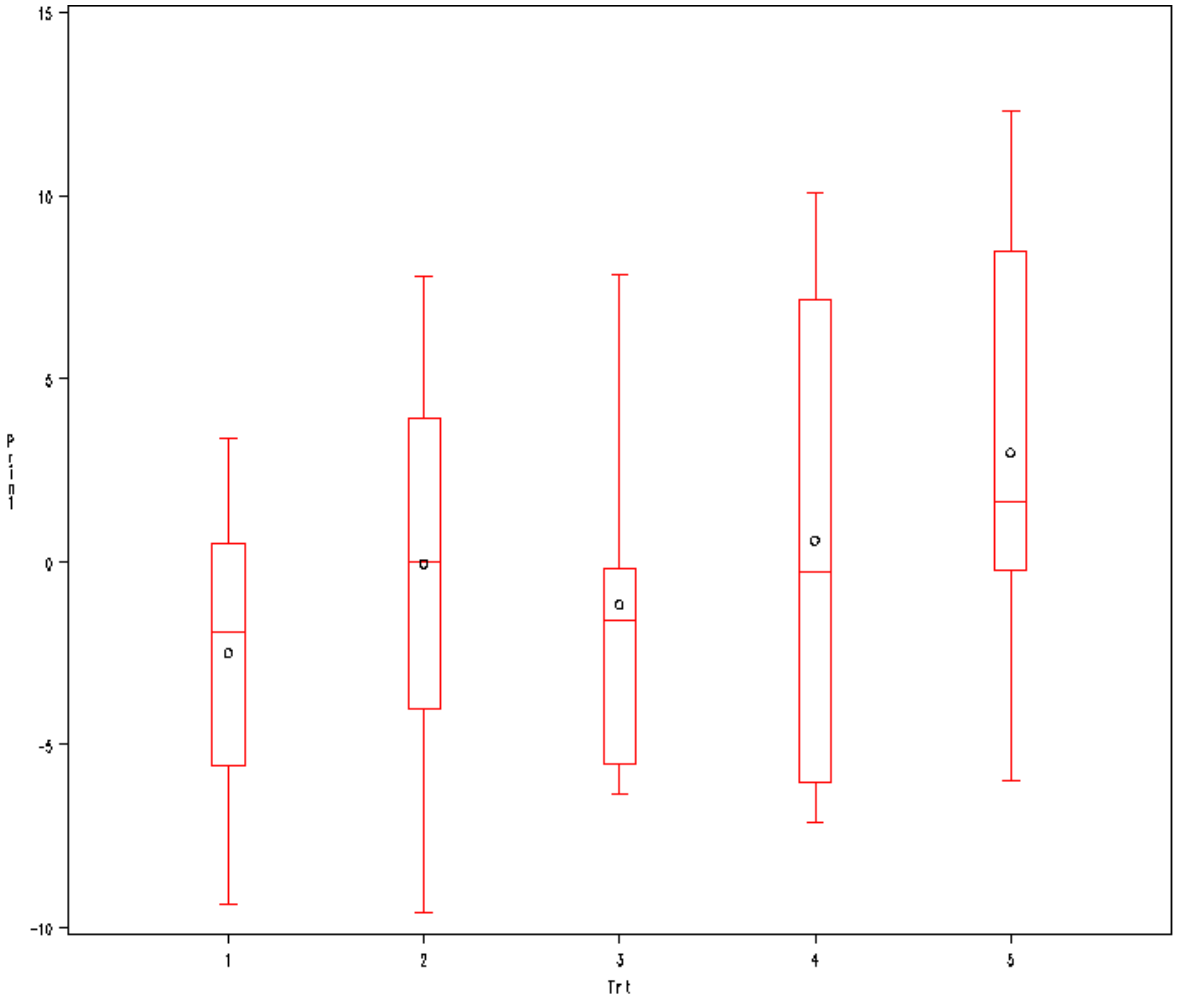
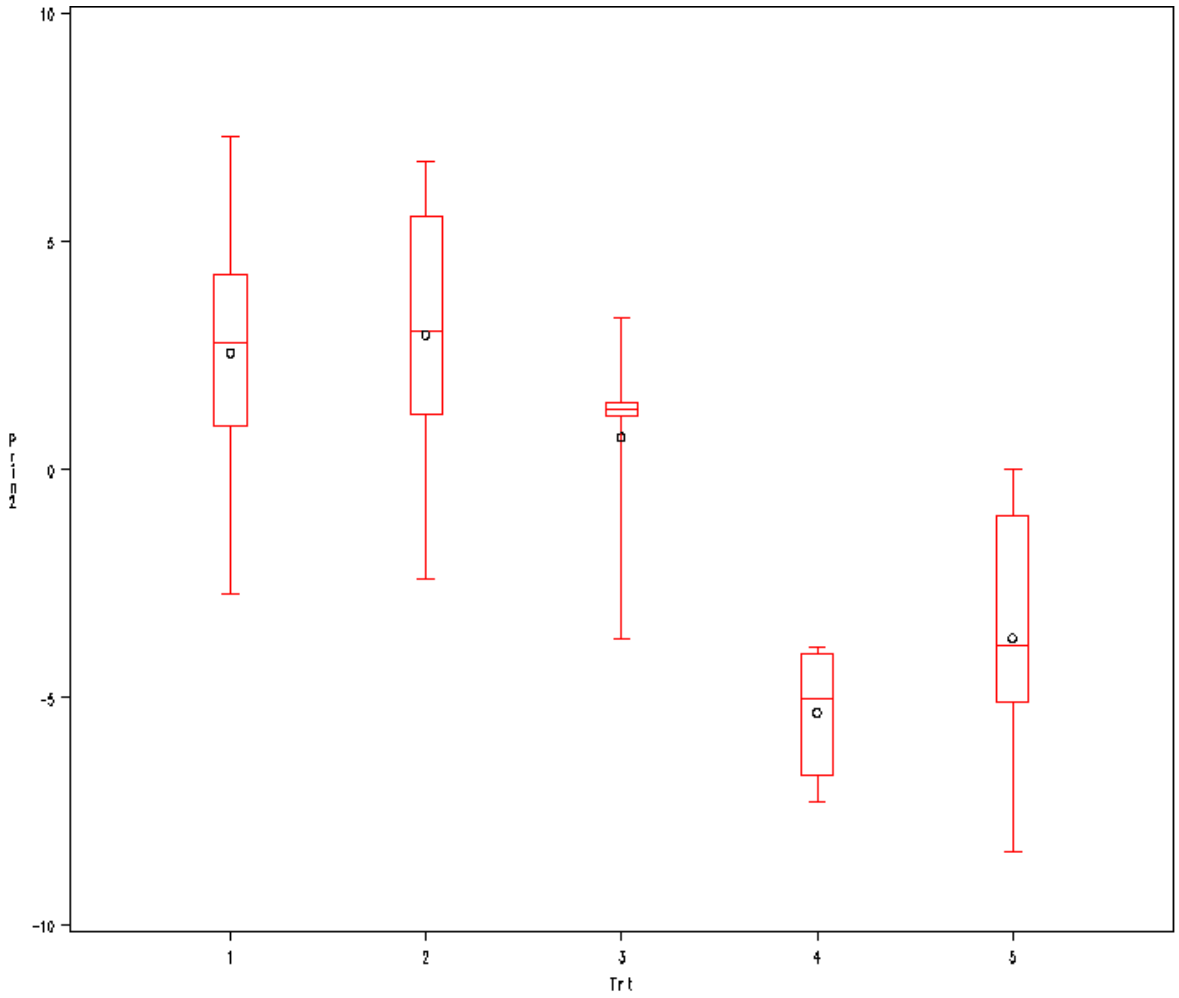


Fig. 5.3 Scree plot of eigenvalues of principal components (PCs) 1-40. PCA analysis creates the number of PCs, and each PC contains eigenvalue or the variance of PC, the proportion and cumulative proportion of the total variance explained. PCs that explained at least 5% of the variation in gene expression were subjected to further analysis.



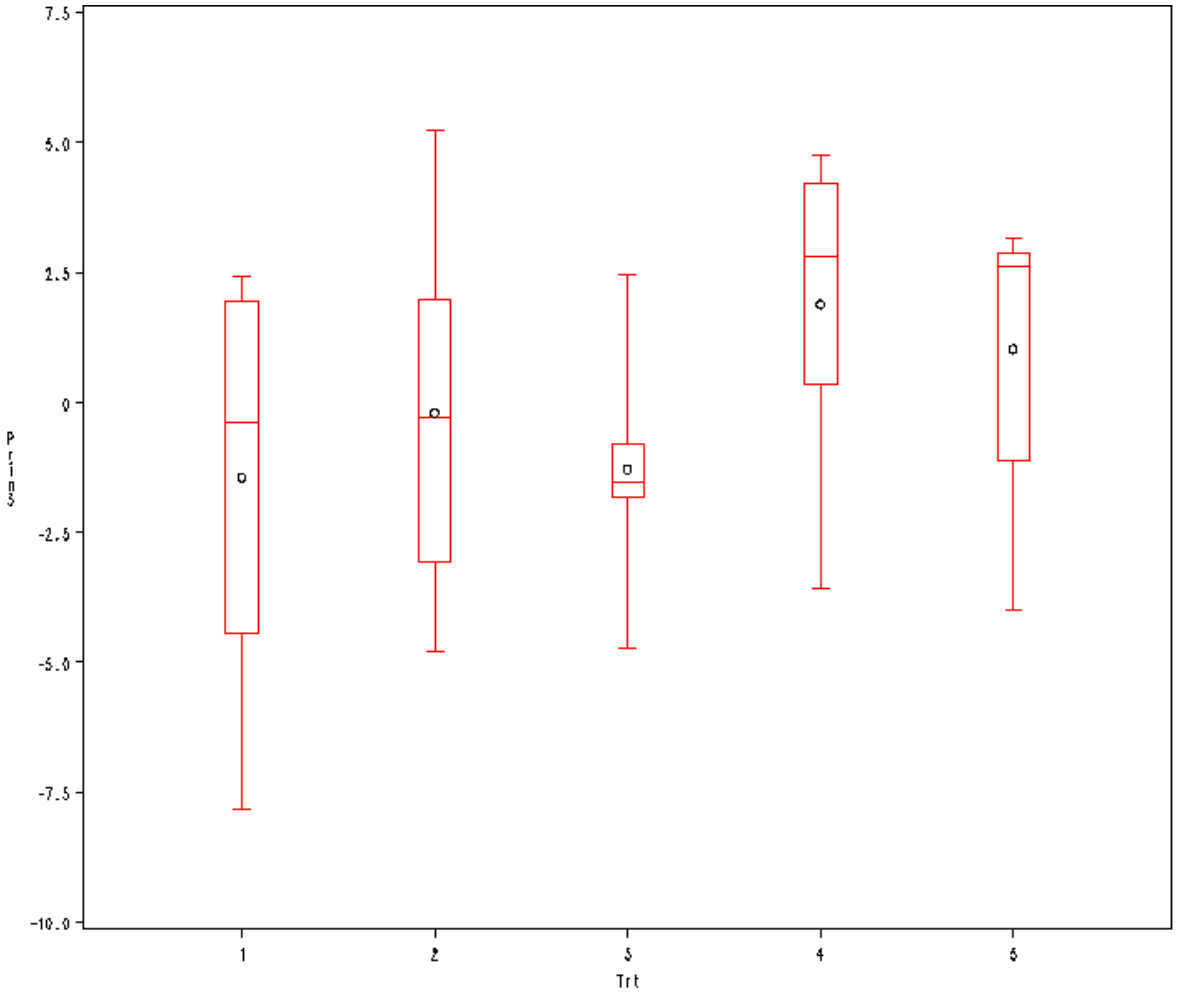
a

Fig. 5.4 Boxplots of PC 1, 2, 3, and 5 demonstrating the treatment effect of GM-CSF and IFN-γ in PC 2 and the difference between CFA and IFN-γ in PC 5. Fig. a (PC 1), b (PC 2), c (PC 3), d (PC 5). PC 1-5 were selected from Scree plot and analysed by ANOVA comparing values among control and treatment groups. PC 2 and 5 were further analysed to identify genes that contribute to the treatment effects.



b

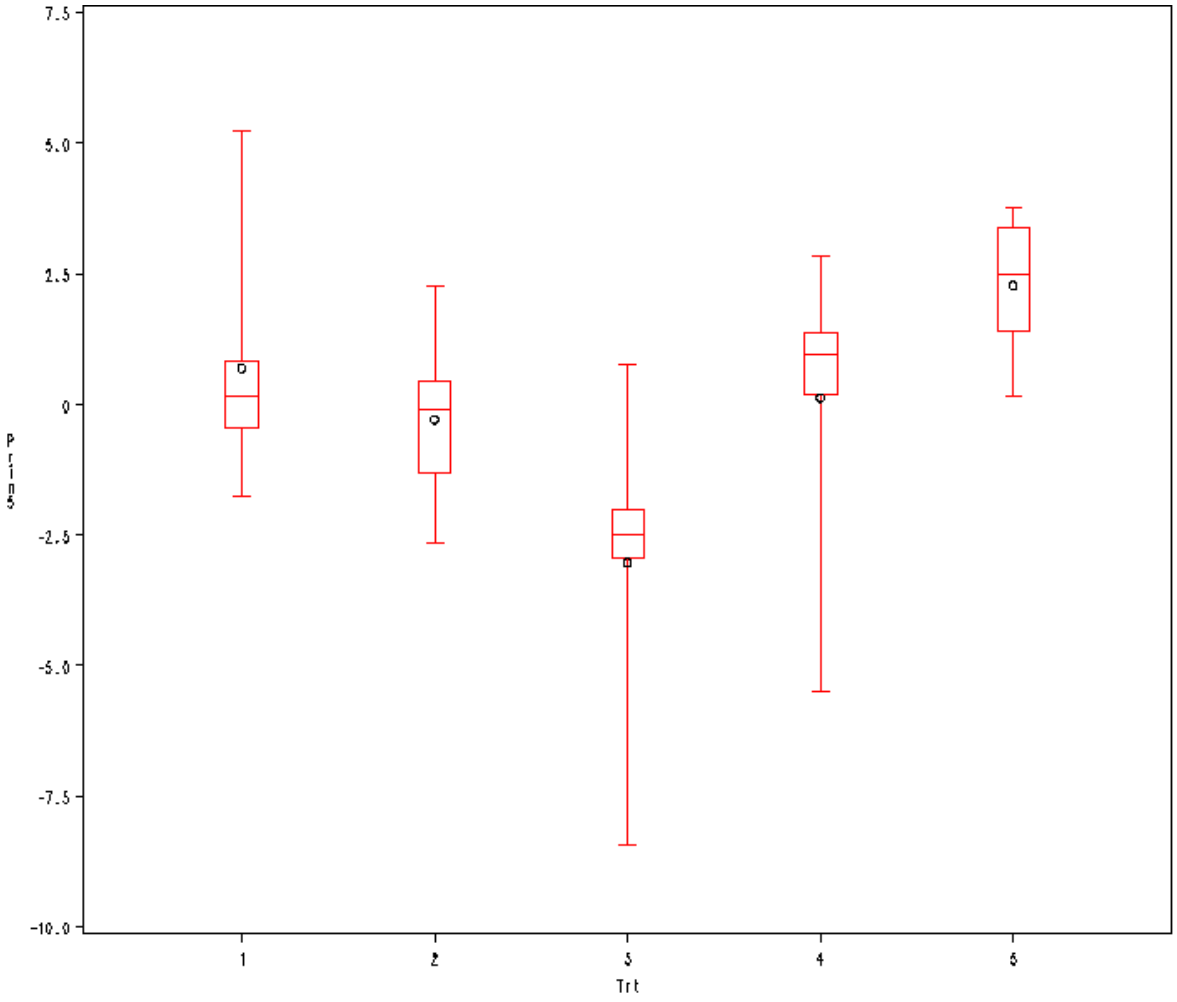
Fig. 5.4 Continued



control diabetes CFA GM-CSF IFN-γ

c

Fig. 5.4 Continued



control diabetes CFA GM-CSF IFN-γ

d

Fig. 5.4 Continued

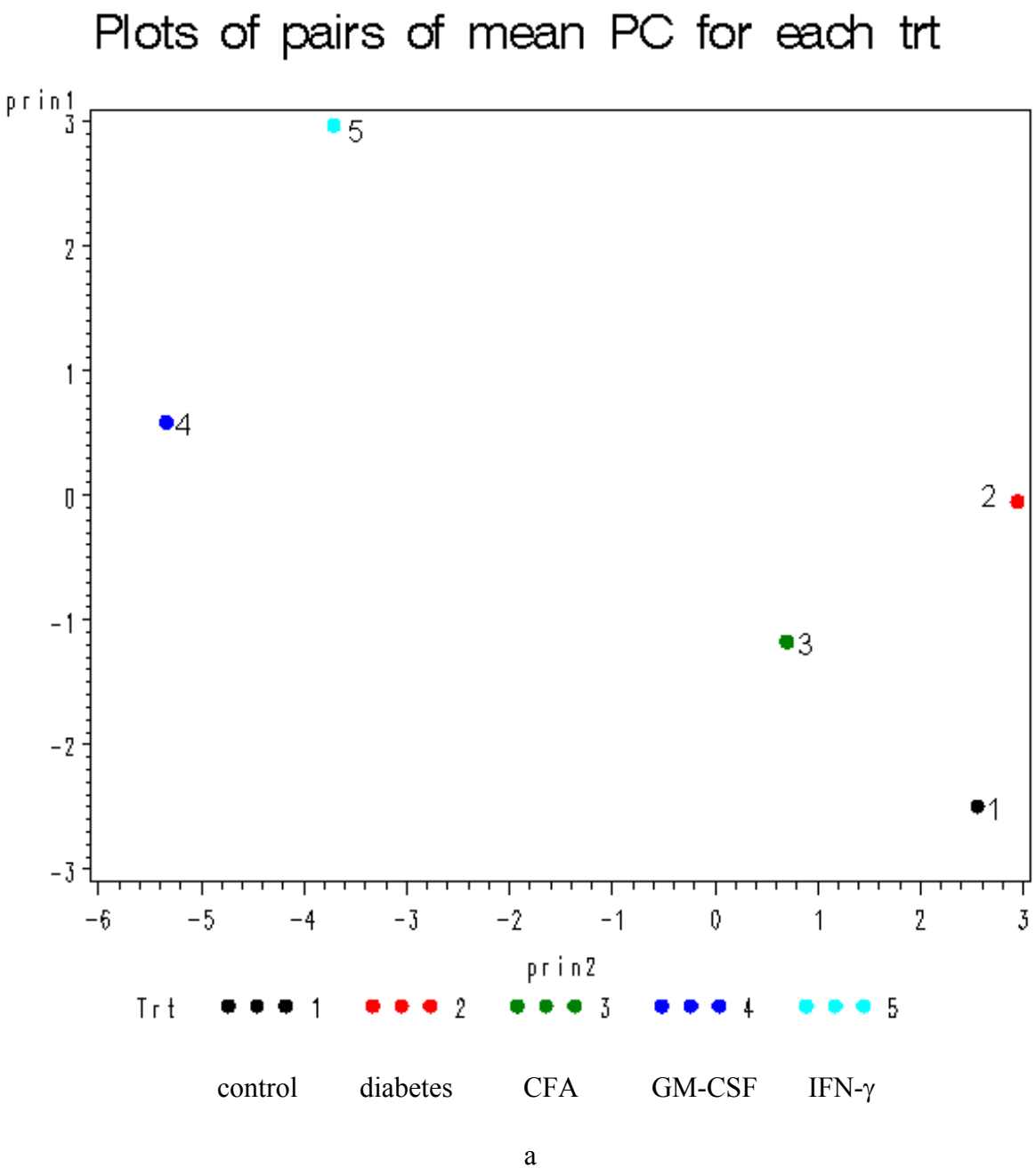
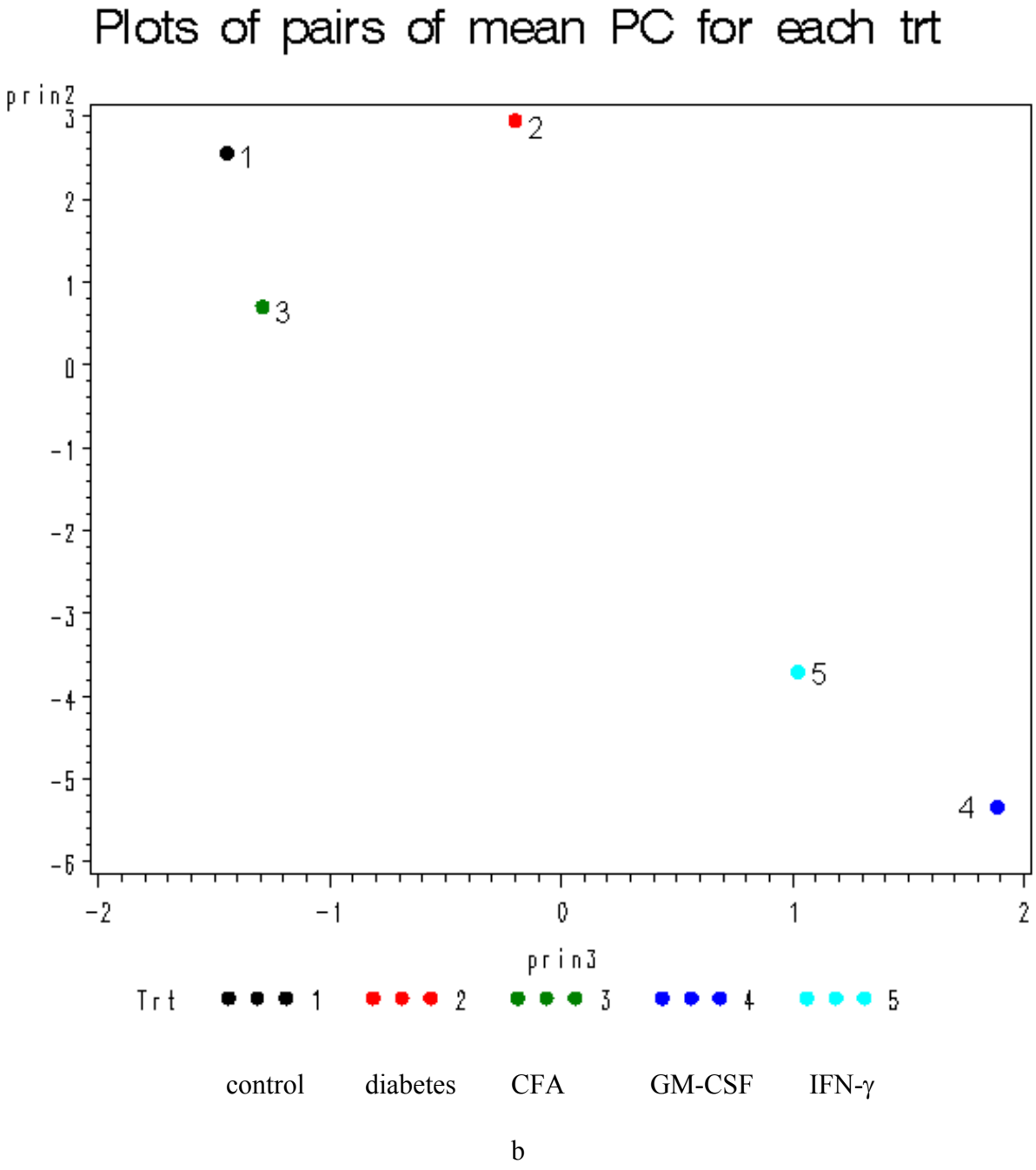


Fig. 5.5 Scatter plots of PCA analysis of splenic gene expression, showing mean of PCs that were significantly affected by experimental treatments. a) PC 1 / PC 2 coordinate space, b) PC 2 / PC 3 coordinate space. Control, diabetic, and CFA groups had relatively similar profiles, while GM-CSF and IFN-γ groups had different profiles from the others and relatively similar to each other.



control diabetes CFA GM-CSF IFN-γ

b

Fig 5.4 Continued

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

Diabetic embryopathy is a serious complication in pregnant women with type I diabetes. The primary causes are insulin deficiency and hyperglycemia, which then produce other intermediate harmful factors, i.e. ketone bodies, somatomedin inhibitors, and advance glycation products, and ultimately interfere with the cellular machinery. These so-called multi-factors affect all body systems; pathological complications of diabetes included retinopathy, nephropathy, neuropathy, delayed wound healing, and diabetic embryopathy. In the case of diabetic embryopathy several strategies, i.e. supplementation of myo-inositol, arachidonic acid, prostaglandin E_2, or antioxidant agents have been tried and were able to prevent fetal abnormalities in some degree. Recent evidence indicates that immune components could be disturbed by diabetes and might have a role in causing birth defects. This combined with the knowledge that immune stimulation reduces chemical-induced teratogenesis, led us to consider the possibility that immune stimulation may reduce diabetic embryopathy.

This study was designed to evaluate the hypotheses that 1) an activated immune system can some overcome adverse effects associated with diabetes and as such result in partial prevention of diabetic embryopathy and 2) a shared profile of placental or splenic gene expression changes may correlate to the reduced birth defect outcome induced by immune stimulation procedures. The experiments used a streptozocin-induced diabetic ICR mouse model to produce fetal abnormalities, and significant numbers of fetuses with defects were obtained. Three methods of immune stimulation, CFA, GM-CSF, and IFN-γ, proved effective for reducing the number of fetal defects, confirming that diabetes might interfere with immune functions. Inhibition of cytokine release from immune cells by diabetes could be a pathway that leads to inappropriate immune response during pregnancy. Effects of diabetes on lowering the level of either myo-inositol or prostaglandins may directly affect immune-related signal transduction processes. Further it has been shown that hyperglycemia blocks macrophage cytokine release without altering their production. The present gene expression studies were designed to evaluate such pathways as mediators of diabetes-induced birth defects in pregnant mice that received or did not receive immune stimulation. Gene expression results demonstrated that diabetes did not down-regulate expression of the selected genes in either placental or splenic cells relative to control. Profiles of changed

placental gene expression that were common to the three immune stimulation procedures, and thus might relate to reduced birth defects caused by each of these procedures, were similarly not identified. In contrast, similar patterns of gene expression change were observed in spleen after all immune stimulation treatments. The activity of both apoptotic and anti-apoptotic genes was up-regulated, as were genes that control cellular proliferation, in events that likely represent increased activity level of splenic leukocytes after the immune stimulations. Expression level for the GM-CSF gene was increased relative to control or diabetic mice by all immune stimulations. This cytokine has previously been associated with reduced defects in chemical-exposed mice, and, after intra-uterine injection with reduced defects in cyclphosphamide-treated mice. Together, these results suggest that GM-CSF should be further evaluated for a role in reduced birth defects associated with diabetic mellitus.

The first hypothesis was proven by significant protection on diabetic embryopathy by the three selected immune activating agents. All agents produced partial protection implying that immune disorders caused by diabetes may be only one of several factors that contribute to the occurrence of diabetic embryopathy, and immune stimulation may not be able to improve these factors. The second hypothesis was not proven, but was supported by similar splenic gene expression profiles of the three immune-stimulated groups. Such was not the case for placental gene expression profiles, where no common pattern of gene expression alteration was seen that might correlate to improve fetal morphologic outcome. Spleen is a major immune organ and has been used commonly for determination of immune status therefore it may be a first line of response to immune stimulations, and consequently affect other organ systems. The similar patterns of splenic gene expression may in part reflect an activating state of this organ and the immune system. While GM-CSF may be part of such activation, this cytokine may also promote proper immune response and cellular interaction of the reproductive system during pregnancy. CFA is a known potent immune stimulator but produced a more limited effect on splenic gene expression than did the other two agents. This phenomenon may caused by several factors. First, relative dosages of the three agents must be considered as there was no reference for potency and the endpoints of present interest. Second, differences in route of administration and absorption property may be important as CFA is oil-based and was injected intra-dermally. This is in contrast to GM-CSF and IFN-γ, which were water-based and were injected intra-peritoneally. Third, GM-CSF and IFN-γ are

purified cytokines that act on specific receptors on target cells, therefore they may be able to activate the immune system more efficiently than CFA, which acts through alerting macrophages to secrete IL-1, which then produces consequent effects on other immune cells.

Different placental gene expression profiles of the three immune-stimulated groups that can be interpreted as immune stimulation may not be related to anti-teratogenic effects directly or through placenta. However placenta has been known for its importance and not only provides oxygen and nutrients to developing fetuses but also a variety of hormones, cytokines and growth factors, therefore a role of placenta should not be ruled out. The number of selected genes used to construct microarrays in this study was quite small compared with commercial microarrays, so we may have missed important placental genes that could be key mediators. Another consideration is that our interpretations were based on the hypothesis that three different immune activators should exert their effects through a common mechanism, and such may not appropriate in the case of placenta. Different mechanisms at the level of placenta could possibly result in the same beneficial effects for embryonic development because action of closely-related cytokines or growth factors are normally redundant and overlapped in their action and can replace for each others. For example, lacking of GM-CSF can be compensated by the action of CSF-1 and G-CSF. Timing of specimen collection could also affect placental gene expression profiles because GD 17 is late in pregnancy and several days away from sensitive time of embryonic development to diabetic effect, therefore collection at GD 12 or GD 14, for instance, may have demonstrated a common placental effect that was not seen at GD 17.

Success of immune stimulations in this study suggests that the immune system was compromised by diabetes, and that such affects embryonic development. This study also demonstrated that a simple treatment with immune activators prevents a serious complication of diabetes. Combining regimens of immune stimulation with glucose control and other methods of nutrient supplementation may further lower the risk of birth defects in diabetic animals. These studies may hopefully be extended to one day also lead to reduced risk of birth defects in diabetic humans.

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Veterinary Immunology

PRESENTATIONS AT MEETINGS

Punareewattana, K., Holladay, S.D., Smith, B.J., and Blaylock, B.L. Topical permethrin exposure alters skin immune responses and produces systemic immune effects. Virginia-Maryland Regional College of Veterinary Medicine 11th Annual Research Symposium. May 1999.

Punareewattana, K., Holladay, S.D. Immuno-Stimulation reduced severity of diabetic embryopathy. Virginia-Maryland Regional College of Veterinary Medicine 14th Annual Research Symposium. June 2002.

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Punareewattana, K., Holladay, S.D., Smith, B.J., and Blaylock, B.L. 1999. Topical permethrin exposure alters skin immune responses and produces systemic immune effects. The Toxicologist (38th Annual Meeting) 48(S-1): 180.

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MANUSCRIPTS

Punareewattana, K., Smith, B.J., Blaylock, B.L., Robertson, J.L., Gogal, R.M., Prater, M.R., Longstreth, J., Snodgrass, H.L., and Holladay, S.D. 2000. Topical Permethrin Exposure Causes Thymic Atrophy and Persistent Inhibition of the Contact Hypersensitivity Response in C57Bl/6 Mice. International Journal of Toxicology. 19(6): 383-389.

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