THE ROLE OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR) ISOFORMS IN THE PATHOGENESIS OF TYPE 1 DIABETES IN MALE NON-OBESE DIABETIC (NOD) MICE

by

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Thesis submitted in fulfillment of the requirements for the degree of Master of Science

January 2008

Dedication

To all who believed that I will finish this thesis,
Even when I myself do not believe

Acknowledgements

I give thanks to God who has been my refuge and strength, an ever-present help throughout my studies and my life. The Lord is my strength and my shield; my heart trusts in him, and I am helped. My heart leaps for joy and I will give thanks to him in song. There is no way I could have survived without His grace. Thanks be to God.

I would also like to thank my dad, Mr. Goh Hin Leong for being there with me when I am down, bearing all my negative thoughts even at the expense of his own well being. With his patience, loving support and counsel, I am able to make it this far. No matter what the circumstances, I am proud to have a father like him. Way to go, dad!

I would also like to show my heartfelt appreciation and gratitude to my supervisor, Associate Professor Dr. Nik Soriani Yaacob and my co-supervisor, Professor Dr. Norazmi Mohd. Nor for their faithful guidance, support and patience, even at times of trial when I fail them miserably. I came to USM to study science from them. In the end, I learned more than just science; I learned invaluable lessons of life that I will not forget.

I would like to thank the administrative staff of PPSK and INFORMM, especially Mr. Jahangir, Mr. Lukmi, Mr. Jamaruddin and Mr. Zaki who had assisted me with the facilities that I needed in my studies. I want to thank my seniors, Mr. Ariffin, Dr. Zulkarnain, Ms. Halisa and Ms. Khoo Boon Yin for all the knowledge and support they provided me in this study. Not to forget also the staff of NMN and NSY research group, Ms. Rohayu and Mr. Norhisyam who faithfully assisted in all the administrative work.

Thank you also to my fellow lab peers, Rafeezul, Nurul Asma, Maryam, Teo Wan Huai, Rohimah and Venugopal. It is an honor to be able to work with them. Finally, but not least, to my juniors Fatmawatee and Norzilla, I am happy to know you all and I wish you all the best in your studies. Thank you also Wong Vic Cern, for being a friend and an unofficial counselor whom I can trust for support. To those whom I did not mention in this acknowledgement but who had assisted me in my studies in any way, my appreciation goes out to you. I thank God for all the help you have provided.

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LIST OF ABBREVIATIONS

Abbreviation

AF	Activating function
APC	Antigen presenting cells
AP-1	Activator protein-1
azPC	azeloayl phosphatidylcholine
BB Rat	Bio Breeding Rat
BCG	Bacillus Calmette – Guerin
BCP	Bromo-chloro propane
CBP/p300	Cyclic-AMP response element binding protein/p300
C/EBP	CCAAT/Enhancer binding protein
CFA	Complete Freund's adjuvant
cFLIP	Caspase-8 homologous FAS associated death-domain-like interleukin-1β converting enzyme-inhibitory protein
C_T	Threshold cycle
DC	Dendritic cells
DEPC	Diethyl-pyrocarbonate
dhT	5α-dihydrotestosterone
EDTA	Ethylene diamine tetraacetic acid
FITC	Fluorescein isothiocynate
FRK/RAK	Fyn related kinase/Gut tyrosine kinase (RAK)
GAD	Glutamic acid decarboxylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GED	Guanidinoethyl disulphide
HLA	Human leukocyte antigen
H&E	Hematoxylin and eosin
HPRT	Hypoxantin ribosyltransferase
HSA	Heat stable antigen
IBD	Inflammatory bowel disease

ICA	Islet cell antibodies
ICAM	Intracellular adhesion molecule
ICOS	Inducible co-stimulator
Idd	Insulin-dependent diabetes
IDDM	Insulin dependent diabetes mellitus
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAK/STAT	Janus kinases/Signal transducers and activators of transcription
JNK/ SAPK	c-Jun NH_2-terminal kinase/Stress-activated protein kinase
LB	Luria-Bertoni
LDL	Low density lipoprotein
LipCl_2MDP	Liposomal dichloromethylene diphosphate
LPS	Lipopolysacharide
LTB4	Leukotriene B4
MAPK	Mitogen activated protein kinase
MHC	Major histocompatability
NCo-A	Nuclear co-activator
NCo-R	Nuclear co-repressor
NKT cells	Natural killer T cells
NMA	L-N^G-monomethyl arginine
NO	Nitric oxide
NOD	Non-obese diabetic
NON	Non-obese non-diabetic
NOR	Non-obese diabetes resistant
PBS	Phosphate buffered saline
PE	Phycoerythrin
PKA	Protein kinase A
PMA	Phorbol 12-myristate 13-acetate
PM	Peritoneal macrophage
PPAR	Peroxisome proliferators-activated receptor

PPRE	PPAR response element
RANK	Receptor activator of NF-κB
RANTES	Regulated upon activation, normal T-cell expressed and presumably secreted
RT-PCR	Reverse transcription-polymerase chain reaction
RXR	Retinoid X receptor
SCID	Severe compromised immune deficient
SLE	Systemic lupus erythematosus
SNPS	Single nucleotide polymorphisms
SOCS	Suppressor of cytokine signaling
SR-A	Scavenger receptor-A
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TCR	T cell receptor
Th	T helper
TNF	Tumour necrosis factor
TRANCE	TNF-related activation induced cytokine
VCAM	Vascular cell adhesion molecule
WHO	World health organization
WHR	Waist to hip ratio
15d-PGJ2	15-deoxy-prostaglandin J2

PERANAN ISOFORM RESEPTOR PENGAKTIF-PROLIFERASI PEROKSISOM (PPAR) DALAM PATOGENESIS DIABETES JENIS 1 MENCIT NOD JANTAN

Abstrak

PPAR merupakan faktor transkripsi yang boleh meredakan tindakbalas imun apabila diaktifkan oleh ligan. Di dalam kajian ini, ekspresi isoform PPAR dan sitokin telah diukur dalam mencit model Diabetis jenis 1 yang dikenali sebagai mencit diabetik tanpa obes (NOD) dan spesies kawalannya iaitu mencit resistan diabetes tanpa obes (NOR). Sel makrofaj peritoneal (PM) serta sel T $CD4^+$ dan $CD8^+$ dari mencit jantan NOD dan NOR yang berusia 5, 10 dan 35 minggu (usia diabetik) telah diperolehi untuk cerapan PCR masa sebenar dan semi-kuantitatif. Kajian histologi juga dijalankan pada pankreas mencit-mencit tersebut. PCR masa sebenar tidak menunjukkan perbezaan yang signifikan dalam pengekspresan PPAR-α, γ1, γ2 antara mencit NOD dan NOR pada usia 5 minggu. Tetapi pada usia 10 minggu, pengekspresan PPAR-α bertambah manakala PPAR-γ1 mengurang secara signifikan pada sel PM. Pada usia diabetik, tidak ada pengekspresan signifikan yang dapat dicerap dalam semua jenis sel antara mencit NOR dan NOD diabetik kecuali PPAR-γ2, yang mana pengekspresannya lebih tinggi dalam sel T $CD8^+$ mencit NOR. Walaupun isoform PPAR mempunyai kesan anti-inflamasi, pengekspresannya di dalam kebanyakan sel mencit NOD tanpa diabetes adalah lebih rendah secara signifikan, terutamanya PPAR-γ1. Semi-kuantifikasi PCR menunjukkan profil sitokin pro-inflamasi dan Th1 pada semua sel mencit muda NOD berbanding NOR, dengan pengekspresan IL-2, TNF-α dan IFN-γ yang signifikan. Cerapan mencit usia diabetik adalah di luar jangkaan kerana pengekspresan sitokin pro-inflamasi dan Th1 adalah lebih tinggi secara signifikan di dalam semua sel mencit NOR berbanding NOD diabetik dan tanpa diabetes. Walau bagaimanapun, pengekspresan sitokin Th2 juga adalah lebih tinggi secara signifikan di dalam sel T $CD4^+$ mencit NOR. Mencit NOD tanpa diabetes mempunyai pengekspresan signifikan IL-2 yang lebih rendah pada sel PM serta IL-1β dan IL-12 pada sel T $CD4^+$ berbanding mencit NOR dan NOD diabetik. Kajian histologi menunjukkan pankreas yang normal dalam mencit NOR dan insulitis gred rendah pada mencit NOD muda dan tanpa diabetes. Pankreas mencit NOD diabetik mempunyai gred insulitis yang paling tinggi. Secara keseluruhannya, pengekspresan konstitutif isoform PPAR tidak menunjukkan kesan anti-inflamasi di dalam patogenesis Diabetes jenis 1 mencit NOD jantan. Pengekspresan sitokin pro-inflamasi dan Th1 adalah selaras dengan insulitis yang dicerap pada mencit NOD muda. Kekurangan pengekspresan beberapa sitokin pro-inflamasi dan Th1 dalam mencit NOD tanpa diabetes mungkin menyebabkan insulitis gred rendah dan ketiadaan penyakit. Bagi mencit NOR pula, pengekspresan sitokin pro-inflamasi dan Th1 yang rendah pada usia muda serta sitokin Th2 yang tinggi pada usia diabetik merupakan salah satu faktor yang boleh memberi kerentanan terhadap penyakit Diabetes jenis 1.

THE ROLE OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR) ISOFORMS IN THE PATHOGENESIS OF TYPE 1 DIABETES IN MALE NOD MICE

Abstract

Peroxisome Proliferator-Activated Receptor (PPAR) is a transcription factor that was observed to suppress the immune response when activated with ligands. In this study, the expression of PPAR isoforms (PPAR-α, γ1 and γ2) and cytokines were evaluated using an animal model of autoimmune diabetes called the non-obese diabetic (NOD) mouse with a control strain called the non-obese diabetes resistant (NOR) mouse. Peritoneal macrophages (PM), $CD4^+$ and $CD8^+$ T cells were harvested from 5, 10 and 35 week (diabetic age) male mice for real time and semi-quantitative PCR. Histological observations were also performed on the pancreas. Real time PCR quantification revealed no significant difference between the expression of PPAR-α, γ1 and γ2 in cells of 5-week-old mice, but in PM cells of 10-week-old mice, the expression of PPAR-α was significantly higher and PPAR-γ1 was significantly lower in NOD mice. At diabetic age, no significant difference of expression was detected between NOR and diabetic NOD mice in all cell types except PPAR-γ2, which was higher in CD8 T cells of NOR mice. Unexpected results were observed when non-diabetic NOD mice were compared to NOR and diabetic NOD mice. Although PPAR has an immune suppressive effect, most of the cell types from non-diabetic NOD mice had significantly lower PPAR isoform expression, especially PPAR-γ1. Semi-quantification of cytokines in all cell types indicate a pro-inflammatory and Th1 profile in young NOD mice with significant IL-2, TNF-α and IFN-γ expression. At diabetic age, another unexpected result was observed when the expression of pro-inflammatory and Th1 cytokines were significantly higher in all the cell types of NOR compared to diabetic and non-diabetic NOD mice. However, the expression of Th2 cytokines were also significantly higher in $CD4^+$ T cells of NOR mice. Non-diabetic NOD mice had a significantly lower expression of IL-2 in PM cells and IL-1β with IL-12 in $CD4^+$ T cells compared to NOR and diabetic NOD mice. Histological studies observed normal pancreas in NOR mice, low grade insulitis in young and non-diabetic NOD mice and high grade insulitis in diabetic NOD mice. Overall, the constitutive expression of PPAR isoforms did not reflect the expected role in suppressing the pathogenesis of Type 1 diabetes of male NOD mice. The pro-inflammatory and Th1 cytokine profile concurs with the development of insulitis in NOD mice at young age. At diabetic age, the lack of some pro-inflammatory and Th1 cytokine expression in non-diabetic NOD mice could be one of the factors for low grade insulitis and inhibition to the onset of diabetes. For NOR mice, lower expression of pro-inflammatory and Th1 cytokines at young age and high Th2 cytokines at diabetic age correlated with disease resistance as would be expected.

CHAPTER 1

INTRODUCTION

1.1 Autoimmune Diseases

Autoimmune diseases are caused by the pathogenic effect of autoantibodies or autoreactive T cells that provoke inflammation, functional alterations and anatomical lesions. The pathogenesis are caused by defects in T and B cell selection that differ in the autoantigen recognized and hence, the injury of the target organ (Gorodezky *et al.*, 2006). There are four criteria used to characterize an autoimmune disease (Bach, 1997, Janeway *et al.*, 2001):

a. The patients' antibodies or T cells can transfer the disease.
b. The disease course can be slowed or prevented by immunosuppressive therapy.
c. The disease is associated with manifestations of humoral or cell mediated autoimmunity directed against self tissues or organs.
d. Disease can be experimentally induced by sensitization against an autoantigen present in an organ or tissue, which presupposes the knowledge of the target autoantigen.

Diseases associated with autoimmune phenomena tend to distribute within a spectrum. At one pole are organ specific diseases such as Type 1 diabetes (IDDM), Hashimoto's thyroiditis and Addison's disease, where autoantibodies and chronic invasive inflammatory cells' destructive lesions are directed against a single organ in the body. On the other end of the spectrum are the non-organ specific autoimmunity typified by Systemic Lupus Erythematosus (SLE), where autoantibodies are directed to antigens throughout the body, resulting in immune complex mediated lesions that are widely disseminated (Kukreja & McLaren, 1999). Autoimmune diseases also frequently occur at a certain age, like Type 1 diabetes primarily occurring in childhood, myasthenia gravis and multiple sclerosis in midlife and rheumatoid arthritis in old age (Cooper & Stroehla, 2003).

The role of T cells in autoimmunity is important because T cells are involved in mediating the immune response to autoantigens presented by antigen presenting cells (APC). Several autoimmune diseases are genetically linked to the Major Histocompatibility (MHC) class I and II genes for humans and mice (H-2), whose expressed protein molecules in antigen presenting cells (APC) bind to self-antigens to be presented to helper ($CD4^+$) and cytotoxic ($CD8^+$) T cells to induce an immune response (Graves & Eisenbarth, 1999). There are multiple interacting factors that lead to the development of autoimmunity. The factors include abnormalities in lymphocytes and APC, genes that predispose to autoimmunity, infections, tissue injury, hormone and drug influence (Abbas *et al.*, 2000). Currently, an article by Prelog (2006) discussed about the effects of aging as a risk factor for autoimmunity. Immunosenescence (aging of the immune system) is characterized by changes in T cell subsets, cellular and molecular level alterations and thymus atrophy, resulting in the decline of B and T cell function. It has been hypothesized that involution of the thymus resulting in a decline of naïve T cells and the accumulation of memory T cells, activated by neoantigens, may contribute to the development of autoimmune diseases (Prelog, 2006).

1.2 Type 1 diabetes

1.2.1 Introduction

There are two major types of diabetes, Type 1 diabetes, formerly known as Insulin Dependent Diabetes Mellitus (IDDM) and Type 2 diabetes, also formerly known as Non-insulin Dependent Diabetes Mellitus (NIDDM). Type 2 diabetes occurs primarily due to insulin resistance and secretion disorder. As a result, higher levels of insulin are needed for cells to uptake and metabolize glucose (Ekoe & Zimmet, 2001b). Disorders in Type 2 diabetes could be caused by environmental factors like diet, sedentary lifestyle and genetic factors like gene mutations that impair insulin receptors. Type 2 diabetes is the most common class among patients. Usually, they are treated with drugs that sensitize cells to insulin action or inhibition of gluconeogenesis (Hundal *et al.*, 2000; Mayerson *et al.*, 2002) and control of glucose intake in diet.

Type 1 diabetes is an organ specific autoimmune disease affecting the pancreas. Over a period of time, cells of the immune system would infiltrate the pancreas, invade the

islet of Langerhans (insulitis) and selectively destroy the β-islet cells that produce insulin. The autoimmune process is silent over months to several years, until the number of β-islet cells are no longer sufficient to maintain normal glucose homeostasis (Ekoe & Zimmet, 2001b; Cnop *et al.*, 2005; Seissler & Scherbaum, 2006). The low level of insulin would lead to hyperglycemia and cause long-term complications and dysfunction of several organs and tissues. Insulin injections are needed for lifetime treatment.

Most of the studies on Type 1 diabetes were carried out on animal models like the Bio Breeding (BB) Rat, non-obese diabetic (NOD) mice and transgenic mice due to the small number of human patients available and access are only from blood samples (Bach, 1997).

1.2.2 The importance of T cells in the pathogenesis of Type 1 diabetes

The autoimmune process of Type 1 diabetes is dependent on $CD4^+$ and $CD8^+$ T cells. Type 1 diabetes is thought to be mediated and propagated by the effects of pro-inflammatory and Th1 cytokines secreted by $CD4^+$ T cells, which induces inflammation and recruits other cell types that are the final effectors of β-islet cell destruction (Rabinovitch & Suarez-Pinzon, 1998; Raz *et al.*, 2005). NOD mice were observed to have an age progressive accumulation of $CD4^+$ T cells expressing the Th1 cytokine profile in the infiltrated pancreas of Type 1 diabetes (Gregori *et al.*, 2003). The study by Gregori *et al.* (2003) also observed that the progression of diabetes in NOD mice depends on reduced activity of a suppressive $CD4^+$ T cell subset called regulatory T cells ($CD4^+$, $CD25^+$) and increased pathogenecity of effector T cells ($CD4^+$, $CD25^-$). Compared to young NOD mice (8 weeks), regulatory T cells isolated from old NOD mice (16 weeks) did not efficiently inhibit the development of induced Type 1 diabetes in Severe Combined Immune Deficient (SCID) NOD mice when injected together with splenocytes from pre-diabetic and diabetic NOD mice. In *in-vitro* studies, effector T cells from old NOD mice were also less susceptible to regulation by regulatory T cells when stimulated with alloantigens. Therefore, development of autoimmune diabetes depends on the dynamic interaction between effector and regulatory T cells (Gregori *et al.*, 2003). On the genetic side, an association has been observed between polymorphism of the CD4 gene promoter in T cells

with Type 1 diabetes. Mutation studies had identified 3 frequently occurring single nucleotide polymorphisms (SNPs) in Danish parents with Type 1 diabetes offspring. Further studies of the SNPs identified four frequent haplotypes with the A4TGC haplotype producing higher promoter activity in reporter assays. This haplotype in the CD4 promoter confers risk of Type 1 diabetes by increasing CD4 surface expression, leading to more efficient activation of autoreactive T cells and eventually β-islet cell destruction (Kristiansen *et al.*, 2004).

The role of $CD8^+$ T cells in the pathogenesis of Type 1 diabetes is quite controversial. Early studies of transplanting splenic T cells from diabetic to irradiated NOD mice show the synergistic requirements of $CD4^+$ and $CD8^+$ T cells to cause diabetes (Christianson *et al.*, 1993; Nagata *et al.*, 1994). But Wong *et al.* (1996) had isolated diabetogenic $CD8^+$ T cell clones that are capable of causing diabetes in irradiated NOD mice without the help of $CD4^+$ T cells. The ability of $CD8^+$ T cells to cause diabetes is donor age dependent. The isolated cells were believed to represent a population involved in the early phases of the disease (Wong & Janeway, 1999a). $CD8^+$ T cells were required for a pathogenic response to islet cells, but once $CD4^+$ T cell response develops, $CD8^+$ T cells are not necessarily needed to cause diabetes (Haskins & Wegmann, 1996). Graser *et al.* (2000) created transgenic NOD mice that express TCR genes from autoreactive $CD8^+$ T cells isolated from invaded pancreatic islets of normal NOD mice. The transgenic mice were observed to have a high rate of diabetes development. Even when induced with mutations that eliminated $CD4^+$ T cells, diabetes development still occurs at an accelerated rate. However, the frequency of $CD8^+$ T cell clones that can independently cause Type 1 diabetes is lower in normal NOD mice compared to the transgenic mice. Thus, most of the $CD8^+$ T cell clones that contribute to the development of Type 1 diabetes require the helper functions of $CD4^+$ T cells. But there is variability in the helper function of $CD4^+$ with $CD8^+$ T cells in contributing to the development of Type 1 diabetes (Graser *et al.*, 2000).

Contemporary studies mainly focus on the role and importance of regulatory T cells (Aoki *et al.*, 2005). Regulatory T cells are important controllers of autoimmunity by suppressing the expansion of autoimmune effecter T cells. The expression of a gene called Foxp3 is required for the generation and activity of those cells and mice with Foxp3 knockout genes have a deficit of regulatory T cells (Chen *et al.*, 2005). The importance of

regulatory T cells in suppressing Type 1 diabetes had been proven in many experiments (Green *et al.*, 2002; Tang *et al.*, 2004; Herman *et al.*, 2004; Tarbell *et al*., 2004; Jaeckel *et al.*, 2005; Chen *et al*., 2005). However, the mechanism of suppression is still subject to further studies. A microarray analysis by Chen *et al*. (2005) observed that regulatory T cells isolated from the insulitic lesion of diabetic NOD mice had a different gene expression profile compared to regulatory T cells isolated from the mesenteric and pancreatic lymph nodes. Among the genes that were differently expressed were IL-10, CD103, S100a67 and chemokine receptors like CCR5, CXCR3, CCR2 and CCR6. The anti-inflammatory gene expression profile seems to be amplified in regulatory T cells isolated from the insulitic lesion. The study by Herman *et al*. (2004) observed that regulatory T cells also had a significantly higher expression of IL-10 in addition to inducible co-stimulator (ICOS) gene. Blockade of ICOS action by antibody treatment leads to rapid diabetes at the onset of insulitis. In the cell signaling aspect, the generation of regulatory T cells was dependent on the signaling between TNF-related activation induced cytokine (TRANCE) protein in T cells and receptor activator of NF-κB (RANK) in APC. Blockade of TRANCE-RANK signaling causes a decrease in the number of regulatory T cells in the pancreatic tissue, resulting in the generation of autoreactive $CD8^+$ T cells in young mice and progression to diabetes (Green *et al.*, 2002).

1.2.3 The importance of other cell types in the pathogenesis of Type 1 diabetes

The pathogenesis of Type 1 diabetes does not solely rely on T cells, as there were also other cells present during insulitis. The other cells that play a role in the pathogenesis of Type 1 diabetes include APC like macrophages, natural killer T (NKT) cells, dendritic cells (DC) and B-lymphocytes (Kukreja & McLaren, 1999). Macrophages had been identified to produce inflammatory cytokines and free radicals that are toxic to β-islet cells. The role of macrophages and its chemical secretions had been determined to be important in the promotion of the Th1 immune response and the differentiation of cytotoxic β-islet T cells. In NOD mice treated with liposomal dichloromethylene diphosphate ($LipCl_2MDP$) (selectively toxic to macrophages), there was a decrease in the production of macrophage derived IL-12 and a shift of the immune balance to the Th2 phenotype, which prevented the

destruction of β-islet cells (Jun *et al.*, 1999). However, the T cells in $LipCl_2MDP$ treated NOD mice did not lose their ability to develop into β- islet cytotoxic cells. Established T cells were able to recover their function as soon as being transplanted into new SCID NOD mice with intact macrophages (Jun *et al.*, 1999).

The presence of NKT cells have been observed to play an important role in the prevention of Type 1 diabetes and these cells are deficient in NOD mice (Godfrey *et al.*, 1997). NKT cells are lymphocytes that express both the surface markers of T (such as α/β-TCR) and NK (such as NK1.1, CD16 and Ly49A) cells. The research by Hammond *et al.* (1998) isolated a subset of cells from the thymus of congenic NOD mice ($\alpha/\beta^{-}TCR^{+}CD4^{-}CD8^{-}$) that is enriched with NKT cells. When injected into young female NOD mice, substantial resistance to development of Type 1 diabetes was observed compared to mice injected with whole thymocytes and PBS buffer. The cell subset was also injected together with splenocytes from diabetic NOD mice to determine its effects on the induction of Type 1 diabetes in irradiated adult NOD mice. The results show significant resistance towards Type 1 diabetes development compared to irradiated NOD mice receiving splenocytes alone, even when the number of cells in the subset was reduced. When the mice were treated with antibodies against IL-4 and IL-10, there was an increase of Type 1 diabetes development. By this, the researchers propose that the deficiency of NKT cells in NOD mice contributes to the pathogenesis of Type 1 diabetes by permitting a disproportionate Th1 response to emerge (Hammond *et al.*, 1998). A similar study by another research group also observed the prevention of Type 1 diabetes in SCID NOD mice injected with CD1d-restricted non-classical NKT cells and splenocytes from diabetic NOD mice (Duarte *et al.*, 2004). An elucidating study has observed that activated NKT cells provided diabetes protection by promoting the migration and maturation of DC in pancreatic lymph nodes, where they suppress autoreactive T cells (Chen *et al.*, 2005).

DC had been identified to play an important role in the expansion of regulatory T cells that prevents the onset of diabetes by suppressing autoreactive T cells. The study by Tarbell *et al.* (2004) used DC and a β-islet cell mimetope (BDC peptide) as the antigen to activate and proliferate regulatory T cells *in-vitro*. The generated cells were observed to suppress the proliferation of non-regulatory T cells *in-vitro* and the pathogenesis of Type 1

diabetes *in-vivo*. In diabetes induction experiment, when the number of DC proliferated-regulatory T cells transplanted into SCID NOD mice were decreased until a certain threshold and autoreactive splenocytes from normal diabetic NOD mice were increased, there was still significant inhibition of Type 1 diabetes development. Therefore, autoantigen-specific, DC expanded regulatory T cells functions efficiently *in-vivo* to suppress Type 1 diabetes mediated by autoreactive T cells (Tarbell *et al.*, 2004).

The role of B cells in Type 1 diabetes however is poorly defined. The study by Carillo *et al.* (2005) had detected autoantibodies secreted specifically against the pancreatic nervous system in most of the hybridoma cell lines generated from islet infiltrating B cells of diabetic NOD mice. On the other hand, Wong *et al.* (2004) observed that the antigen presenting properties of B cells seem to play a more important role in the pathogenesis of Type 1 diabetes compared to autoantibody secretion. They generated a transgenic mouse that produces B cells with antigen presenting capability but could not secrete antibodies. They observed that the transgenic mice had a significantly higher incidence of developing Type 1 diabetes compared to the negative control strain that secretes antibodies (Wong et al., 2004). Investigative studies had proposed that defects in the MHC gene expression of B cells could contribute to the pathogenesis of Type 1 diabetes (Noorchasm *et al.*, 1999; Hussain & Delovitch, 2005).

1.3 Epidemiology of Type 1 Diabetes

1.3.1 Diagnosis and etiology

The most important factor and hallmark for diagnosing diabetes mellitus is hyperglycemia (Graves & Eisenbarth, 1999). Studies in populations with high prevalence of diabetes show that normal blood glucose values are bimodally distributed with a cut off point of 11.1 mmol/l (Ekoe & Zimmet, 2001a). This observation also agrees with the results of a study on Malaysians, which has a low prevalence of the disease (Lim *et al.*, 2002). The World Health Organization (WHO) recommends a fasting plasma glucose value of more than 7.0 mmol/l as an indicator for diabetes mellitus (Ekoe & Zimmet (b), 2001). The net effects of genetics, environmental factors and immune dysregulation influence the development of this disease (Skyler *et al.*, 2001, Gorodezky *et al.*, 2006; Seissler & Scherbaum, 2006).

On the genetic side, the HLA class II region has been found to play an important role in the susceptibility of this disease. It contributes 50% of the inherited risk for Type 1 diabetes (Gorodezky *et al*., 2006). The HLA class II allele DQ2, DQ8, DR3 and DR4 have been known to confer susceptibility to diabetes. In contrast, HLA DQ6 confers resistance to the disease (Graves & Eisenbarth, 1999; Skyler *et al.*, 2001). The role of genetics in Type 1 diabetes is further demonstrated in inheritance of the disease and a high concordance rate for Type 1 diabetes in monozygotic twins (35-50%) than in dizygotic twins (5-10%). However, there are other indicators that suggest an environmental factor like the other 65-50% discordance rate of diabetes in monozygotic twins. There is also a seasonal variation in the disease onset where peak incidences are observed at certain times of the year (Graves & Eisenbarth, 1999; Green & Kyvik, 2001; Skyler *et al.*, 2001). Other environmental factors have been suggested and reviewed that includes viral infection (Rubella and Coxsackie B), neonatal nutrition (cow's milk), chemical toxins (nitrosamine), stress and sex hormones (Bach, 1997; Graves & Eisenbarth, 1999; Skyler *et al.*, 2001).

1.3.2 Disease distribution

Type 1 diabetes is a disease with variable geographic and ethnic distribution. The highest worldwide prevalence occurs in Finland and Sardinia and the lowest prevalence appears in orientals (Gorodezky *et al.*, 2006). It is prevalent among the Caucasian race, particularly in Northern Europe, compared to populations in Asia and South America. However, significant increases were also detected among Asians in China and Japan, Mestizos in Peru and Polynesians in Hawaii. The number of patients with Type 1 diabetes is increasing worldwide (about 3% per year) in both low and high incidence populations (Onkamo *et al.*, 1999).

In Singapore, a study conducted by Lee *et al.* (1998) also showed that although the incidence of Type 1 diabetes was rare, the number of patients were increasing, being 1.4 per 100000 children in 1992, 2.4 per 100000 in 1993 and 3.8 per 100000 in 1994. The age-standardized incidence rate of Type 1 diabetes was 2.46 per 100000 in 0 – 12 year-old children. There was a preponderance of this disease to females (male to female ratio is 1:1.85) and this was quite similar when compared to other Asian populations like Thailand and Hong Kong. This was in contrast to Western populations where the incidence of

disease was equal between both sexes. The age group that had the highest incidence was 10-12 years old and the overall average age for the onset of Type 1 diabetes in boys and girls were 5.6 and 7 years respectively.

It was also observed that the frequency of Type 1 diabetes varies according to the ethnic group in a same geographic area. In the USA, Hispanics and African Americans had a lower prevalence than Caucasians. In China, a low risk country, the Zhuang ethnic had a lower prevalence than the Mongols (Gorodezky *et al.*, 2006). In South East Asia, data from Singapore shows that the Indian population had a higher risk with an incidence of 5.78 per 100,000 children, followed by Chinese (2.25/100,000) and Malays (1.23/100,000) (Lee *et al.*, 1998). However, the data had to be interpreted carefully due to the small number of patients. The incidence of Type 1 diabetes among the Chinese in Singapore, who were mainly of Southern Chinese descend, was similar to that of Hong Kong and Japan. When compared among other Asian countries, the overall incidence in Singapore is slightly higher among countries with oriental races but still far lesser than Caucasian countries.

Table 1.1 Compiled data of Type 1 diabetes incidence in several countries with different populations (Lee *et al.*, 1998; Onkamo *et al.*, 1999)

Country	Incidence per 10^5	Age group
Hong Kong	1.7	< 15 years
Japan	1.65-2.0	< 15 years
Korea	0.6	< 15 years
Shanghai	0.72	< 15 years
Australia	11-22	< 15 years
Finland	30.3	< 15 years
France	8.0	< 15 years
Sweden	24.9	< 15 years
Singapore	2.46	< 12 years
Singapore Chinese	2.25	< 12 years
Singapore Malay	1.23	< 12 years
Singapore Indian	5.78	< 12 years

The data on Type 1 diabetes in Malaysia is quite limited. Malaysia is a low disease prevalence country with an incidence of 0.3 per 100000 persons aged less than 16 years (Tan *et al.*, 2005). In a study on glycemic control of diabetic patients, Ismail *et al.* (2000) compiled some data on the number of Type 1 and 2 diabetic patients from hospitals around

peninsular Malaysia. Out of 926 patients, 329 had Type 1 diabetes. Ethnic distribution of patients varies between hospitals. When compiled according to ethnicity, the patients had a mean age of onset at 17.5 – 18.9 years. In a study of blood glucose levels conducted by Lim *et al.* (2002), a total of 19218 subjects were recruited all over East and West Malaysia. In the sample, it was observed that the prevalence of diabetes (Type 1 and 2) is equal among males and females (7.0 and 7.1 % respectively). Indians had a higher percentage of diabetes prevalence compared to Malays, Chinese and other indigenous ethnics. Unfortunately, the study did not specifically focus on Type 1 diabetes and most of the diabetic subjects were of the Type 2 group.

1.3.3 Complications and mortality

Type 1 and 2 diabetes may present with the characteristic symptoms of thirst, polyuria, polydypsia, blur vision, weight loss and infections. In the most severe form, ketoacidosis may develop, leading to coma and death. Long-term complications include nephropathy (causing renal failure), retinopathy (with potential blindness) and neuropathy with risk of foot ulcers, amputation, charcot joints and autonomic dysfunction (Ekoe & Zimmet, 2001a). Diabetic individuals are also at higher risk of cardiovascular, peripheral vascular and cerebrovascular disease. In Singapore, the most common cause of death from diabetic complications is ischaemic heart disease (mainly Malays and Indians), cerebrovascular disease, chronic renal failure (mainly Chinese), infections (mainly respiratory tract infections) and diabetic ketoacidosis (mainly Type 1 Diabetic patients) (Cutter, 1998). Type 1 and 2 diabetes was the sixth most important cause of death and third most common reason for attendence in government outpatient clinics (Lee, 2000).

Good glycemic (blood glucose level) control can prevent the development of diabetic complications. In Malaysia, a study was done by Ismail *et al.* (2000) to determine the factors that affect glycemic control in Malaysia. Generally, overall glycemic control was poor among Malaysian patients. The authors identified 3 factors that affect glycemic control in Type 1 and 2 diabetes. They were availability of nurse educators, ethnicity and the waist to hip ratio (WHR) of patients. In the study, hospitals that lack educator nurses had the highest number of patients with poor glycemic control. The study also observed that the Chinese had better glycemic control compared to Indians and Malays, partially

because of protective genes and lifestyle. Intra-abdominal fat is associated with insulin resistance and therefore, patients with high WHR usually have poor glycemic control. In terms of socioeconomic status, household income was an important determinant of glycemic control in Type 1 diabetes due to the cost of disease treatment. Educational background does not influence the glycemic control of Malaysian diabetics. Data from a later study by Tan *et al.* (2005) on the association between ethinicity, depression and quality of life with glycemic control of Type 1 diabetes patients also concurs with the results of the study by Ismail *et al*. (2000). In addition, the study also observed that good diabetes control is associated with good functional families, which brings a better quality of life.

1.4 Cytokines

1.4.1 The role of cytokines

The chemical balance in Th1 and Th2 immune response is important in the development of Type 1 diabetes. While the Th1 immune response promotes cell-mediated immunity that may damage tissue, the Th2 immune response provides help for B-lymphocytes in the production of antibodies, particularly IgG1 and IgE in the mouse (Wong & Janeway, 1999a). Examples of pro-inflammatory and Th1 cytokines are interleukins (IL-1, IL-2 & IL-12), Tumour Necrosis Factors (TNF-α & TNF-β) and Interferon's (IFN-α & IFN-γ) (Kukreja & MacLaren, 1999). In Type 1 diabetes, most of the pancreas infiltrating T cells secretes cytokines of the Th1 group (Trembleau *et al.*, 2003). The study by Lejon & Fathman (1999) isolated an antigen reactive $CD4^+$ T cell that had a high expression of the CD4 molecule from invaded β-islets of diabetic NOD mice. The cells were observed to be very potent in transferring diabetes and had a Th1 cytokine profile with reduction in IL-4 expression. Since the inflammatory process of Type 1 diabetes was mediated by the effects of pro-inflammatory and Th1 cytokines, it was hypothesized that skewing the cytokine cascade from a Th1 to a Th2 profile could prevent the disease (Raz *et al.*, 2005). Experimental manipulations like the administration of Complete Freund's Adjuvant (CFA) and Bacillus Calmette – Guerin (BCG) in NOD mice can divert the destructive pathway of β-islet cells to a non-destructive pathway due to the

increase of Th2 cytokines like IL-4 and IL-10 (Calcinaro *et al.*, 1997; Tominaga *et al.*, 1998; Serreze *et al*., 2001; Lee *et al.*, 2003).

Chemicals secreted by immune cells that promote the Th1 phenotype immune response and inflammation were well known to damage β-islet cells that produce insulin and cause Type 1 diabetes (Pilstrom *et al.*, 1997; Rabinovitch & Suarez-Pinzon, 1998; Yadav & Sarvetnick, 2003; Cnop *et al.*, 2005). It also prevents the replication and regeneration of new β-islets and this reduces the number of insulin producing cells in the pancreas (Meier *et al.*, 2006). In a study of immune cell infiltration, cytokine expression and β-islet apoptosis in the diabetic rat model (Lew.1AR1/Ztm-iddm), it was observed that the expression of IL-1β and TNF-α by immune cells increased progressively with islet infiltration, leading to the destruction of β-islets (Jorns *et al.*, 2005). In *in-vitro* studies, a combination of IL-1β, TNF-α and IFN-γ shows enhanced damage to cultures of β-islet cells of diabetes prone and resistant BB Rats (Wachlin *et al.*, 2003). In a dose dependent manner, it also decreases the population and replication rate whilst increasing the apoptotic frequency of cultured rat insulinoma and human β-islet cells (Meir *et al.*, 2006). IFN-γ is known to increase the expression of MHC I and II genes, which allows better recognition of the peptide antigens presented by these molecules (Wong & Janeway, 1999a). The up-regulation of MHC I genes might render β-islet cells susceptible to $CD8^+$ T cell mediated destruction.

IL-12 is mainly produced by activated APC like macrophages and plays a pivotal role in the differentiation and expansion of the Th1 arm of the immune response, which in turn, is required for eliciting organ specific autoimmunity. Besides having an effect on the development of $CD4^+$ Th1 cells, IL-12 is also required for the generation of $CD8^+$ T cells from naïve T cells (Yadav & Sarvetnick, 2003). In the study by Trembleau *et al.* (2003), IL-12 administration induces the secretion of IFN-γ and accelerates the development of Type 1 diabetes in NOD mice. It also accelerates the development of Type 1 diabetes in NOD mice with knockout IFN-γ genes, dispensing the role of IFN-γ. In that experiment, the dual role of IFN-γ (pathogenic and protective) was observed. The pathogenic role of IFN-γ was due to the increased production of nitric oxide (NO) by an enzyme called inducible nitric oxide synthase (iNOS). NO is a toxic mediator that destroys β-islet cells

and the production of NO is dependent on IFN-γ secretion. On the other hand, the protective role is mainly caused by the increased apoptotic frequency of $CD4^+$ T cells due to high IFN-γ levels induced by IL-12, which inhibits the development of Type 1 diabetes (Trembleau *et al.*, 2003).

Besides IL-12, macrophages also produce IL-15 and IL-18, which are mediators of innate immunity. Both IL-15 and IL-18 play a pivotal role in the pathogenesis of chronic inflammatory autoimmune diseases such as collagen-induced arthritis. In experiments where diabetes was induced using streptozotocin, mice treated with soluble murine IL-15 receptor α-chain had a significantly reduced glycemic level compared to controls. It was also observed that IL-18 knockout mice were significantly more resistant to diabetes induced by streptozotocin and do not develop typical islet infiltration (Lukic *et al.*, 2003). IL-15 acts as a growth factor and an activator of $CD8^+$ memory T cells whereas IL-18 acts synergistically with IL-12 to increase the production of IFN-γ by Th1 cells (Lukic *et al.*, 2003). Figure 1.1 shows a proposed scheme on the effects and mechanisms of immune cells and cytokines in β-islet cell destruction.

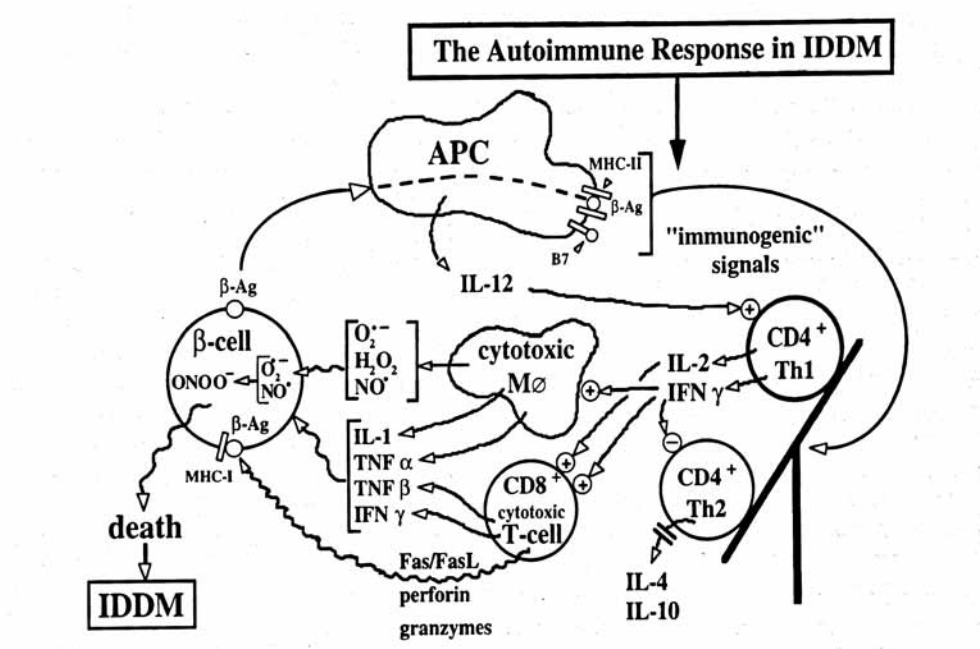

Figure 1.1 Pathogenesis of Type 1 Diabetes (Rabinovitch and Suarez-Pinzon, 1998)

A proposed scheme of immune cells and cytokines that are involved in the autoimmune destruction of β-islet cells. Antigen in the β-islet cells activate APC which in turn activate $CD4^+$ T cells, predominantly of the Th1 subset. β-islet cells are destroyed by two mechanisms, that is cell lysis through interaction with cytotoxic macrophages, $CD8^+$ T cells and non-specific inflammatory mediators like free radicals and cytokines.

1.4.2 Mediators of cytokine induced β-islet cell destruction

Cytokines can cause cellular dysfunction like decrease in islet insulin content, suppression of glucose stimulated insulin secretion, generation of enhanced amounts of NO, damages to cell membranes (Wachlin *et al.*, 2003), induction of DNA strand breaks and apoptosis (Delaney *et al.*, 1997; Cnop *et al.*, 2005). Hence, pioneering research on the mechanism of cytokine induced β-islet cell destruction in Type 1 diabetes mainly focused on damaging mediators like oxygen and nitrogen free radicals. Cytokines like IL-1, TNF-α and IFN-γ had been observed to induce the formation of superoxide, NO and peroxynitrite in β-islet cells, which is auto-destructive (Rabinovitch & Suarez-Pinzon, 1998). NO is mainly produced by iNOS, which catalyzes L-arginine to N^{ω}-hydroxy-L-arginine and finally to form L-citruline and NO using NADPH as a reducing agent (Beshay *et al.*, 2001; Pagliaro, 2003). Peroxynitrite, a product formed by the combination of NO and superoxide

free radicals, is a very potent oxidant and cytotoxic mediator that has been shown to cause necrosis in cultured human and rat β-islet cells (Delaney *el al.*, 1997). It has also been identified to irreversibly inhibit respiration in the mitochondria, which leads to cellular damage (Pagliaro, 2003).

Cytokine induced peroxynitrite formation in cultured human β-islet cells were observed to be dependent upon the increase of superoxide generation, and is independent of NO production, although it increases the level of NO. This was supported by the destruction of cultured β-islet cells that were treated with L-N^G-monomethyl arginine (NMA), an inhibitor of iNOS that reduces the production of NO. On the other hand, destruction of cultured β-islet cells was prevented by the addition of a peroxynitrite scavenger and superoxide inhibitor, guanidinoethyl disulphide (GED) (Lakey *et al.*, 2001). In *in-vivo* studies, GED was observed to delay the onset and significantly reduce the incidence of Type 1 diabetes in female NOD mice (Suarez-Pinzon *et al.*, 2001).

However, the role of NO could not be ignored in cytokine induced cell destruction, as it was still needed for the production of peroxynitrite and the regulation of certain genes. It is also produced by β-islet cells and can induce damage in response to cytokines (Thomas *et al.*, 2002). In a clinical setting, the study by Hoeldtke *et al.* (2003) observed that there was a significant conversion of NO to peroxynitrite in the blood plasma of Type 1 diabetes patients. Peroxynitrite has been observed to cause endothelial dysfunction, vasoconstriction and dysfuntional vasopressor responses in human diabetes.

1.4.3 Gene regulation and signaling pathways of β-islet cell destruction

Contemporary research has mainly focused on the molecular mechanism of gene regulation and cell signaling. Microarray technology has allowed detailed observations of genome expression in a pathogenic event. This technology has been used to observe the gene expression of cultured β-islet cells exposed to cytokines and its dependence on NO to apoptotic destruction. In the study by Kutlu *et al.* (2003), insulin producing INS-1E cells were cultured and exposed to IFN-γ, IL-1β and with or without NMA at 6 different time intervals. In the microarray analysis, a total of 698 genes were affected by cytokines at one time point. Although cytokine induced apoptosis of INS-1E cells were independent of NO

production, 50% of the genes affected by cytokines were also affected by NO production after 6-8 hours of culture (based on the influence of NMA on gene expression), suggesting a role for NO in the late effects of cytokine induced cell destruction. During NO production, besides the up regulation of iNOS and AS (the enzyme that recycles citrulline into arginine, allowing continuous NO production), a new gene, guanosine triphosphate cyclohydrolase I (GTPCH), was also identified to be up regulated. GTPCH is involved in the production of tetrahydrobiopterin, which is a cofactor for the iNOS enzyme. When the genes were clustered according to their functions, genes involved in metabolism was observed to be the most affected by cytokines, especially lipid metabolism with more than half of the genes were NO dependent. Other major affected clusters include cytokine processing and signal transduction genes and transcription factors. Cytokines also decreased the expression of genes related to differentiate β-islet cell functions and preservation of cell mass like Pdx-1, Isi-1, insulin, GLUT2 and glucokinase. It also up regulates pro-apoptotic genes like Bid and Bak (Kutlu *et al.*, 2003). The network of genes affected by the combination of IL-1β and IFN-γ are summarized in Figure 1.2.

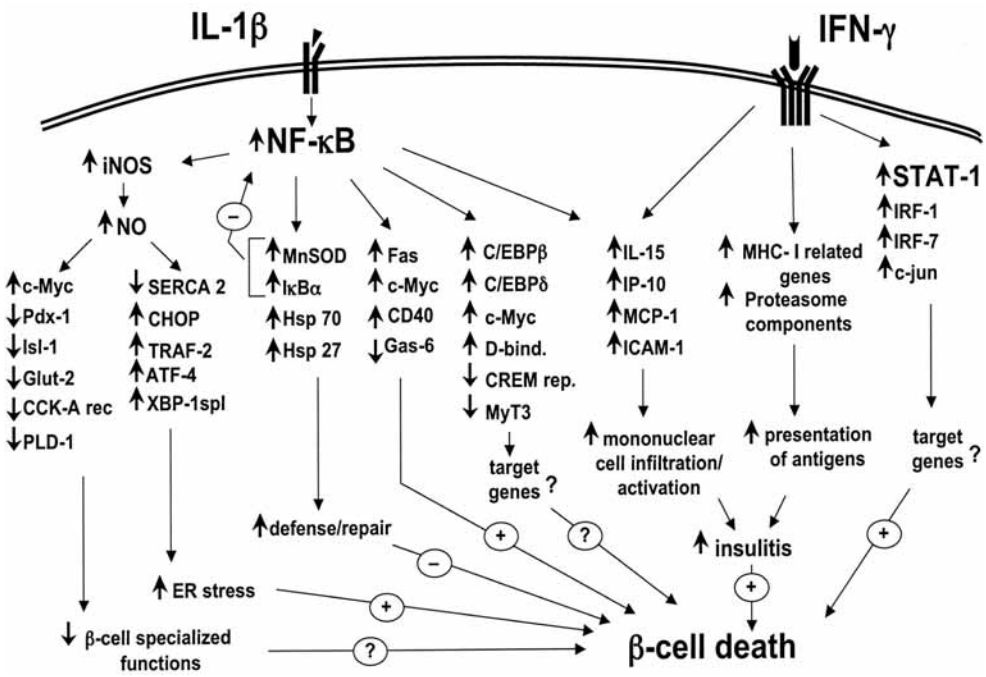

Figure 1.2 The network of genes up regulated (up arrow) and down regulated (down arrow) by IL-1β and IFN-γ that contributes to the apoptosis of β-islet cells (Cnop *et al.*, 2005)

Cluster of genes regulated by IL-1β and IFN-γ compiled from multiple microarray references (including Kutlu *et al*.) in the review by Cnop *et al.* (2005). The transcription factors NF-κB and STAT-1 seems to be the main regulator of gene expression that leads to β-islet cell death.

Cytokines could induce cell damage through many signaling pathways like JAK/STAT (Cnop *et al.*, 2005), JNK/SAPK (Kim *et al.*, 2005) and FRK/RAK (Welsh *et al.*, 2004). The activation of tyrosine kinase JAK by IFN-γ would cause the phosphorylation of STAT-1, which potentiates the effects of iNOS expression. Excessive activation of the JAK/STAT signaling may lead to cell death and is regulated by negative feedback mechanisms such as the up regulation of the Suppressor Of Cytokine Signaling (SOCS) gene. SOCS-3 had been observed to prevent cytokine induced islet cell death by reducing the level of NO and inhibiting the activity of STAT-1 (Karlsen *et al.*, 2001).

Activation of the JNK/SAPK signaling by TNF-α and IFN-γ in cultures of isolated mice pancreatic β-islet cells and insulinoma cell line (MIN6N8) resulted in apoptosis that

correlates with the translocation of Bax into the mitochondria, the release of cytochrome c and the activation of caspase-3. JNK/SAPK signaling also induces the expression of p53 and p21 protein, which correlates with the loss of mitochondria membrane potential and production of reactive oxygen species that finally leads to apoptosis. Apoptosis and the expression of p53 protein were prevented when the cell cultures were treated with SP600125, a specific JNK inhibitor (Kim *et al.*, 2005). The FRK/RAK signaling pathway had also been observed to increase the susceptibility of β-islet cells to cytokine induced cell death. β-islet cells isolated from FRK/RAK knockout mice pancreas were observed to be more resistant to cell death when cultured with IL-1β and IFN-γ compared to normal mice. In normal β-islet cells treated with inhibitors or transfected with small interfering RNA (siRNA) to interfere with FRK/RAK expression, cytokine induced cell death was also significantly reduced (Welsh *et al*, 2004). The review by Cnop *et al.* (2005) proposed a few mechanisms of cytokine induced gene expression and signaling patterns that result in β-islet cell death as shown in Figure 1.3.

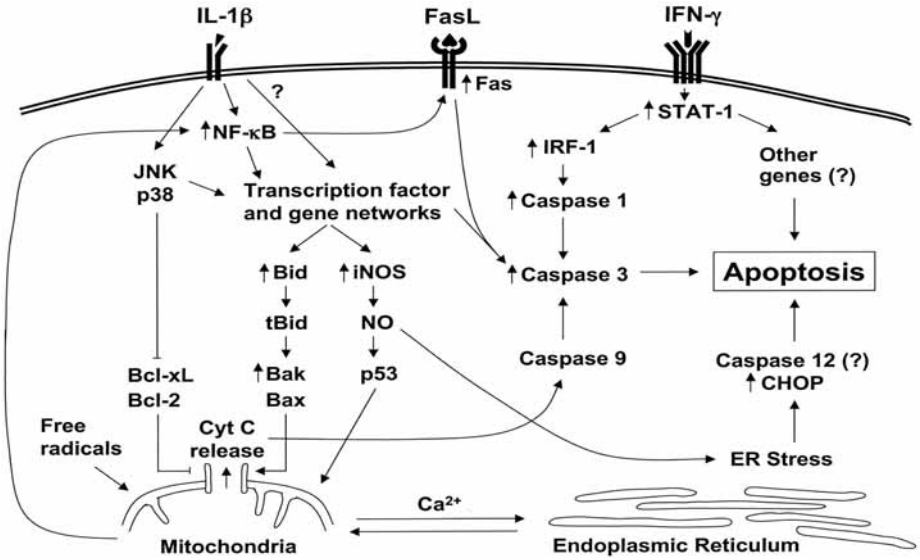

Figure 1.3 Proposed mechanism of gene expression and signaling pathways leading to cytokine induced cell death (Cnop et al., 2005)

β-islet cell apoptosis is mediated by three pathways that includes activation of stress proteins like JNK, p38 MAPK, ERK and NF-κB. This triggers reticulum endoplasmic stress and the release of death signals from the mitochondria.

1.5 Peroxisome Proliferator-Activated Receptor (PPAR)

1.5.1 Introduction and structural functions

The peroxisome proliferator-activated receptor (PPAR) is a transcription factor that belongs to the nuclear hormone receptor superfamily. PPAR is mainly involved in cellular energy usage and adipogenesis by regulating a broad range of genes involved in glucose and lipid metabolism and adipocyte differentiation. Currently, there are 3 known subtypes of PPAR, that is PPAR-α, PPAR-δ/β and PPAR-γ. Each subtype of PPAR has a different tissue distribution, is very similar in molecular structure and is encoded by different genes mapped to human chromosomes 22, 6 and 3 respectively (Bar-Tana, 2001; Rotondo & Davidson, 2002). In the mouse, PPAR-α, PPAR-δ/β and PPAR-γ is located in chromosomes 15, 17 and 6 respectively (Yousef & Badr, 2004). The PPAR protein consists of 5 domains: a ligand independent transactivation domain (Domain A/B), a DNA binding domain (Domain C), a hinge region (Domain D) and a ligand-binding domain (Domain E/F) as shown in Figure 1.4 (Kota *et al.*, 2005).

The ligand independent transactivation domain (Domain A/B) is a domain that contains the Activation Function 1 (AF1), which is transcriptionally active in the absence of ligands. The DNA binding domain (Domain C) is highly conserved in all the isoforms, supporting the fact that all three PPAR isoforms bind to the same DNA response element, the PPAR Response Element (PPRE). PPRE consist of a direct repeat of the hexanucleotide DNA sequence **AGGTCA** separated by one or two nucleotides, termed Direct Repeat 1 and 2 (DR1 & DR2). The hinge region (Domain D) is the docking site for co-factors. The ligand binding domain (Domain E/F) is less conserved, allowing each subtype to have its own activating ligand. The domain consists of 12 α-helical regions named H1 to H12 and are also a binding site for co-activator proteins (Zhang & Young, 2002; Kota *et al.*, 2005; Lazar, 2005). The ligand binding domain also contains the Activating Function 2, which mediates conformational changes through a conserved hydrogen-bonding network to create an interacting surface with co-activator proteins upon binding with a ligand (Cronet *et al.*, 2001; Blanquart *et al.*, 2003).

Natural ligands for PPARs are mostly fatty acids and their derivatives. The eicosanoid derivatives from the lipoxygenase pathway such as Leukotriene B4 (LTB4) and

oxidized phospholipids are natural ligands for PPAR-α. The natural activating ligands for PPAR-γ are prostaglandins like 15-deoxy-Prostaglandin J2 (15d-PGJ2), a product of arachidonic acid metabolism and hexadecyl azeloayl phosphatidylcholine (azPC), a product of oxidized low-density lipoproteins (Zhang & Young, 2002). Synthetic ligands include lipid-lowering fibrates for PPAR-α and thiazolidinediones, a class of drugs termed glitazones (insulin sensitizers for the treatment of Type 2 Diabetes) for PPAR-γ.

Figure 1.4 The linear structure of PPAR (Kota *et al.*, 2005)

The PPAR protein consists of 5 domains, a ligand independent transactivation domain (Domain A/B), a DNA binding domain (Domain C), a hinge region (Domain D) and a ligand-binding domain (Domain E). Domain A/B is an activating domain that is ligand independent. Domain C is implicated in DNA binding of target gene. Domain D is a binding region for co-factors and Domain E/F is also an activating domain but is ligand dependent.

1.5.2 Activation and transcriptional control

In an inactive state, PPARs are bound with a nuclear co-repressor (NCo-R) containing histone deacetylase activity, which inhibits transcriptional activities (Kota *et al.*, 2005). Upon binding with a ligand, the NCo-R is released and the free PPAR would form a heterodimer with another nuclear receptor protein, the Retinoid X Receptor (RXR) (Zhang & Young, 2002). The PPAR:RXR heterodimer would be translocated into the nucleus and bind to the PPRE located in the promoter of target genes (Moraes *et al.*, 2006). Activated PPARs could also recruit accessory proteins like Nuclear Co-Activators (NCo-As), Cyclic-AMP Response Element Binding Protein/P300 (CBP/p300) and CCAAT/Enhancer binding proteins (C/EBP) (Rosen *et al.*, 2002) that are essential for the initiation of gene transcription.

Besides natural and synthetic activating ligands, the transcriptional activities of PPARs are also regulated by post-translation mechanisms including phosphorylation and

ubiquination. The activities of PPARs are activated or deactivated following phosphorylation by the Mitogen Activated Protein Kinase (MAPK) or Protein Kinase A (PKA) signaling pathway as shown in Figure 1.5. The phosphorylation of PPAR-α is increased in response to insulin through the MAPK pathway and this correlates with the enhancement of transcriptional activity (Shalev *et al*., 1996; Blanquart *et al.*, 2003). PPAR-γ is also a phosphoprotein, which however, differs with PPAR-α, in that phosphorylation inhibits its transcriptional activities (adipogenesis) in mice and rat fibroblast (Hu *et al.*, 1996).

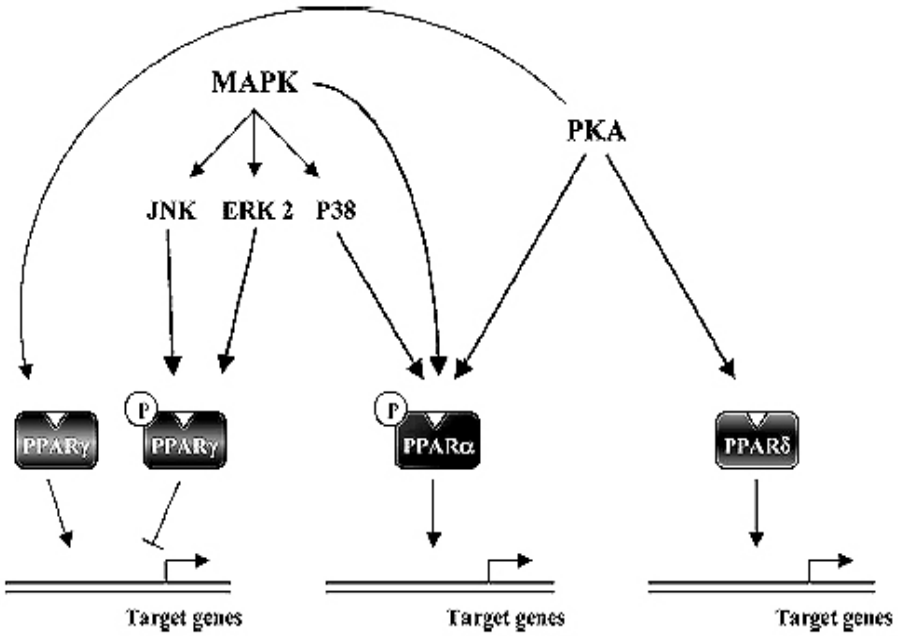

Figure 1.5 Kinase pathways implicated in the phosphorylation and in the regulation of ligand independent PPAR transcriptional activity (Blanquart *et al.*, 2003)

Kinase signaling is one of the ligand independent activation mechanisms of PPAR proteins. MAP Kinase phosphorylation increases the activity of PPAR-α but inhibits the activity of PPAR-γ. Protein Kinase A (PKA) increases the activity of all PPAR isoforms. PPAR isoforms are regulated by physiological changes leading to the production of kinase activators.

PPARs are short-lived proteins that are degraded in the ubiquitin-proteosome pathway as a regulation to its transcriptional activities (Blanquart *et al.*, 2003). The proteins degraded in this pathway are covalently modified on lysine residues by fixation of ubiquitins in a three-step process as shown in Figure 1.6. First, ubiquitin is activated by a ubiquitin-activating enzyme. The activated ubiquitin is subsequently transferred to a ubiquitin carrier protein. Finally, ubiquitin-protein ligase catalyzes the covalent binding of ubiquitin from its carrier protein to the target protein. Multi-ubiquitinated proteins are degraded by the 26S proteosome. This system controls the PPAR protein levels in cells and also the intensity of a response to a ligand (Hauser *et al.*, 2000; Blanquart *et al.*, 2002).

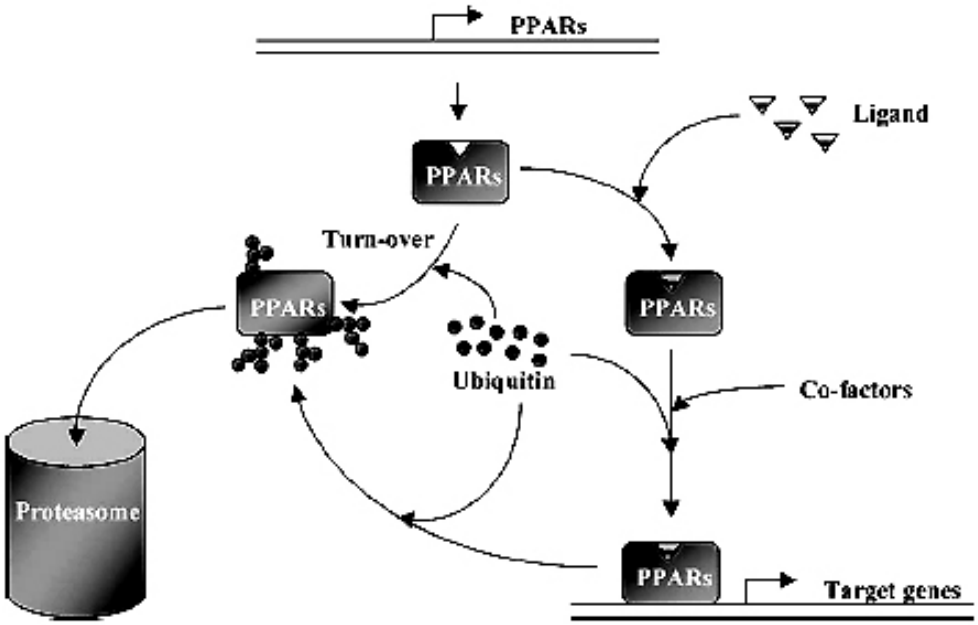

Figure 1.6 Possible mechanisms of PPAR degradation by the ubiquitin-proteosome system (Blanquart *et al.*, 2003)

PPAR proteins are degraded by the ubiquitin-proteosome pathway. Ligand activated PPARs are stable due to decreased ubiquitination. However, upon binding with co-factors, ubiquitination is increased again in order to control the response.

1.5.3 Isoforms of PPAR and its known functions

PPAR has been implicated in several biological roles that include aging, immunity, obesity, cell cycle control and fertility (Youssef & Badr, 2004). PPAR-α is mainly expressed in hepatocytes, enterocytes, smooth muscle cells, monocytes, macrophages, endothelial and kidney cells. It is usually involved in lipid metabolism by hepatic cells. It regulates the uptake, binding, activation and oxidation of fatty acids, synthesis of ketone bodies and apolipoproteins, control of gluconeogenesis and deamination of amino acids (Bishop-Baily & Wray, 2003).

PPAR-γ is found in brown and white tissue adipocytes. It is mainly involved in adipocyte function and proliferation. In humans, PPAR-γ might play a role in the differentiation of cells, the sensitization to insulin and atherogenesis. PPAR-γ ligands have been reported to increase the adipose mass of humans *in-vivo*. It also promotes the differentiation of macrophages into foam cells via the expression of CD36, which would lead to arteriosclerosis. CD36 serves as a plasma membrane scavenger receptor for oxidized low-density lipoprotein (LDL). On the other hand, PPAR-γ has been shown to inhibit the proliferation of human cancer cell cultures and represses the production of inflammatory cytokines (Bar-Tana, 2001). In fertility, PPAR-γ appears to play an important role in the development of embryos (Cui *et al.*, 2002; Yousef & Badr, 2004).

No specific function has yet been identified for PPAR-δ although it has a wide distribution. Research on the role of PPAR-δ is lacking, partially because specific ligands are not readily or commercially available. PPAR-δ is involved in the control of cellular development and the pathogenesis of tumours such as in colorectal cancer (Rotondo & Davidson, 2002). PPAR-δ knockout mice had been observed to be more susceptible in developing colon polyps when treated with azoxymethane compared to mice that express PPAR-δ (Harman *et al*., 2004). The review by Yousef & Badr (2004) mentions a study in their group using a dual PPAR agonist (L783483), which activates PPAR-δ and γ. Treatment with the agonist caused a two-fold reduction in carrageenan-induced paw edema of rats, compared to rosiglitazone (a potent PPAR-γ agonist) alone. This suggests that PPAR-δ may also play a role in modulating inflammation (Youssef & Badr, 2004). PPAR-δ/β had also been observed to play an important role in signaling pathways that leads to the

development of placenta in pregnant mice as mice with PPAR-δ/β knockout genes exhibit embryonic growth retardation. The important role of PPAR-δ/β has been identified in the differentiation and metabolic functions of giant trophoblast cells, which is essential for embryo implantation, remodeling of the uterine stroma and hormone secretion that promotes placenta formation (Nadra *et al.*, 2006).

1.6 PPAR and Immunity

1.6.1 Role of PPAR-α

Apart from transactivating genes, PPARs can trans-suppress inflammatory gene activation. PPAR-α knockout mice have been shown to exhibit a prolonged inflammatory response when treated with LTB4. LTB4 is a PPAR-α ligand that induces its own degradation by stimulating the β-oxidation pathway (Bishop-Bailey & Wray, 2003). In an animal study by Cuzzocrea *et al.* (2006), the role of PPAR-α was observed in two types of acute inflammation (carrageenen induced paw edema and lung pleurisy). In paw inflammation, mice with the PPAR-α knockout gene had significantly enhanced paw edema and higher myloperoxidase activity (indicator for polymorphonuclear (PMN) leukocyte accumulation) compared to wild type mice. Histological studies of paw tissues observed significant pathological changes, which includes more inflammatory cell infiltration and higher staining for FAS ligand in PPAR-α knockout mice. In lung inflammation, PPAR-α knockout mice had significantly higher pleural exudates and inflammatory cell infiltration (characterized by myeloperoxidase activity) in the lung compared to wild type mice. Histological studies also indicate augmentation of lung tissue damage and higher FAS ligand staining in the vascular wall and pneumocytes of PPAR-α knockout mice. Cytokine measurements detected a significantly higher level of IL-1 and TNF-α expression in lung tissue and pleural exudates of PPAR-α knockout mice. The authors propose that PPAR-α modulates the degree of acute inflammation by reducing FAS ligand activation, suppressing the formation of pro-inflammatory cytokines and decreasing PMN leukocyte infiltration (Cuzzocrea *et al.*, 2006).

PPAR-α also inactivates NF-κB and represses the expression of inflammatory mediators induced by extracellular inflammatory stimuli like cytokine-induced Vascular