The role of macrophages in early phases of type 2 diabetes mellitus

Gunaseelan, Divya

2018

Gunaseelan, D. (2018). The role of macrophages in early phases of type 2 diabetes mellitus. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/83668>

<https://doi.org/10.32657/10220/46661>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**THE ROLE OF MACROPHAGES IN EARLY PHASES
OF TYPE 2 DIABETES MELLITUS**

DIVYA GUNASEELAN
Interdisciplinary Graduate School
NTU Institute for Health Technologies

2018

**THE ROLE OF MACROPHAGES IN EARLY PHASES
OF TYPE 2 DIABETES MELLITUS**

DIVYA GUNASEELAN

**Interdisciplinary Graduate School
NTU Institute for Health Technologies**

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2018

STATEMENT OF ORIGINALITY

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

24-AUG-2018

.....

Date

.....

DIVYA GUNASEELAN

SUPERVISOR DECLARATION STATEMENT

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University Singapore and that the research data are presented honestly and without prejudice.

23/10/2018

.....

Date

.....

Asst. Prof. YUSUF ALI

ACKNOWLEDGEMENTS

“If I have seen further than others, it is by standing upon the shoulder of giants”

-Isaac Newton

First and foremost, I would like to sincerely thank my supervisor Asst. Prof. Yusuf Ali for providing me the opportunity to work under his guidance, without whom this thesis would not have been possible. His thoughtful suggestions, patience and constant encouragement have enabled me to do my research. I am extremely grateful to him for putting his faith in me and enabling me to complete this thesis on time.

I would like to thank my co-supervisor Assoc. Prof. Joachim Loo for enlightening me on the thought-provoking aspects of my project and allowing me to be a part of his group discussions, that proved to be a knowledge sharing platform. I thank my mentor, Prof. Bernhard Boehm for his useful insights, advice and guidance. I am grateful to Assoc. Prof. Christiana Ruedl, for her generosity in providing me with the DTR mice for my study.

I truly appreciate the guidance and timely help of Dr. Baek Jongsuep in completing my experiments. I also thank Vanessa Tay for being one of the best teachers and training me in most of the experimental procedures. I deeply appreciate Dr. Manesh Chittezhath and for sharing his knowledge, his guidance and help. My appreciation also extends to my laboratory colleagues Cho Mar, Rosi Chelliah, Dr. Surabhi Devaraj, Dr Xiaofeng Zheng, Calvin Ho, Dr Xiaofeng Zheng, Jueying Chua and Minni Chua.

It was an honour to be a part of NTU Singapore and I sincerely thank Interdisciplinary Graduate School (IGS) and HealthTech NTU for providing me an opportunity to be a part of this institution, funding my research and providing me with the financial support. I also thank Lee Kong Chian School of Medicine for providing me with all the research equipment and instruments. My sincere thanks to the IGS team and Ms. Suriani Binte Rabu for all the help and support.

I thank my husband for his constant support, patience and continuous encouragement. I also thank my parents for their blessings and my brother for his best wishes for the completion of this Ph.D. I am indebted to my friends Jasmine Chong, Muthu Kumar K, Kaarunya Sampathkumar, Uthra Balasubramanian, Gopal Ammanath, Akshay Kumar Suresh, Ramya R and Ankita Pan for being there in difficult times and ensuring that I got through them.

Finally, I thank God who never let me lose hope during difficult times and for enabling me to reach my destination at a good note.

ABSTRACT

Inflammatory response is a hallmark process of type 2 diabetes mellitus (T2DM) development and progression. In overt hyperglycaemia, low-grade inflammation of the pancreatic islets is known to cause β -cell dysfunction paving way for the pathogenesis of T2DM. Macrophages are vital elements of the immune system that play a major role in both innate and adaptive immune responses. Increased macrophage infiltration in islets of Langerhans has been documented in humans and rodents with T2DM, thus exposing the islets to a pro-inflammatory milieu and thereby contributing to the disease. Though the detrimental role of islet macrophages in overt T2DM has been widely studied, the role of macrophages in islet cell remodelling during the (very) early stage of pre-diabetes, i.e. the compensatory phase, is hardly known. Elucidating the role of macrophages, and more so islet resident macrophages, along with their contribution to islet remodelling in early stages of T2DM can offer a window of opportunity to delay the progression of the disease. We established an animal model of pre-diabetes by exposing mice to a very short term high-fat diet (HFD) and utilized different established macrophage markers, to understand the dynamics of islet macrophages during this phase of pre-diabetes. We found a significant increase in islet macrophage cells within HFD fed mice at 21-days when compared to mice treated with the iso-calorie low-fat diet (LFD). To further determine whether macrophages play a direct role in islet dysfunction during the initial compensatory phase, we used diphtheria toxin receptor (DTR) mice to deplete macrophages during HFD feeding. Our results suggest that presence of macrophages may be important to promote compensatory islet hyperplasia (growth of islet size) at a much earlier stage of mouse T2DM pathogenesis.

As macrophages seem to play a dual role in the pathogenesis of diabetes, with early anti-inflammatory role predominating while the later phase is marked by pro-inflammation and islet destruction, it is important to understand which phenotype/ or subtype modulation of macrophages (from pro-inflammatory M1-like phenotype to an anti-inflammatory M2-like phenotype) will keep islets healthy and functioning. To this end, a recent report showed that a small molecule drug, I-BET151, which is a bromodomain and extra-terminal (BET) protein inhibitor, can perform dual function i.e. to modulate macrophages to adopt an anti-inflammatory phenotype and also aid in pancreatic β -cell regeneration and was then subsequently found to be particularly effective against type 1 diabetes mellitus (T1DM) development in non-obese diabetic (NOD) mice. As I-BET151 is emerging as a potential drug for diabetes treatment, we studied the effect of this drug on macrophage re-programming to M2-like phenotype and the effect of macrophage secreted factors on β -cell, *in vitro*. We found reduced gene expression of pro-inflammatory cytokines in macrophages treated with I-BET151. We also found that I-BET151 has a direct effect on β -cell gene expression levels than indirectly through I-BET151 treated macrophage secreted factors. Marked reduction in gene expression levels of T2DM-associated susceptibility genes were observed in the β -cells as a result of exposure to I-BET151 on murine β -cells. Our novel finding suggests that while I-BET151 may be effective for modulating macrophage phenotype and function, it may also have unintended consequences on the health of the β -cell.

As literary evidences suggest that low dose of I-BET151 can elicit anti-inflammatory response in macrophages we also devised a poly(lactic-co-glycolic) acid (PLGA) based nanoparticle system for the controlled and sustained release of I-BET151 with the aim of modulating macrophage function without the side-effect of increasing β -cell

dysfunction. We achieved 72% encapsulation efficiency of the compound and sustained release of low dose I-BET151 over a period of 48h. In summary, we show that islet macrophages increase in a mouse model that recapitulates human pre-diabetes and that this increase contributes to islet growth, function and thus compensation. It is also reported herein that attempts to modulate macrophage phenotype by BET protein inhibitors was not macrophage-specific and may also exert “undesirable consequences” on the β -cells. Therefore, drug development targeting BET protein must proceed with caution.

SUMMARY

Changes in pancreatic β -cells (insulin secreting cells) occur early, and usually precede the development of frank type 2 diabetes mellitus (T2DM). Islet resident macrophages (anti-inflammatory) have been shown to assist in β -cell development during the embryonic stages however its role in pre-diabetic β -cells remains unknown. In this thesis, I determined the role of islet macrophages in facilitating pre-diabetic islet remodelling, failure of which results in frank T2DM. Through the use of mouse models, we suggest a new paradigm for islet macrophages whereby increased islet macrophage numbers in a pre-diabetic mouse model promotes islet remodelling in pre-diabetes. This work lends greater appreciation and possible therapeutic emphasis on islet macrophages for preserving islet and β -cell function for the prevention of frank T2DM.

TABLE OF CONTENTS

STATEMENT OF ORIGINALITY	i
SUPERVISOR DECLARATION STATEMENT	ii
ACKNOWLEDGEMENTS	iii
ABSTRACT	v
TABLE OF CONTENTS	viii
LIST OF FIGURES.....	xv
LIST OF TABLES.....	xx
LIST OF ABBREVIATIONS.....	xxi
INTRODUCTION	1
1.1 Prevalence of diabetes.....	1
1.2 Pathophysiology of T2DM	2
1.3 Clinical presentation and diagnosis of T2DM	5
1.4 Risk factors of T2DM	5
1.5 Pancreatic β -cells and the islets of Langerhans	7
1.6 Role of low-grade inflammation in insulin resistance	11
1.7 Role of low-grade inflammation in pancreatic β -cell dysfunction	12
1.8 Macrophages and their role in T2DM.....	15
1.9 Role of macrophages in pancreatic islet cell remodelling	17
1.10 Macrophage modulators.....	20
1.11 Macrophage modulation by epigenetics and role of BET protein inhibitors	
23	
1.12 Controlled drug release using PLGA nanoparticles.....	27

1.13	Overarching hypothesis	29
1.14	Specific aims	29
MATERIALS AND METHODS.....		30
2.1	Materials	30
2.1.1	Animal studies	30
2.1.2	Specialized animal diets.....	30
2.1.3	Paraformaldehyde (PFA) solution (4%)	30
2.1.4	List of antibodies.....	31
2.1.5	Cell culture.....	31
2.1.6	Primer design	32
2.1.7	List of primer sequences used for qPCR.....	34
2.1.7.1	Primer sequences for mouse-specific qPCR	34
2.1.7.2	Primer sequences for rat-specific qPCR.....	35
2.1.7.3	Primer sequences for human-specific qPCR.....	36
2.1.8	RIPA buffer.....	36
2.1.9	GSIS buffer	37
2.1.9.1	1.5G Buffer.....	37
2.1.9.2	16.7G Buffer.....	37
2.1.9.3	25mM KCl in Ca-10.....	38
2.1.10	PLGA nanoparticles	38
2.2	Methods.....	38
2.2.1	Intraperitoneal glucose tolerance test (IPGTT).....	38
2.2.2	Blood collection for plasma insulin	38
2.2.3	Plasma insulin ELISA.....	39
2.2.4	Insulin tolerance test	39

2.2.5	Tissue fixation.....	40
2.2.6	Tissue sectioning.....	40
2.2.7	Tissue immunofluorescence staining.....	40
2.2.8	Confocal microscopy	41
2.2.9	Image analysis.....	41
2.2.10	RNA isolation from cell lines	42
2.2.11	cDNA synthesis.....	42
2.2.12	Real-time polymerase chain reaction.....	43
2.2.13	qPCR analysis	43
2.2.14	Cell viability assay	44
2.2.15	Glucose stimulated insulin secretion on cultured cells	44
2.2.16	Protein isolation	45
2.2.17	Protein estimation	45
2.2.18	Statistical method.....	46
RESULTS-I DEVELOPMENT OF PRE-DIABETIC MOUSE MODEL		47
3.1	Introduction.....	47
3.2	Methods.....	49
3.2.1	Animal studies: high-fat/ low-fat diet feeding	49
3.2.2	Immunofluorescence staining: Islet vascularization	52
3.3	Results.....	53
3.3.1	Effect of acute-term high-fat and low-fat diet feeding on metabolic profiles of C57BL/6 mice	53
3.3.2	Increased islet area in pancreatic islets of mice fed with HFD for 21 days 57	
3.3.3	Effect of HFD on islet vascularization.....	58

3.4	Discussion	61
 RESULTS-II EFFECT OF ACUTE TERM HIGH-FAT DIET FEEDING ON		
PANCREATIC ISLET MACROPHAGES.....		63
4.1	Introduction.....	63
4.2	Methods.....	66
4.2.1	Immunofluorescence staining: Islet macrophages	66
4.2.2	Animal studies: DTR mice model.....	66
4.2.3	Immunofluorescence staining: Islet vascularization	68
4.3	Results.....	68
4.3.1	Changes in islet macrophage population in HFD fed mice	68
4.3.2	Confirming the ablation of macrophages in DTR ⁺ mice	74
4.3.3	Reduced islet area in islets of macrophage depleted mice.....	77
4.3.4	Reduced islet vascularization in macrophage ablated mice.....	79
4.3.5	Effect of acute-term high-fat feeding on metabolic profiles of macrophage ablated mice	82
4.4	Discussion	86
 RESULTS-III MODULATION OF MACROPHAGES TO ANTI-		
INFLAMMATORY PHENOTYPE AND THEIR EFFECT ON PANCREATIC		
β-CELL FUNCTION		90
5.1	Introduction.....	90
5.2	Methods.....	93
5.2.1	Activation of mouse macrophages by lipid A.....	93
5.2.2	Verification of macrophage activation by lipid A	94
5.2.3	Treatment of β-cells with macrophage CM	94

5.2.4	Treatment of macrophages with I-BET151	96
5.2.5	Macrophage wash after treatment with I-BET151.....	96
5.2.6	β -cell treatment with I-BET151 treated RAW 264.7 CM.....	96
5.2.7	INS1E cells wash after treatment with I-BET151	97
5.2.8	Human β -cell treatment with I-BET151	97
5.2.9	β -cell treatment with BET protein inhibitors JQ1 and I-BET762	98
5.2.10	INS1E pre-treatment with actinomycin D	98
5.3	Results.....	100
5.3.1	Increased pro-inflammatory gene expression levels in RAW 264.7 cells upon exposure to lipid A.....	100
5.3.2	Reduced expression of key β -cells genes upon treatment with activated macrophage pre-conditioned media.....	102
5.3.3	Reduced expression of <i>Il-1β</i> and <i>Ccl2</i> in RAW 264.7 cells treated with I- BET151	107
5.3.4	Marked reduction in specific T2DM susceptibility genes upon I-BET151 CM treatment	111
5.3.5	IBET151 does not affect cell viability	119
5.3.6	Comparison of the direct effect of I-BET151 on pancreatic β -cells of different origins.....	121
5.3.7	I-BET151 induced gene expression changes abrogated following a wash in INS1E cells	125
5.3.8	Effect of I-BET151 on glucose stimulated insulin secretion	127
5.3.9	Reduced expression of T2DM susceptibility genes in INS1E cells is due to BET protein inhibition in general	129

5.3.10	Effect of I-BET151 on reducing β -cell gene expression is through transcription	132
5.4	Discussion	134
RESULTS-IV CONTROLLED DELIVERY OF I-BET151 USING PLGA NANOPARTICLES.....		140
6.1	Introduction.....	140
6.2	Methods.....	141
6.2.1	I-BET151 dose dependent treatment on murine β -cells	141
6.2.2	Fabrication of I-BET151 loaded nanoparticles	142
6.2.3	Field emission scanning electron microscopy	142
6.2.4	Particle size and zeta potential measurement.....	142
6.2.5	Drug release study.....	143
6.2.6	High-performance liquid chromatography (HPLC).....	143
6.3	Results.....	143
6.3.1	Dose dependent effects of I-BET151 on INS1E cells	143
6.3.2	Verification of dose dependent effects of I-BET151 on MIN6 cells	146
6.3.3	Synthesis and characterization of I-BET151 encapsulated PLGA nanoparticles	149
6.3.4	Zeta potential	151
6.3.5	I-BET151 release study from PLGA nanoparticles	152
6.4	Discussion	153
DISCUSSION AND FUTURE PERSPECTIVES		155
7.1	Discussion	155
7.2	Clinical significance.....	160

7.3	Novelty of research	161
7.4	Future perspectives	162
7.5	Conclusion	163
REFERENCES		164

LIST OF FIGURES

Figure 1.1. Current mode of T2DM development.	4
Figure 1.2. Pancreas and structure of pancreatic islets.	9
Figure 1.3. Mechanism of development of T1DM and T2DM.....	10
Figure 1.4. Immune cell infiltration in adipose and pancreatic islets during T2DM progression.	14
Figure 1.5. Pancreatic islet macrophages during T2DM progression.....	19
Figure 1.6. Macrophage polarization stimuli, markers and function.	22
Figure 1.7. The dynamic process of epigenetics.	26
Figure 1.8. Dual effect of I-BET151 on macrophages and pancreatic β -cells.....	27
Figure 1.9. Overarching hypothesis.	29
Figure 3.1. Experimental design to determine islet changes in mice fed with HFD.	51
Figure 3.2. Unstained mouse pancreatic islet.	53
Figure 3.3. Body weight changes upon acute-term of HFD feeding.	54
Figure 3.4. Glucose excursion profiles and FBG of HFD/LFD fed mice.	55
Figure 3.5. Fasting plasma insulin and GSIS of acute-term HFD/LFD fed mice.....	56
Figure 3.6. Islet size and pancreatic β -cell population changes in acute-term HFD fed mice.....	58
Figure 3.7. Islet vascularization changes in acute-term HFD fed mice.	60
Figure 4.1. Targeted Macrophage ablation using the CD169 expressing DT receptor (DTR) mice model.	65
Figure 4.2. Experimental design for determining role of macrophages in islet dynamics in response to HFD.	67

Figure 4.3. Representative images showing increased F4/80 ⁺ macrophage population in HFD fed mice islets over time.	70
Figure 4.4. Increased F4/80 ⁺ macrophage population in HFD fed mice islets over time.	71
Figure 4.5. Representative images showing increased CD68 ⁺ macrophage population in HFD fed mice islets on day 21.	72
Figure 4.6. Increased CD68 ⁺ macrophage population in HFD fed mice islets on day 21.	73
Figure 4.7. Representative images of macrophage ablation in HFD fed CD169-DTR ⁺ mice liver.	75
Figure 4.8. Representative images of macrophage ablation in HFD fed CD169-DTR ⁺ mice islets.	76
Figure 4.9. Quantification of macrophage ablation in HFD fed CD169-DTR ⁺ mice islets.	77
Figure 4.10. Reduced islet area and β -cell population in macrophage depleted mice.	78
Figure 4.11. Representative images of islet vascularization in macrophage ablated mice.	80
Figure 4.12. Reduced islet vascularization in macrophage ablated mice.	81
Figure 4.13. Body weight changes of macrophage depleted mice upon acute-term of HFD feeding.	82
Figure 4.14. Insulin tolerance test of acute-term of HFD fed macrophage depleted mice.	83
Figure 4.15. Fasting plasma insulin and GSIS of acute-term HFD fed macrophage ablated mice.	84

Figure 4.16. Glucose excursion profile with respect to body weight and FBG of acute-term HFD fed macrophage depleted mice.	86
Figure 5.1. Overall Experimental plan to study the effect of I-BET151 on macrophages and the CM effect on pancreatic β -cells.	92
Figure 5.2. Schematic workflow of macrophage activation using lipid A and CM treatment on β -cells.....	95
Figure 5.3. Schematic workflow of macrophage exposure to I-BET151 and I-BET151 CM treatment on β -cells.	99
Figure 5.4. Pro-inflammatory genes and transcription factor, <i>Nf-κB</i> , expression of RAW 264.7 cells upon activation by lipid A.	101
Figure 5.5. β -cell signature gene expression upon treatment with lipid A activated macrophage CM in MIN6 cells.....	105
Figure 5.6. β -cell signature gene expression upon treatment with lipid A activated macrophage CM in INS1E cells.	106
Figure 5.7. Reduced IL-1 β and CCL2 gene expressions in mouse macrophages treated with I-BET151.	109
Figure 5.8. Gene expression changes in I-BET151 treated macrophage cells abrogated following a wash to remove the drug.	110
Figure 5.9. Modest changes in β -cell signature gene expression levels upon treatment with I-BET151 conditioned RAW 264.7 cell media in INS1E cells.	115
Figure 5.10. Reduced expression of T2DM susceptibility genes in INS1E cells upon I-BET151 CM treatment.	116
Figure 5.11. Reduced <i>Glut2</i> expression in MIN6 cells upon I-BET151 treated RAW 264.7 cell CM treatment and direct treatment of I-BET151.....	117

Figure 5.12. Significant reduction in <i>Glut2</i> and <i>Hnf4a</i> gene expression in MIN6 cells upon I-BET151 treated RAW 264.7 cell CM treatment and direct treatment of I-BET151.....	118
Figure 5.13. No significant cell death in β -cells and macrophages treated with I-BET151.....	120
Figure 5.14. Reducing trend in gene expression levels of <i>Gck1</i> and <i>Glut2</i> in three different β -cell lines.	122
Figure 5.15. Changes in T2DM susceptibility genes in EndoC- β H1 cells upon direct treatment of I-BET151.....	124
Figure 5.16. Gene expression changes in I-BET151 treated INS1E cells abrogated following a wash to remove the drug.....	126
Figure 5.17. Glucose stimulated insulin secretion in β -cells treated with I-BET151.....	128
Figure 5.18. Reduced expression of β -cell signature genes and T2DM susceptibility genes in INS1E cells treated with BET protein inhibitor JQ1.....	130
Figure 5.19. Reduced expression of β -cell signature genes and T2DM susceptibility genes in INS1E cells treated with BET protein inhibitor I-BET762.....	131
Figure 5.20. Effect of I-BET151 on INS1E cells is through transcription.	133
Figure 5.21. Workflow of validating experimental design using lipid A treated macrophages.	137
Figure 5.22. Workflow to validate CM-mediated gene expression changes in β -cells. Summary of major findings and possible inferences from the I-BET151 study.....	138

Figure 5.23. Direct effect of I-BET151 on β -cells. Different experiments were performed to determine the direct effects of I-BET151 on β -cells. The list of experiments, their outcomes and inferences are as shown.	139
Figure 6.1. Dose dependent effects of I-BET151 on INS1E cells.	145
Figure 6.2. Dose dependent effects of I-BET151 on MIN6 cells.	148
Figure 6.3. Representative SEM image of I-BET151 encapsulated PLGA nanoparticles.	150
Figure 6.4. Dynamic laser scattering of I-BET151 encapsulated PLGA nanoparticles.	150
Figure 6.5. Zeta potential of I-BET151-loaded PLGA nanoparticles.	151
Figure 6.6. The release profiles of I-BET151 from PLGA nanoparticles in 1x PBS.	152
Figure 7.1. Overview of research.	160

LIST OF TABLES

Table 1.1. Blood glucose levels of healthy adults and pre-diabetic and T2DM patients.	5
Table 2.1. List of antibodies used in this study.....	31
Table 2.2. Mouse primers for qPCR.	34
Table 2.3. Rat primers for qPCR.	35
Table 2.4. Human primers for qPCR.	36
Table 3.1. Comparison of diet formula between HFD and LFD. This	48
Table 5.1. β -cell signature gene description and functions.....	103
Table 5.2. Functions of T2DM susceptibility genes involved in pancreatic β -cell function.	114

LIST OF ABBREVIATIONS

ADA	-	American Diabetes Association
ANOVA	-	Analysis of Variance
ARF	-	Animal Research Facility
ATCC	-	American Type Culture Collection
BCA	-	Bicinchoninic Acid
BET	-	Bromodomain and Extra-Terminal
BLAST	-	Basic Local Alignment Search Tool
BMDM	-	Bone Marrow-Derived Macrophage
BRC	-	Biological Resource Centre
BRD	-	Bromodomain
BSA	-	Bovine Serum Albumin
CaCl ₂	-	Calcium chloride
CCK-8	-	Cell Counting Kit - 8
CCL2	-	Chemokine (C-C motif) Ligand 2
CCR2	-	C-C Chemokine Receptor type 2
CD	-	Cluster of Differentiation
cDNA	-	Complementary DNA
CM		Conditioned Media
Ct	-	Cycle Threshold
CXCL	-	Chemokine (C-X-C motif) Ligand
DAPI	-	4',6-diamidino-2-phenylindole
DLS	-	Dynamic Light Scattering
DMEM	-	Dulbecco's Modified Eagle Medium

DNA	-	Deoxyribonucleic Acid
DT	-	Diphtheria Toxin
DTR	-	Diphtheria Toxin Receptor
EDTA	-	Ethylenediaminetetraacetic Acid
EGF	-	Epidermal Growth Factor
ELISA	-	Enzyme-linked Immunosorbent Assay
ER	-	Endoplasmic Reticulum
FBG	-	Fasting Blood Glucose
FBS	-	Foetal Bovine Serum
FFA	-	Free Fatty Acid
GLP-1	-	Glucagon-like peptide-1
GSIS	-	Glucose Stimulated Insulin Secretion
GWAS	-	Genome-Wide Association Study
HAT	-	Histone Acetyltransferases
HbA1c	-	Hemoglobin A1c
HDAC	-	Histone Deacetylases
HEPES	-	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	-	High-Fat Diet
HMT	-	Histone Methyltransferase
HOMA-B	-	Homeostasis Model Assessment of β -cell Function
IACUC	-	Institutional Animal Care and Use Committee
IC	-	Immune Complexes
IF	-	Immunofluorescence
IFG	-	Impaired Fasting Glucose
IFN γ	-	Interferon Gamma

IGT	-	Impaired Glucose Tolerance
IL-1RA	-	Interleukin-1 Receptor Antagonist
IL-1 β	-	Interleukin 1 Beta
IP		Intraperitoneal
IPGTT	-	Intraperitoneal Glucose Tolerance Test
JNK	-	c-Jun N-Terminal Kinase
KCl	-	Potassium chloride
KDM	-	Lysine Demethylase
LED	-	Light Emitting Diode
LFD	-	Low-Fat Diet
LPS	-	Lipopolysaccharide
MCP1	-	Monocyte Chemoattractant Protein 1
MgCl ₂	-	Magnesium chloride
MHC	-	Major Histocompatibility Complex
MIP	-	Maximum Intensity Projection
MODY	-	Maturity Onset Diabetes of the Young
MR	-	Mannose Receptor
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
NCBI	-	National Center for Biotechnology Information
NEFA	-	Non-Esterified Fatty Acid
NOD	-	Non-Obese Diabetic
NUT	-	Nuclear Protein of the Testis
OCT	-	Optimal Cutting Temperature Compound
OGTT	-	Oral Glucose Tolerance Test

PBS	-	Phosphate Buffered Saline
PBST	-	Phosphate Buffered Saline with 0.05% Tween 20
PCA	-	Polycyanoacrylate
PFA	-	Paraformaldehyde
PLA	-	Poly(lactic Acid
PLGA	-	Poly(lactic-co-glycolic) Acid
PP	-	Pancreatic Polypeptide
PRMT	-	Protein Arginine Methylation
PVA	-	Polyvinyl Alcohol
qPCR	-	Real-Time Polymerase Chain Reaction
RBG	-	Random Blood Glucose
RIPA	-	Radioimmunoprecipitation Assay
RNA	-	Ribonucleic Acid
RNI	-	Reactive Nitrogen Intermediates
ROI	-	Reactive Oxygen Intermediates
ROS	-	Reactive Oxygen Species
RT	-	Room Temperature
SDS	-	Sodium Dodecyl Sulphate
SEM	-	Scanning Electron Microscope
SEM	-	Standard Error of Mean
SGLT2	-	Sodium-glucose cotransporter 2
SMAD7	-	Mothers Against Decapentaplegic Homolog
SPF	-	Specific Pathogen Free
SR-A1	-	Scavenger Receptor Class A1
T1DM	-	Type 1 Diabetes Mellitus

T2DM	-	Type 2 Diabetes Mellitus
TGFβ1	-	Transforming Growth Factor Beta 1
TLR	-	Toll-Like Receptor
TNF-α	-	Tumour Necrosis Factor Alpha
TZD	-	Thiazolidinediones
WHO	-	World Health Organization
WST-8	-	2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt

INTRODUCTION

1.1 Prevalence of diabetes

The battle against the rising incidence of diabetes has been in spotlight over many decades, but yet the disease remains as a leading cause of impairment to daily living and death all over the world. A total of 1 in every 11 adults in the world have been estimated to have diabetes in 2015 alone [1]. The World Health Organization (WHO) has reported 422 million cases of diabetes in the world in 2014, which is approximately a 4-fold increase within a span of 34 years [2]. Despite extensive research and developments, diabetes mortality rates remain high with an estimation of the disease becoming the 7th leading cause of death by 2030 [3]. Out of the total diabetic population in the world, 90% suffer from T2DM [4]. The American diabetes association (ADA) lists numerous diabetic complications including nephropathy, neuropathy, retinopathy, stroke, cardiovascular disease and hypertension [5], rendering it as a complex disease with a dire need for immediate attention. Singapore is no exception. With gaining affluence amongst its citizens, Singapore is rapidly becoming a nation with the highest incidence of diabetes in the developed world. There are currently more than 400,000 diabetics in Singapore out of a population of 5.7 million people [6, 7]. More than 90% of diabetics in Singapore suffer from T2DM. One in every three persons, aged 65 and above, residing in Singapore suffers from diabetes. If left unchecked, the number of diabetics in Singapore is expected to rise to 1 million by the year 2050 [6]. It is also fathomable that there is a sizeable number of pre-diabetics (slightly elevated fasting glycemia) in Singapore who are undiagnosed. Diabetes has been known as an invisible disease because the hard-to-discern pre-diabetes phase can occur up to 10 years before the onset of T2DM.

1.2 Pathophysiology of T2DM

The regulation of glucose in blood is controlled by a feedback machinery involving pancreatic β -cells and the insulin-sensitive tissues, namely the liver, muscle and adipose tissue. Upon consumption of food, the carbohydrates are broken down to glucose and absorbed from the gastrointestinal tract into the blood stream. The glucose is then absorbed by the insulin-sensitive tissues with the help of insulin secreted by β -cells, to be metabolized to release energy or to be stored as a reserve in the form of glycogen. The degree of insulin sensitivity in these tissues determines the amount of insulin secretion by β -cells [8], and a balance in this process maintains the blood glucose levels within the physiological range (Figure 1.1-A). In T2DM, both insulin sensitivity in peripheral tissues and insulin secretion by β -cells are severely affected as they are tightly regulated together [9] causing chronic elevated blood glucose levels.

Three main factors contribute to T2DM, (i) Insulin resistance in peripheral tissues, (ii) defective insulin secretion from β -cells (that does not occur due to autoimmunity) and (iii) increased conversion of glycogen to glucose by the liver (hepatic glucose output) [10]. During the early stages of T2DM, the insulin sensitive tissues develop a resistance towards the available insulin due to environmental factors such as poor lifestyle (obesity) or due to genetic predisposition (a result of complex gene interactions and disorders) (Figure 1.1-B). For the latter, there are a number of T2DM susceptibility genes that have been identified through large-scale genome-wide T2DM association studies (GWAS: covered in greater detail in section 1.4), and a majority of these genes were found to be associated with β -cell function. This pathological defect characterized by a suboptimal glucose uptake by the insulin-sensitive tissues is referred to as insulin resistance. Insulin resistance is largely driven either by less sensitive insulin receptors or by reduced insulin downstream

signalling resulting in lower recruitment of glucose transporters (GLUT4) to the plasma membrane. Either of the affected pathways can be overcome when cells are exposed to higher extracellular insulin concentrations. Hence, as part of the feedback loop and in order to compensate for the increasing peripheral insulin resistance, the β -cells hypersecrete insulin (hyperinsulinemia) to maintain the fasting and post-prandial blood glucose within the normoglycemic range (Figure 1.1-B and Figure 1.1-C). This is known as the islet compensatory phase of T2DM and the compensatory phase can occur for years before the onset of full blown T2DM. Waning of β -cell function marks the decline of the compensatory phase and till date the exact reason for loss of β -cell function in T2DM remains elusive. The loss of β -cell function in T2DM has been suggested to be a result of β -cell exhaustion due to insulin resistance. Other proposed mechanisms include glucotoxicity caused by hyperglycaemia, lipotoxicity caused by enhanced circulating saturated free fatty acids (FFAs) due to loss of adipose insulin signalling, or perhaps even both glucolipotoxicity resulting in enhanced endoplasmic reticulum (ER) stress and oxidative stress pathways[11]. Once the β -cells start to lose function during insulin resistance, the blood glucose levels start to elevate (hyperglycaemia) causing impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) (Figure 1.1-B and Figure 1.1-D). IFG is the elevated blood glucose level after an overnight fast and IGT signifies the elevated blood glucose after an oral glucose tolerance test (OGTT) [12], described in section 1.3. This stage of normoglycaemia with hyperinsulinemia is known as pre-diabetes in humans; a hitherto underappreciated state of diabetic pathogenesis.

Figure 1.1. Current mode of T2DM development. Under physiological conditions, insulin-sensitive tissues, liver, muscle and adipose, take up the blood glucose with the aid of insulin. (A) Feedback between insulin sensitivity and insulin response by pancreatic β -cells to maintain normoglycaemia. (B) Fasting and post prandial blood glucose levels in response to insulin resistance and insulin response by β -cells. (C) Increased insulin secretion by β -cells to compensate for insulin resistance. (D) β -cell dysfunction due to prolonged insulin resistance. Figures adapted from [13] and [10], and modified accordingly.

1.3 Clinical presentation and diagnosis of T2DM

Increased thirst (polydipsia) and frequent urination (polyuria) are hallmark symptoms of full-blown T2DM, [14, 15]. Excess build-up of plasma glucose causes osmotic imbalance leading to increased diffusion of water from tissues into the blood stream causing dehydrated tissues resulting in increased thirst and frequent urination [14, 16]. Other major symptoms include increased hunger, weight loss and fatigue due to reduced metabolism.

The important methods used in the diagnosis of T2DM are the measurement of (i) glycated haemoglobin (HbA1c) [17], (ii) random blood glucose (RBG), (iii) fasting blood glucose (FBG), and (iv) OGTT [18]. The value ranges of the aforementioned tests for a healthy adult, pre-diabetic and T2DM patients have been described in Table 1.1.

Diagnostic test	Healthy	Pre-diabetes	T2DM
HbA1c	4.0-5.6%	5.7-6.4%	> 6.5%
RBG	< 140 mg/dl (< 7.7 mM)	140-199 mg/dl (7.8-11.0 mM)	≥ 200 mg/dl (≥ 11.1 mM)
FBG	< 100 mg/dl (< 5.5 mM)	100-125 mg/dl (5.6-6.9 mM)	> 125 mg/dl (> 7.0 mM)
OGTT	< 140 mg/dl (< 7.7 mM)	140-199 mg/dl (7.8-11.0 mM)	≥ 200 mg/dl (≥ 11.1 mM)

Table 1.1. Blood glucose levels of healthy adults and pre-diabetic and T2DM patients. The blood glucose range to diagnose pre-diabetes and T2DM are listed [14, 19-21].

1.4 Risk factors of T2DM

T2DM is a heterogenous disease which develops from the intricate interactions between environmental and genetic risk factors. Though it is unclear as to why some subjects develop only pre-diabetes and not T2DM while other subjects with pre-diabetes develop

T2DM, several key risk factors have been identified predicting the chances of both pre-diabetes and T2DM.

The major and most well-recognized risk factor of T2DM is obesity [22]. In obese individuals, increased secretion of non-esterified fatty acids (NEFAs, also known as free-fatty acids) have been associated with development of insulin resistance [23], which is reversible if the NEFA secretions are contained [24]. HFD feeding has been suggested to instigate impaired glucose tolerance and reduced glucose stimulated insulin secretion (GSIS) from β -cells in mice islets [25] and canines [26, 27]. Kahn SE has suggested a model for the effect of high dietary intake of fat on β -cells suggesting that HFD together with genetic defects can cause β -cell dysfunction involving (i) defective insulin secretion and (ii) increased amyloid deposits leading to loss of β -cells, both causing severe hyperglycaemia leading to (iii) desensitization of β -cells to glucose [28]. Overall, lifestyle changes involving consumption of HFD is a high-risk factor for the development of T2DM.

Along with environmental factors, hereditary components play a major role in development of T2DM. The risk of developing T2DM varies from individual to individual due to the genetic factors involved. It has been found that there is a 6-fold increased risk of developing T2DM if one parent has the disease, compared to the risk of developing T2DM in normal population [29].

After numerous attempts to find genetic links candidate markers for T2DM association, GWAS in humans have revealed several gene loci that predispose individuals to develop T2DM [30]. The strongest association to T2DM was found to be the polymorphisms in the gene *TCF7L2*. Genetic variations in *TCF7L2* gene are associated with impaired insulin

secretion in pancreatic β -cells [31]. In the GWAS conducted by Kooner and colleagues on T2DM susceptibility in individuals descended from South Asian population revealed six novel loci that included 2 major genes *Hnf4a* and *Grb14*. Polymorphisms in *Hnf4a* genes were associated with β -cell dysfunction adversely affecting insulin secretion [32] and *Grb14* (adapter protein) knockdown in mice was shown to enhance insulin sensitivity despite reduced insulin secretion [33]. Genetic variations in *Xrcc4*, *Manf* and *Glis3* genes have not only been associated with T2DM, but also T1DM [34]. Polymorphisms in *Xrcc4* genes are known to adversely affect *Glis3* expression, which in turn affects the expression of *Manf* and these events lead to pancreatic β -cell stress and apoptosis. In our research, we focus on the expression levels of these genes as the genetic variations and expressions are closely related to the functioning of β -cells.

Ethnicity is also an important risk factor of T2DM. It has been shown that Asians residing in western countries have twice the susceptibility to T2DM when compared to the population of European descent [35]. South Asian population have been shown to be more prone to insulin resistance than Caucasians, even at a young age [36]. The greater susceptibility of Asians to T2DM compared to Caucasians has been linked to β -cell capacity (HOMA-B scores) and on the ability of the β -cell and pancreatic islets to adapt to mounting insulin resistance [37]. The other risk factors of T2DM include age, pre-diabetes and gestational diabetes [38].

1.5 Pancreatic β -cells and the islets of Langerhans

The pancreas is made up of two major cellular components i.e. the exocrine cells and the endocrine cells. The exocrine cells (acinar cells) of the pancreas secrete enzymes through the pancreatic ducts that are required for digestion, whereas the endocrine cells secrete

hormones mainly required for regulating blood glucose levels [39]. The endocrine cells in the pancreas are organized into islands of cells known as islets of Langerhans or pancreatic islets (Figure 1.2), that are distributed throughout the pancreas. The pancreatic islets contain five types of cells that secrete different hormones, β -cells (insulin), α -cells (glucagon), δ -cells (somatostatin), ϵ -cells (ghrelin) and PP-cells (pancreatic polypeptide) [40]. The architecture of these aforementioned cells differs between humans and mice, for eg., in mice, the β -cells are mainly located at the core of the pancreatic islets with the α -cells and δ -cells are located at the periphery. Whereas in humans, the distribution of islet cells are heterogenous [41]. Also, the ratio between β -cells and α -cells in mice (60-80%) is higher than in humans (50-70%) [42].

The α -cells and β -cells produce counter-acting hormones glucagon (elevates blood glucose) and insulin (reduces blood glucose), to maintain the blood glucose to an optimal level required by the body [43]. Damage to or dysfunction of the β -cells causes chronic hyperglycaemia (elevated blood glucose) leading to diabetes. The two major types of diabetes are T1DM and T2DM. T1DM (insulin-dependent) is an autoimmune disease wherein the β -cells are destroyed by the immune system causing acute insulin deficiency; whereas in T2DM (non-insulin-dependent), obesity and genetic predisposition result in resistance of the body tissues to utilize the available insulin (Figure 1.3) [44]. In T2DM, the islets undergo several transformations in order to adapt to the insulin demand and cater the excess insulin production, known as islet remodelling.

Figure 1.2. Pancreas and structure of pancreatic islets. The pancreatic islets are islands of cells that perform endocrine function and are present throughout the pancreas. The exocrine cells (acinar cells) of the pancreas secrete enzymes for digestion and the endocrine cells, present in pancreatic islets secrete hormones to maintain glucose homeostasis. Figures modified from [45] and [46].

Figure 1.3. Mechanism of development of T1DM and T2DM. In a healthy individual, the pancreatic β -cells secrete insulin which binds to the insulin receptor on the insulin sensitive cells, to facilitate glucose uptake and maintain normal blood glucose level. In T1DM, autoimmune destruction of β -cells results in failure of insulin production and hence causing elevated blood glucose. In T2DM, due to insulin resistance in peripheral tissues, the available insulin cannot be utilized causing elevated blood glucose. Figures adapted from [47].

1.6 Role of low-grade inflammation in insulin resistance

The pathogenesis of T2DM is known to involve chronic low-level inflammation and numerous studies have shown a link between obesity, insulin resistance and inflammation [48-50]. It is important to note that this chronic low-level inflammation is different from classical inflammation caused by invading pathogens. Classical inflammation is acute, local and involves tissue injury, whereas chronic low-grade inflammation is systemic and no tissue injury is involved [51].

Chronic low-level inflammation is known to play a major role in β -cell dysfunction [50]. These inferences were primarily drawn due to increased circulating pro-inflammatory markers such as TNF α [52], C-reactive protein (CRP) [53], IL-1 β [54, 55] and IL-6 [52, 55] in patients with T2DM. Increased expression of TNF α in adipose tissue have has been known to invoke insulin resistance in T2DM [56] by suppressing insulin transmembrane signalling insulin sensitive cells [57]. Elevated levels of pro-inflammatory markers could indicate immune cell activation [58]. Evidences from animal and human models have revealed the role of immune cells such as macrophages, T-cells and B-cells [59].

Increased dietary fat and obesity has been implicated with the activation of inflammatory response through NF- κ B and JNK pathways in adipose tissues, liver and macrophages [60]. Increased adiposity also causes elevated levels of monocyte chemoattractant protein (MCP1), also known as CCL2, that recruits monocytes, dendritic cells and memory T-cells, thus recruiting these cells to adipose tissue [61]. A key receptor of MCP-1, CCR2, is a known regulator of monocyte and macrophage recruitment; and CCR2 deficient mice have been shown to be protected from insulin resistance induced by HFD and reduced

recruitment of macrophages to adipose tissue and in turn, reduced expression of pro-inflammatory cytokines [62].

Inflammation has not only been implicated in adipose tissue to cause insulin resistance but also in liver. Lipid accumulation in the hepatocytes causes the activation of inflammatory responses, both local and systemic, causing increased pro-inflammatory gene expression levels and hepatic inflammation [63]. This causes the development of insulin resistance in the hepatocytes and the resident macrophages of liver tissue, Kupffer cells, get activated. The Kupffer cells are normally present in high numbers in the liver tissue (approximately 5%), causing increased inflammatory effect on hepatic cells that what is observed in adipose tissue, as the adipose tissue macrophages are relatively lesser in number [60]. These pro-inflammatory pathways in adipose and liver tissues pave way for insulin resistance in skeletal muscles and also lead to the formation atherosclerotic plaques, causing further complications in T2DM [60].

1.7 Role of low-grade inflammation in pancreatic β -cell dysfunction

During T2D pathogenesis, the preservation of β -cell function is key to preventing overt T2D. Once lost, β -cell function and/or mass cannot be regained, thereby offering no remission [64]. For pathways involved in β -cell function, inflammation has been suggested to be a key player. As described in section 1.2, increased insulin resistance in peripheral tissues causes excessive insulin production which eventually leads to β -cell dysfunction, and this loss of function has been proven to be accompanied by the different components of inflammatory processes. Adverse effects of hyperglycaemia has been shown to include β -cell death and reduced capacity to proliferate [65]. The underlying mechanism in some studies show that the death of β -cells is due to increased expression and secretion of

IL-1 β , which in turn upregulates the Fas receptor mediated apoptosis [66, 67]. Another mechanism that has been suggested is the activation of signalling pathways by pro-inflammatory cytokines such as IL-1 β and IFN γ (interferon gamma) that elicits dysfunction of β -cells by accumulation of nitric oxide and death of β -cells by necrosis [68]. In summary, a number of inflammation-mediated mechanisms have been shown to be directly involved in β -cell dysfunction thereby contributing to T2DM progression.

In T2DM, elevated cytokine and infiltration of immune cells have also been observed in the pancreatic islets. Investigation of pancreas of all animal models of T2DM have revealed immune cell infiltration in the islets [69]. Increased blood glucose levels and prolonged exposure to glucose have been implicated in elevated metabolic activity of the pancreatic islets, causing production of reactive oxygen species (ROS). This oxidative stress triggers a series of sequentially orchestrated events to recruit immune cells such as macrophages to the islets, further leading to secretion of IL-1 β in cytotoxic levels [70].

Figure 1.4. Immune cell infiltration in adipose and pancreatic islets during T2DM progression. Increased glucose and FFAs cause a pro-inflammatory milieu in the adipose tissues causing the release of pro-inflammatory cytokines and chemokines. This leads to the recruitment of macrophages and other inflammatory cells to the adipose tissue, causing insulin resistance. The increased glucose and FFAs in the presence of insulin resistance causes reduced production of interleukin-1 receptor antagonist (IL-1RA) by β -cells causing increased IL-1 β in the islet microenvironment leading to the recruitment of macrophages and other inflammatory cells. This process of islet inflammation occurs in the decompensatory phase of β -cell function in T2DM leading to β -cell dysfunction. Figure adapted from [71].

To summarize, inflammation and immune cell infiltration are key factors contributing to the pathology of T2DM, by affecting both adipose tissue and pancreatic β -cells. In obesity, increased pro-inflammatory cytokines in the adipose tissue microenvironment causes recruitment of macrophages and T-cells (Figure 1.4) to the adipose tissue that further secrete inflammatory cytokines leading to insulin resistance. Whereas in islets, inflammation plays a detrimental role by increased IL-1 β in islet microenvironment and recruitment of macrophages and other immune cells to the islets (Figure 1.4), once the β -cells start losing their ability to compensate for the insulin demand.

1.8 Macrophages and their role in T2DM

Macrophages are important innate immune cells that are part of the body's defence mechanism against invading pathogens. Independent of the host defence function, macrophages also play a key role in tissue homeostasis [72]. They are mononuclear phagocytes [73] that digest the erythrocytes and cellular debris produced due to apoptosis (programmed cell death) and necrosis (cell death due to toxicity, hypoxia or stress), apart from pathogens [72]. Monocytes, originating from hematopoietic stem cells of the bone marrow, are the progenitors of macrophages that perform inflammatory functions [74]. Apart from this, macrophages are also distributed in all tissues of the body, known as tissue resident macrophages, that perform the tissue homeostatic functions [75, 76]. The tissue resident macrophages are suggested to be derived from three origins, (i) embryonic yolk sac [77], (ii) foetal liver [74] and (iii) monocytes derived from bone marrow [78]. Resident macrophages are given distinct nomenclature according to the organs that they reside in, such as microglia (brain), Kupffer cells (liver), alveolar macrophages (lungs), red pulp macrophages (spleen) and Langerhans cells (skin) [74].

The macrophage has been classified into two extreme phenotypes namely (i) classically activated (M1), which acts as an effector to Th1 immune response and (ii) alternatively activated (M2), which is involved in suppression of immune system and tissue repair [72, 79]. Macrophage populations are highly heterogeneous with the M1/M2 ratio being dynamic in different tissues. The regulatory systems maintain the balance between different activation states in order to prevent the immune system from leading to pathological conditions [79]. The spectrum of activated states, the population, distribution and phenotype of macrophages may vary from tissue to tissue, and also from physiological to disease states. In T2DM, it is hypothesized that increase in weight causes recruitment of monocytes to adipose tissue, thus tipping the M1/M2 macrophage balance to M1 phenotype causing adipocyte dysfunction and insulin resistance [80].

Substantial studies have been carried out to evaluate the interactions between M1 macrophages and the pancreatic β -cells. In 2012, Cucak and colleagues have demonstrated that migration of macrophages active for toll like receptor-4 (TLR-4) occurs into the islet cells in T2DM, causing enhanced cytokine secretion leading to β -cell death [81]. Another study by Eguchi and colleagues suggest that saturated fatty acid palmitate, derived from HFD, induces migration of macrophages into pancreatic islets followed by release of cytokines that are detrimental to β -cell function [82].

However, M2 macrophages are known to aid in vascularization and tissue repair by secretion of several growth factors and chemokines [83]. Xiao and colleagues observed that M2 macrophages secrete growth factors EGF and TGF β 1 resulting in the upregulation of SMAD7, which in turn stimulates β -cell replication and proliferation [83]. Tessem and co-workers have performed a study on mice with chronic pancreatitis-like syndrome to

speculate that trophic macrophages could effectively protect pancreatic islets from degeneration [84].

1.9 Role of macrophages in pancreatic islet cell remodelling

Pancreatic islet macrophages are tissue resident macrophages that are under the research limelight as the islets have surfaced as a site for sterile inflammation in the pathology of T2DM and obesity [69, 85]. The islet macrophages have varied phenotypes and several surface markers have been identified in murine models and human donors with T2DM. Some of the major markers of islet associated macrophages include CD68, F4/80, CD11b, CD11c, CD206, CD163 and Ly-6c [86]. A majority of these markers are known to be co-expressed by the macrophages in the pancreatic islets.

The earliest studies on islet macrophage phenotypes identified accumulation of CD68⁺ macrophages in both human subjects [87, 88] and rodent models [89] of T2DM. Islet associated macrophages in mice have been shown to co-express F4/80, CD11b and CD11c [90, 91], whereas those in humans are known to co-express CD11b and CD11c [92]. M1 macrophages that co-express CD11b and Ly-6c have been found to be recruited into islets exposed to the saturated fatty acid palmitate [82]. The other major co-expressed macrophage markers found in the resident islet macrophages of T2DM mouse model *db/db* at 8 weeks into the disease are F4/80 and CD68 [93]. The macrophages that were recruited to the islets at the same stage of the disease were shown to express only CD68 and not F4/80 [93]. Both these resident- and recruited islet macrophages identified expressed TLR4 and pro-inflammatory activation of these macrophages could be facilitated by lipopolysaccharide (LPS), indicating M1 phenotype. However, the islet macrophages positive for both CD68 and F4/80 have been shown to express CD206 (scavenger receptor),

which is a signature for M2 macrophages [94]. Accumulating evidences suggest that macrophages adopt pro-inflammatory phenotype within the islets in obesity and T2DM causing β -cell dysfunction, whereas the role of anti-inflammatory islet macrophages is scarcely explored.

In the pathophysiology of T2DM, we have seen that the β -cells secrete more insulin in the pre-diabetic stage to compensate for the defects in insulin uptake in the peripheral tissue and the onset of T2DM occurs when β -cells start to fail to supply enough insulin to meet the demand. With the aforementioned studies, we have also seen that pancreatic islet inflammation and islet macrophage recruitment go hand in hand to cause β -cell dysfunction during the islet decompensatory phase of T2DM, whereas the role of macrophages in early T2DM i.e. during β -cell compensation is still unclear (Figure 1.5).

Figure 1.5. Pancreatic islet macrophages during T2DM progression. During embryonic development macrophages aid in the development of pancreatic islets. At birth, resident macrophages are present within the islets. During obesity and insulin resistance, the islets enlarge due to hyperplasia and produce more insulin to compensate for insulin resistance. It is unknown, whether the macrophages help in β -cell compensation. Once β -cell start to dysfunction (decompensation), the active islet inflammation due to increased pro-inflammatory macrophages cause β -cell dysfunction. In T2DM, accumulation of pro-inflammatory macrophages is detrimental to β -cell function. Figure adapted from [86].

During the compensatory phase, the pancreatic islets undergo hyperplasia and increase in β -cell mass to facilitate excess insulin production. This compensation not only occurs during the pre-diabetic phase, but also during pregnancy [95] and partial pancreatectomy [96] in rodents. Though hyperglycaemia elicits the proliferation of β -cells, it is predicted that macrophages could play a role in supporting the growing islet. As growing β -cell mass could mean increased vascular supply, Dai and team evaluated the islet vascularization in mice models of T2DM and found that the blood vessels within the islets are dilated without increase in their density [97]. Hence, they suggest that the changes in islet vascularization during islet enlargement is largely in correlation with increased insulin demand than expanding β -cell mass [97].

In a review by Morris D.L., a key question posited was whether the islet macrophages may play a beneficial role during β -cell compensation [86]. We decided to tackle this research question by elucidating the role of islet macrophages during β -cell compensation.

1.10 Macrophage modulators

As described in section 2.8, macrophages exist as highly heterogenous populations and they can exist in a spectrum of activated states ranging from the M1 to the M2 phenotype [98]. The M1 macrophages secrete high levels of pro-inflammatory cytokines with a capacity to destroy microbes and tumour cells, whereas the M2 macrophages have low microbicidal activity, secrete anti-inflammatory cytokines, play a role in remodelling of tissues and tumour progression [99]. The macrophages are also remarkably plastic in nature as their functional phenotype varies according to the tissue microenvironment they are present in [100-102] such as liver, spleen and pancreas. Accordingly, the macrophages can

be tailored to adopt specific functional subtypes by providing the appropriate stimuli and this could have a pronounced effect on the immune response elicited by the macrophages.

Classical activation of macrophages and development of classically activated macrophages from monocytes evoke increased pro-inflammatory cytokine secretion and this M1 activation can be facilitated by exposure of macrophages to LPS and IFN γ [103, 104]. Alternative activation of macrophages elicits anti-inflammatory responses from macrophages and this M2 activation is achieved by exposing macrophages to IL-4 or IL-13 [103, 104]. The activation of macrophages to M1 and M2 are dynamic in nature and are reversible to up to an extent [105]. The M2 macrophages have been further subdivided into M2a, M2b and M2c macrophages based on the applied stimuli, cytokine expressions and functions [99, 103]. Vasiliadou and Holen, in their review, have summarized the stimuli for the polarization of macrophages, their markers and functions (Figure 1.6) [103]. Apart from these stimuli, macrophage modulation can also be brought about by epigenetic modifications, which are discussed in the next section.

Figure 1.6. Macrophage polarization stimuli, markers and function. Exposure of monocytes to distinct stimuli leads to different subsets of macrophage polarization. The two major macrophage polarizations are M1 (classically activated) and M2 (alternatively activated). M2 is further subtyped to M2a, M2b and M2c, and they perform different functions. The M1 macrophages are pro-inflammatory macrophages involved in host defence, have bactericidal properties and are cytotoxic to tumour cells. They overexpress pro-inflammatory cytokines and chemokines. M2 macrophages perform tissue homeostatic functions, are effective scavenger cells, promote angiogenesis and enable tumour progression. Figure adapted from [103].

1.11 Macrophage modulation by epigenetics and role of BET protein inhibitors

Epigenetic modifications imply the dynamic process of addition and removal of ligands on the DNA and histones without any amendments to the gene sequence [106]. These modifications occur due to environmental cues, are capable of regulating gene expressions and are heritable [106]. Epigenetic modifications are emerging as potential targets for therapeutic intervention of several diseases. Some of the epigenetic modifications are DNA methylation, histone acetylation and histone methylation [107]. Histones are positively charged proteins around which DNA is wound to exist in a compact and condensed form [108]. The addition and removal of epigenetic marks are facilitated by specific enzymes such as methyltransferases (addition of methyl group), demethylases (removal of methyl group), acetyl transferase (addition of acetyl group) and deacetylases (removal of acetyl group) [109].

Epigenetic mechanisms, mainly histone modifications, regulate gene expression levels by controlling the conversions between heterochromatin (densely packed DNA, no transcription) and euchromatin (loosely packed DNA and transcription can occur) states [110]. The most studied macrophage polarizations by epigenetic mechanisms are the post translational modifications that occur on the histones [111]. Acetylation of histones at the N-terminal lysine is one of the extensively studied epigenetic modifications [112] due to its vital role in both immunological and metabolic pathways [113]. Histone deacetylation by the action of histone deacetylase 3 (HDAC3) has been shown to cause macrophages to adopt M1 phenotype and it also arrests macrophages from adopting M2 phenotype [111], and inhibition of HDAC3 by small molecule inhibitors has been shown to impair the immune inflammatory responses of the macrophages. Another class of proteins, known as BET proteins, are known to elicit the production of pro-inflammatory cytokines (M1

phenotype) in macrophages and their inhibition using small molecule inhibitors has been shown to curtail inflammation [107, 110].

Histone modifications are dynamic processes orchestrated by “writers” (enzymes responsible for addition of modifications to histones), “erasers” (enzymes catalysing removal of modifications) and “readers” (domains recognizing the laid down modifications) (Figure 1.7) [114]. Amongst the histone readers is a major family of proteins comprising of tandem bromodomains and an extra-terminal domain is the BET protein family [115], which is comprised of bromodomain (BRD) proteins namely BRD2, BRD3, BRD4 and BRDT [115-117]. BRDs are evolutionarily conserved regions, which act as ‘epigenetic readers’ to identify the acetylation marks on histones and are responsible for converting the acetylated lysine residues signal into observable phenotypes [116]. BET proteins are also known to aid in the transcriptional initiation and elongation processes. BRD2 along with RNA polymerase 2 and other transcriptional factors forms the mediator complex that enables transcriptional initiation [118]. BRD4 is known to act as scaffold to recruit positive transcription elongation factor to the promoter regions and thus aids in transcriptional elongation [119]. It has also been proposed that BRD4 can act as histone acetyltransferase, an epigenetic writer enzyme, that adds acetyl group to histone, causing de-compaction of chromatin leading to transcriptional activation, though the molecular mechanisms remain unclear [120]. Inhibition of BET proteins has gained remarkable attention due to its ability to modulate transcription of the associated genes and hence have been shown to play a major role in attenuating the progression of cancer [117], arthritis [121], viral infections [122], T1DM [123] and other autoimmune diseases.

BET protein inhibition is facilitated by small molecule inhibitors such as I-BET151 [124], JQ1 [125] and I-BET762 [126], which compete with the amino terminal of the BRD in BET proteins, in binding with acetylation marks of histones [127]. Some of the BET protein inhibitors are already in early phases of clinical trials including I-BET762 for solid tumours, multiple myeloma, NUT midline carcinoma and haematological malignancies [122, 128]. It has been shown that pre-treatment of bone marrow-derived macrophages (BMDMs), *in vitro*, with I-BET762 followed by LPS induction significantly suppresses LPS-inducible (pro-inflammatory) genes [126]. I-BET762 has also been implicated in suppression of LPS-induced inflammation *in vivo*, in which LPS treated C57BL/6 mice were injected with I-BET762 and a significantly reduced secretion of pro-inflammatory cytokines, IL-6, IL-1 β , IFN γ and IL-12p70, was observed [126]. In a recent study, Fu and colleagues suggested that a small molecule drug - I-BET151 - may enable β - cell proliferation and regeneration in NOD mice and also, modulate pancreatic macrophages to adopt an anti-inflammatory phenotype (Figure 1.8), thus preventing the development of T1DM [123]. It has also been shown that I-BET151 increases the expression levels of genes responsible for islet regeneration, β -cell differentiation and function and genes that enhance insulin production in NOD mice [123, 129].

Though inhibition of BET proteins by I-BET151 have been implicated in immunosuppression of LPS stimulated macrophages, the effect of I-BET151 treated macrophage secreted factors on pancreatic β -cell function remains unexplored. As described in section 1.9, inflammation plays a major role in β -cell dysfunction and death during the islet decompensatory phase in the progression of T2DM. If the islet macrophages are modulated to M2 phenotype with reduced inflammatory or anti-

inflammatory phenotype during islet compensation in T2DM, the functions of the β -cells can be preserved and development of T2DM can be prevented. With the aforementioned evidence, I-BET151 could be a potential target to modulate islet macrophage phenotype during early stages of T2DM to prevent the progression of the disease.

Figure 1.7. The dynamic process of epigenetics. Epigenetic writers are enzymes that catalyse the addition of epigenetic marks on histone tails. Examples of writer enzymes are HATs, histone methyltransferases (HMTs) and protein arginine methyltransferases (PRMTs). Epigenetic readers are proteins with domains to recognize the epigenetic marks, which are recruited to the specific marks on histones. Examples of epigenetic readers are BRDs and chromodomains. Epigenetic marks are reversible and can be removed by a class of enzymes called epigenetic erasers. Classic examples of epigenetic erasers are Histone deacetylases (HDACs) and lysine demethylases (KDMs). Figure adapted from [130].

Figure 1.8. Dual effect of I-BET151 on macrophages and pancreatic β -cells. I-BET151 has been suggested to have dual role in T1DM. The drug could cause the macrophages to adopt anti-inflammatory phenotype and thus limit the recruitment of T- and B-cells to the islets and thereby reducing islet inflammation. I-BET151 is also shown to have proliferative effects on pancreatic β -cells. Together the effects of I-BET151 prevents the development of T1DM in NOD mice. Figure adapted from [131].

1.12 Controlled drug release using PLGA nanoparticles

Targeted and controlled delivery of therapeutic agents to the diseased tissues or cells has been used to enhance the effect of the drug, reduce side effects, minimize toxicity and control dosage, while protecting the healthy tissues against damage [132, 133]. In recent years, nanoparticles have contributed to a great effect in acting as carriers to deliver these

drugs at the target sites in a controlled and efficient manner. Nanoparticle based drug delivery systems offer several advantages such as improved bioavailability of drug, augmented half-life, improved solubility and high drug load [132], reinforced therapeutic efficiency [134], thus rendering them as useful drug carriers. In addition, nano-drug carriers enable re-consideration of delivering bioactive drugs that were earlier considered to be unusable for direct delivery [134].

The utilization of polymer based nanoparticles in drug delivery is attributed to their enhanced encapsulation, controlled release, high biodegradability and bioavailability and minimal toxicity [135]. Several polymeric substances such as poly(lactic co-glycolic acid) (PLGA), polylactic acid (PLA), poly(cyanoacrylate) (PCA), gelatin, alginate and chitosan have been used in synthesis of nanoparticles for drug delivery [135, 136]. Polymer based nanoparticles have been used for drug delivery to macrophages in various studies such as to enhance bactericidal properties [137, 138], to deplete macrophages in inflamed regions as in arthritis [139-141], to induce polarization to M1 phenotype to enhance immunostimulation and anti-tumour properties in cancers [142] and to use macrophages as drug carriers to regions such as tumours or brain, without intermediate effect of drug on macrophages [143, 144].

Polymeric nanoparticles have also been used to program macrophages to anti-inflammatory M2 phenotype. Tran and colleagues have reported macrophage repolarization to M2 phenotype by combining gene therapy and targeted drug delivery by delivering “plasmid DNA expressing IL-4 or IL-10 hyaluronic acid-poly(ethyleneimine) nanoparticles” [145]. Owing to the ease in surface modification of polymeric nanoparticles, they can also be used to target specific macrophage subsets for drug delivery. Delivering

I-BET151 using a polymer based nanoparticle is a potential drug delivery system that could enable controlled drug delivery without any toxic effects, if any, on the surrounding tissues.

1.13 Overarching hypothesis

Islet macrophages could aid in islet remodelling during compensatory phase of early T2DM.

Figure 1.9. Overarching hypothesis. Illustration of how islet macrophages may aid in islet remodelling and may mitigate the progression of T2DM.

1.14 Specific aims

In this research, we aim to

1. Develop a mouse model to recapitulate islet compensation in pre-diabetes
2. Study the effect of macrophage depletion on islet compensation
3. Determine the role of I-BET151, a novel modulator of macrophage function, on pancreatic islet and β-cell physiology

MATERIALS AND METHODS

2.1 Materials

2.1.1 Animal studies

Male C57BL/6 mice were purchased from InVivos, Singapore. Male mice were chosen for this study as the estrous cycle in female mice may interfere with metabolic studies. They were maintained under 12h light and dark cycle in either a specific pathogen free (SPF) environment in the Biological Resource Centre (BRC), A*STAR or in a non-SPF Animal Research Facility (ARF) at the Lee Kong Chian School of Medicine (LKCMedicine), Nanyang Technological University (NTU), Singapore. Female CD-169 transgenic mice generated and maintained at the NTU Singapore animal facility [146] were a kind gift from Assoc. Prof. Christiana Ruedl (School of Biological Sciences, NTU Singapore). All experiments were conducted according to the ethical guidelines of Institutional Animal Care and Use Committee (IACUC# 140905 & ARF-SBS/NIE-A0373). The mice had *ad libitum* access to food and water at all times. Mice were fed with regular chow diet (Altromin 1324 mod., 18% protein, 6% fat, Germany) unless otherwise indicated.

2.1.2 Specialized animal diets

To induce diabetes, specialised animal diets were procured (Research Diets Inc, NJ, USA). Mice were either fed with high-fat diet (#D12492) or the calorie-matched control low-fat diet (#D12450J) accordingly. High-fat diet containing 60 kcal% fat, 20 kcal% carbohydrate and 20 kcal% protein and low-fat diet containing 10 kcal% fat, 70 kcal% carbohydrate and 20 kcal% protein, were stored at -80°C and thawed to 4°C, for at least 24h before use. Diets were provided *ad libitum*.

2.1.3 Paraformaldehyde (PFA) solution (4%)

An aqueous solution of 32% PFA (Electron Microscopy Sciences, USA) was diluted to 4% PFA solution using 1x PBS in a fume hood and stored as 10 ml aliquots at -20°C until required.

2.1.4 List of antibodies

Antibody	Supplier
Guinea Pig Anti-Mouse Insulin	Dako, Agilent technologies, USA
Rat Anti-Mouse F4/80	Bio-Rad, USA
Rat Anti-Mouse CD68	Bio-Rad, USA
Goat Anti-Mouse/Rat CD31/PECAM-1	R&D Systems, USA
Alexa Fluor 568 Goat Anti-Guinea Pig	Life Technologies, USA
Alexa Fluor 647 Donkey Anti-Guinea Pig	Life Technologies, USA
Alexa Fluor 488 Goat Anti-Rat	Life Technologies, USA
Alexa Fluor 568 Donkey Anti-Goat	Life Technologies, USA
DAPI	Thermo Fisher Scientific, USA

Table 2.1. List of antibodies used in this study. This table summarizes the list of primary and secondary antibodies used for immunofluorescence (IF) staining of mouse tissue sections.

2.1.5 Cell culture

All cells were cultured at 37°C in a humidified incubator supplemented with 5% CO₂. The cell lines used in this research are RAW 264.7 (mouse macrophage cell line), INS-1E (rat pancreatic β -cell line), MIN6 (mouse pancreatic β -cell line) and Endoc- β H1 (human pancreatic β -cell line). All the cell culture media in this section were filtered after preparation using 500 ml sterile disposable membrane filter (Corning, USA).

RAW 264.7 cells were purchased from ATCC and were used at passage numbers ranging from 10 to 19. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) - high glucose (Nacalai Tesque, Japan), supplemented with 10% foetal bovine serum (FBS) (Gibco, USA) and 100 U/ml Penicillin-100 µg/ml streptomycin (Gibco, USA). MIN6 cells were purchased from ATCC and were used at passage numbers ranging from 33 to 42. MIN6 cells were cultured in RAW 264.7 media with an additional supplementation of 0.1% β-mercaptoethanol. INS-1E cells were kindly provided by Dr. Claes B. Wollheim, University Medical Center, Switzerland [147], and were used at passage numbers between 87 and 96. The cells were cultured in RPMI 1640 (Nacalai Tesque, Japan) supplemented with 5% FBS, 0.01% β-mercaptoethanol, 1% 1M HEPES (1st BASE, Singapore) and 100 U/ml Penicillin-100 µg/ml streptomycin.

Endoc-βH1 cells (EndoCells, France) and were used at passage numbers from 12 to 24. Endoc-βH1 cells were cultured in DMEM-low glucose (Gibco, USA) supplemented with 0.02 g/ml BSA fraction V (Sigma, USA), 50 µM β-mercaptoethanol, 10 mM nicotinamide (Sigma, USA), 5.5 µg/ml transferrin (Roche, Germany), 6.7 ng/ml selenite (Sigma, USA) and 100 U/ml Penicillin-100 µg/ml streptomycin. The culture flasks or well plates were coated with a solution of EndoC- βH1 media containing 1:100 Matrigel (Sigma, USA) and 2 µg/mL fibronectin (Sigma, USA) overnight at 37°C, prior to seeding of the cells.

2.1.6 Primer design

All primers were designed to be intron spanning to avoid amplification of genomic DNA, except for single exon genes. Primer design was carried out primarily using Universal Probe Library Assay Design Center (Roche Diagnostics Asia Pacific Pte Ltd., Singapore). If the primers of the required genes were not available in the aforementioned website,

Primer-BLAST (Primer designing tool, NCBI, USA) was used. Primer-BLAST was performed for all primer sets against the corresponding organism's whole mRNA and only those primer sets that produced a single 100% match were selected so as to ensure that false regions in the mRNA are not amplified. All primers were purchased from Integrated DNA Technologies Pte. Ltd., Singapore, in LabReady (100 μ M, pH 8.0) form. The forward and reverse primers were diluted into a single solution of RNase-free water at a final concentration of 1 μ M and stored at -20°C until further use.

2.1.7 List of primer sequences used for qPCR

2.1.7.1 Primer sequences for mouse-specific qPCR

Genes	Forward Primer	Reverse Primer
Tnf-α	GGTCCCCAAAGGGATGAG	TGGGCTACAGGCTTGTCCT
Il-1β	CAGGCAGGCAGTATCACTCA	GTCACAGAGGATGGGCTCTT
Ccl2	CATCCACGTGTTGGCTCA	GATCATCTTGCTGGTGAATGAGT
Nf-κB	ATGGCAGACGATGATCCCTAC	CGGAATCGAAATCCCCTCTGTT
Vegf-b	GAAGCCAGACAGGGTTGCC	GATGGATGATGTCAGCTGGGG
Arg1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTGATTAGGGACATC
β-actin	TGTGATGGTGGGAATGGGTCAG	TTTGATGTCACGCACGATTTCC
Gck1	CTGGATGACAGAGCCAGGAT	GCTGGAACCTCTGCCAGGAT
Ins1	CAGCAAGCAGGTCATTGTTTCAAC	TGGGTGGGTTTGGGCTCC
Ins2	CCATCAGCAAGCAGGAAGCCTATC	CCCCACACACCAGGTAGAGAGCC
MafA	GAGGTCATCCGACTGAAAC	CACTTCTCGCTCTCCAGA
NeuroD1	AACAACAGGAAGTGGAAC	TTTCTTGTCTGCCTCGTG
Pdx1	GAAATCCACCAAAGCTCAC	AAGAATTCCTTCTCCAGCTC
Glut2	GGATGAAGAGGAGACTGAAG	CATTGCTTTGATCCTTCCAAG
Tcf7l2	AACGATTGAACACCAGATTGC	CCTTGAGGGTTTGTCTGCTC
Grb14	GCCTGCGATAACACCAATAGT	CAAACATCTCGGGCTGTGA
Hnf4α	CAGCAATGGACAGATGTGTGA	TGGTGATGGCTGTGGAGTC
Glis3	AGCATGCAAGCAGATCAGTC	CCTGTAGCTCTGAGACCACT
Xrcc4	AAATGGCTCCACAGGAGTTG	TGTTTTTCAGCAGAGGTGCTC
Manf	CTCAGTGGGGAAACTTTGCT	TTAGCAGTTGCTCCTTTCTCATT

Table 2.2. Mouse primers for qPCR. This table summarizes the mouse β -cell specific genes and T2DM susceptibility genes intron-spanning primers used for quantitative real time PCR experiments.

2.1.7.2 Primer sequences for rat-specific qPCR

Genes	Forward Primer	Reverse Primer
β-actin	GCCTTCCTTCCTGGGTATGG	ACGCAGCTCAGTAACAGTCC
Gck1	ACTCAGCCAGACAGTCCTCAC	GTCTCCGACTTCTGAGCCTTCT
Ins1	TCAGCAAGCAGGTCATTGTTC	GGGTCCTCCACTTCACGAC
Ins2	GTGACCAGCTACAGTCGGAA	GCTTCCACCAAGTGAGAACCA
MafA	ACTTCTTTCTGTGAGCGCGG	GGCCTCAGAGTCCGAACCG
NeuroD1	AACAACAGGAAGTGGAACATGAC	AGACGAGGTCTGGGCTTTTG
Pdx1	AGTGGGCAGGAGGTGCATAC	CGAGGTTACGGCACAATCCT
Glut2	AATTTTCATCATCGCCCTCTG	TGCAGCAATTTTCGTCAAAAG
Tcf7l2	AACGATTGAACACCAGATTGC	GGGCTTGTCTGCTCTGGA
Grb14	GAAGCTCAGCAGTTGATTACCC	CTCTGACTATCCCGTACCAAGAA
Hnf4α	AGCAATGGACAGATGTGTGAGT	AGATCCAGAGCCACTTGGTG
Glis3	AATTTTCATCATCGCCCTCTG	TGCAGCAATTTTCGTCAAAAG
Xrcc4	TCAGCCTGGACTGCAAAAGT	GTGCCCGTCTCAGCTCAT
Manf	GCAAAGAAAATCGGTTGTGC	CTTCTCCACAGGGATATGGTG

Table 2.3. Rat primers for qPCR. This table summarizes the rat β -cell specific genes and T2DM susceptibility genes intron-spanning primers used for quantitative real time PCR experiments.

2.1.7.3 Primer sequences for human-specific qPCR

Genes	Forward Primer	Reverse Primer
β-actin	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG
Gck1	AGATGCTGGACGACAGAGC	TGCCAGGATCTGCTCTACCT
Ins	AGGCTTCTTCTACACACCCAAG	CACAATGCCACGCTTCTG
MafA	AGAGCGAGAAGTGCCAACTC	TTGTACAGGTCCCGCTCTTT
NeuroD1	CTGCTCAGGACCTACTAACAACAA	GTCCAGCTTGGAGGACCTT
Pdx1	CCTTTCCCATGGATGAAGTC	TTCAACATGACAGCCAGCTC
Glut2	CCCTGTCTGTATCCAGCTTTG	TGTTTGCTACTAACATGGCTTTG
Tcf7l2	CTTACCAGCCGACGTAGACC	CTAGCGGATGGGGGATTTGT
Grb14	ATGGTGAAATATCCCCCACA	TCGCTGAAAACTGCAAATG
Hnf4α	TGACAACCTGTTGCAGGAGA	CGTTGGTCCCATATGTTCC
Xrcc4	CTTGGGACAGAACCTAAAATGG	GACGTCTCAGGTAGTGAAGAATCA
Manf	TGCACGGACCGATTTGTAGT	GAAAGCTCCAGGCTTCACA

Table 2.4. Human primers for qPCR. This table summarizes the human β -cell specific genes and T2DM susceptibility genes intron-spanning primers used for quantitative real time PCR experiments.

2.1.8 RIPA buffer

EDTA-free RIPA buffer was prepared for total protein isolation from cells. RIPA buffer was prepared in MilliQ water by adding 50 mM of Tris (pH 7.5) (1st BASE, Singapore), 150 mM NaCl (Merck, USA), 1% IGEPAL[®] CA-630 (Sigma, USA), 0.5% Sodium Deoxycholate (Sigma, USA) and 0.1% SDS (Sigma, USA). The buffer was stored at 4°C until further use. Protease Inhibitor Cocktail (Nacalai Tesque, Japan) and 1x PhosSTOP[™] (Roche, Germany) were freshly added to a final dilution of 1x at the time of the experiment to prevent proteolysis.

2.1.9 GSIS buffer

GSIS buffer is a high calcium concentration buffer (Ca-10) that is routinely used for evaluating glucose-induced insulin secretion (GSIS) [148]. Ca-10 buffer was prepared in distilled water by adding 125 mM Sodium Chloride (NaCl) (Merck, USA), 5.9 mM Potassium Chloride (KCl) (Merck, USA), 1.28 mM Calcium Chloride Dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) (Sigma, USA), 1.2 mM Magnesium Chloride Hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) (Sigma, USA) and 25 mM HEPES (1st BASE, Singapore). The pH was adjusted to 7.4 with 1M NaOH (Merck, USA). The solution was stored at room temperature (RT) until further use.

2.1.9.1 1.5G Buffer

To the required volume of GSIS Buffer, 0.2% BSA, Fraction V (Sigma, USA) and 1.5 mM D+(-) Glucose (Sigma, USA) were added. The buffer was then filtered using sterile 0.22 μm Sartorius™ Minisart™ NML Syringe Filters (Fisher Scientific, USA) and stored at 4°C until further use.

2.1.9.2 16.7G Buffer

To the required volume of GSIS Buffer, 0.2% BSA, Fraction V (Sigma, USA) and 16.7 mM D+(-) Glucose (Sigma, USA) were added. The buffer was then filtered using sterile 0.22 μm Sartorius™ Minisart™ NML Syringe Filters (Fisher Scientific, USA) and stored at 4°C until further use.

2.1.9.3 25mM KCl in Ca-10

A solution of 25 mM KCl was added to 1.5G buffer (Section 2.1.9.1) before filtration. The buffer was then filtered using sterile 0.22 µm Sartorius™ Minisart™ NML Syringe Filters (Fisher Scientific, USA) and stored at 4°C until further use.

2.1.10 PLGA nanoparticles

PLGA (50:50) (IV: 1.18) were purchased from Corbion (Netherlands). Hydrochloric acid (HCl) solution (37% v/v Fuming), Tween-20, Tween-80, Span-80 and polyvinyl alcohol (PVA) (Molecular weight 30-70 kDa) were purchased from Sigma-Aldrich (Steinheim, Switzerland).

2.2 Methods

2.2.1 Intraperitoneal glucose tolerance test (IPGTT)

The mice were placed in fresh cages and were fasted for 6h prior to the start of the experiment. Blood samples obtained by tail snipping method were used to measure blood glucose levels using blood glucose meter (Accu-Chek Performa, Roche Diabetes Care, Inc., Switzerland) at 0 min (FBG) according to the manufacturer's instructions. An intraperitoneal (IP) injection of 20% wt/v D-(+)-glucose (Sigma, USA) prepared in Milli-Q water (2 g/ kg body weight of mouse) was administered. Blood glucose levels were subsequently monitored at 7.5, 15, 30, 60, 90 and 120 min.

2.2.2 Blood collection for plasma insulin

The mice placed in fresh cages and were fasted for 6h prior to the start of the experiment. Blood samples (approximately 40 µl) were collected by tail snipping method in sodium-

heparinized capillary tubes (Marienfeld, Germany) for plasma insulin measurement at time-point of 0 min and stored on ice. An IP injection of glucose 2g /kg body weight. Blood samples from the tail vein were collected at 7.5 and 15 min time-points after glucose administration and stored on ice. Plasma samples were collected by centrifuging the blood samples at 2000 rpm for 5 min at RT. The samples were stored at -80°C, prior to insulin measurements.

2.2.3 Plasma insulin ELISA

The plasma samples were measured using the Mercodia Mouse Insulin ELISA kit (Mercodia, Sweden) according to the manufacturer's protocol. Briefly, samples were thawed on ice for 30 mins before applying a two-fold dilution using the 'calibrator 0' provided by the manufacturer. Samples and known standards were then pipetted in a clear 96-well plate that has been coated with the antibody specific for mouse insulin. Following incubation, washing and detection steps, the absorbance at 450 nm was determined after subjecting the plate to orbital shaking for 10s in Synergy™ HTX Multi-Mode Microplate Reader (BioTek Instruments, USA). Insulin concentration was determined from a logarithmic graph constructed from the readings from the known standards.

2.2.4 Insulin tolerance test

The mice were placed in freshly prepared cages and fasted for 4h prior to the start of the experiment. Blood samples obtained by tail snipping method were used to measure blood glucose levels using blood glucose meter (Accu-Chek Performa, Roche Diabetes Care, Inc., Switzerland) at 0 min (FBG) according to the manufacturer's instructions. An IP injection of insulin (Eli Lilly And Company, USA) prepared in 1x PBS was administered

at a dose of 1 U/kg body weight. Blood glucose levels were monitored at 7.5, 15, 30, 60, 90 and 120 min.

2.2.5 Tissue fixation

The mice were culled at the required time-point and laparotomy was performed. Pancreas followed by the liver were harvested and fixed in 4% PFA solution (Section 2.1.3) at 4°C for 24h. The fixed tissues were transferred to 30% sucrose (Kanto Chemical Co., Inc., Japan) solution and stored at 4°C for 24h or until the tissues sank. The tissues were then embedded in Optimal Cutting Temperature compound (OCT) (Leica Biosystems, Germany) and stored at -80°C.

2.2.6 Tissue sectioning

The frozen tissue embedded in OCT were thawed to -20°C for sectioning. The tissues were sectioned to 10 µm thickness using a Leica CM 3050 S research cryostat (Leica Biosystems, Wetzlar, Germany) at a temperature of -20°C. The obtained tissue sections were placed on positively charged microscope slides (Fisher Scientific, USA) and stored at -80°C until further use.

2.2.7 Tissue immunofluorescence staining

The tissue sections were cooled to RT for 1h to evaporate the water droplets. The slides were rinsed thrice with 0.05% Tween (Sigma, USA) in PBS (PBST) for 5 min/wash. Subsequently, blocking was carried out at RT for 1h in 100-200 µl of blocking solution containing 5% FBS (Gibco, USA) and 2% BSA (Sigma, USA) in PBST. A water repellent outline was drawn enclosing the tissue sections using a PAP marker (Abcam, UK) to pool the antibodies on the tissues. The primary antibodies

(1° Ab) were diluted to the required concentration in blocking solution and was added over the tissue sections. The slides were covered with parafilm to ensure even spreading of the 1° Ab. The slides were then placed in a slide staining tray containing a wet tissue to retain moisture, protected from light and were incubated overnight at 4°C. The slides were washed 3 times with PBST for 5 min/wash. This step was followed by the addition of required concentration of secondary antibodies (2° Ab) solution prepared in blocking solution and incubation at RT for 1h. The slides were washed 3 times in PBST for 5 min/wash. The region surrounding the tissue sections and the PAP outline were wiped clean with lint-free tissues. A drop of Vectashield mounting medium (Vector Laboratories, Inc., USA) was added on the tissue section and covered with coverslip (Marienfeld, Germany). The slides were stored in the dark at 4°C until imaged.

2.2.8 Confocal microscopy

The IF stained slides were imaged using LSM800 with Airyscan inverted confocal microscope (Zeiss, Germany) using 63x Plan Apochromat/NA 1.4 oil objective lens at 0.5x digital magnification. The images were acquired using lasers of wavelengths: 405 nm, 488 nm, 561 nm and 640 nm according to the experiment protocol, using Zen (blue) 2.3 software (Zeiss, Germany). z-stack images were obtained manually at 1 µm interval between the slices and maximum intensity projection (MIP) images were created using Zen (blue) 2.3 software.

2.2.9 Image analysis

All confocal images were analysed and required quantification was carried out using open source image processing software Fiji 2.0, (National Institutes of Health, MD, USA). Cell counter plugin in Fiji 2.0 was used to quantify the number of cells by counting the number

of DAPI nuclear stain in a given image. The free hand selection tool was used to mark the outline of islets according to the insulin stain and area was measured using measurement option. The obtained data was graphically represented using Prism 7.0c (GraphPad, USA) software.

2.2.10 RNA isolation from cell lines

RNA isolation from cell lines was carried out using RNeasy[®] Plus Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Briefly, the cells were washed with 1x PBS, followed by lysis using 350 µl of buffer RLT from the kit containing 1% β-mercaptoethanol. The lysed cells were stored at -80°C, according to manufacturer's protocol, until RNA isolation was carried out. The final elution of RNA from the provided spin column was carried out using 35 µl of RNase free water. The obtained RNA samples were quantified using NanoDrop[™] One Microvolume Spectrophotometer (Thermo Scientific, USA) and converted immediately to cDNA.

2.2.11 cDNA synthesis

Out of the obtained total RNA samples, an amount of 1 µg were reverse transcribed to single stranded cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), according to the manufacturer's protocol. An amount of 700-1000 ng of total RNA was used per 20 µl reaction of cDNA synthesis. The reaction mixture was subjected to 25°C for 5 min, 37°C for 120 min and 85°C for 5 min in Veriti Thermal Cycler (Applied Biosystems, USA). The cDNA samples were stored at -20°C until further use.

2.2.12 Real-time polymerase chain reaction

Real-time polymerase chain reaction (qPCR) was carried out using cDNA to quantify the gene expression levels. The primers used for qPCR reactions are listed in Table 2.1 to Table 2.4. β -actin was used as housekeeping control for all gene expression studies. Each 10 μ l reaction mix contained 5 μ l of Fast SYBR[®] Green Master Mix (Applied Biosystems, USA), 1.5 μ l of diluted primer stock (section 2.1.6), 15-50 ng (depending on the amount purified) of total RNA converted to cDNA and required volume of RNase-free water. Initially, a master mix was prepared for each gene with all components mentioned above without the cDNA (according to the number of cDNA samples available) and mixed well. The master mix was added to MicroAmp[™] Fast Optical 96-Well Reaction Plate with Barcode (0.1 mL) (Thermo Fisher Scientific, USA) followed by addition of required volume of cDNA. The plate was sealed with MicroAmp[™] Optical Adhesive Film (Thermo Fisher Scientific, USA) and the contents were spun down using MPS 1000 Mini PCR Plate Spinner (Labnet International, USA). The qPCR reaction was run in QuantStudio[™] 6 Flex Real-Time PCR System and the Ct values were obtained using QuantStudio[™] Software V1.3. The Ct values were exported to Microsoft Excel for analysis.

2.2.13 qPCR analysis

The relative gene expression levels were determined using comparative Ct method, also known as $2^{-(\Delta\Delta Ct)}$ method [149]. Briefly, The Ct value of β -actin was subtracted the gene of interest in a given sample to obtain ΔCt value. The ΔCt value of the control or untreated sample was subtracted from the test sample to obtain $\Delta\Delta Ct$ values. This was done to normalize all the test samples with respect to internal control. The $2^{-(\Delta\Delta Ct)}$ values were then plotted to determine the relative gene expression levels in terms of fold change. To ensure

normal distribution of data for statistical analyses, $2^{-\Delta C_t}$ values of both control and test samples were used to perform required statistical tests and to determine statistical significance [150].

2.2.14 Cell viability assay

The cells were seeded in 96 well plates at a density of 10,000 cells/well and cultured for 24h unless mentioned otherwise. The cells were then subjected to treatment for a time stipulated in each experiment prior to cell viability determination in 100 μ l culture media/well. Cell viability was determined using Cell Counting Kit-8 (CCK-8, Dojindo, Japan) and carried out according to the manufacturer's instruction. Briefly, 10 μ l of CCK-8 solution containing water soluble tetrazolium salt, WST-8, was added to cells and incubated for 4h. The plate was subjected to orbital shaking for 10s and read at 450 nm in Synergy™ HTX Multi-Mode Microplate Reader (BioTek Instruments, USA). DMSO treated cells were used as controls.

2.2.15 Glucose stimulated insulin secretion on cultured cells

In order to verify whether various treatments affect the insulin secretion from pancreatic β -cells in culture, GSIS was carried out. Briefly, 5×10^5 pancreatic β -cells were seeded per well in a 6-well plate and cultured for 36h, unless mentioned otherwise. Following treatments for a period indicated in each experiment, the media was aspirated, and the cells were starved in 1.5G buffer containing 1 μ M I-BET151 for 2h. The buffer was aspirated and washed once with 1x PBS to remove I-BET151 completely. The cells were then treated with 1.5G buffer (without I-BET151) for 30 min. The buffer was then collected from the cells to measure basal insulin secretion. This was followed by cell treatment with 16.7G buffer for 30 min and the buffer was collected to measure the insulin secretion upon high

glucose treatment. The cells were washed with 1x PBS, followed cell lysis using 150 μ l of RIPA buffer (section 2.1.8) per well, for protein isolation. All the samples collected were stored at -80°C, until further use. DMSO treated cells were used as control.

2.2.16 Protein isolation

The lysed cells were thawed on ice followed by sonication in a water bath sonicator for 5 min. The cells were placed on ice for 10 min and centrifuged at $8000 \times g$ for 10 min at 4°C. The supernatant containing the total protein from cells was carefully removed without dislodging the pellet. The protein samples were stored at -80°C.

2.2.17 Protein estimation

The isolated total protein samples were quantified using PierceTM BCA Protein Assay Kit, according to the manufacturer's protocol. Briefly, BSA standards ranging from 0-1500 μ g/ml were prepared by diluting the BSA stock of 2 mg/mL containing 0.9% saline and 0.05% sodium azide in RIPA buffer. The protein test samples were diluted 5-fold, and 10 μ l of the samples and standards were added to each well in a 96-well plate. The working reagent was prepared according to the manufacturer's protocol and 200 μ l was added to each well. The plate was wrapped in aluminium foil and incubated at 37°C for 30 min. After cooling to RT, the plate was subjected to orbital shaking for 10s and read at 562 nm in SynergyTM HTX Multi-Mode Microplate Reader (BioTek Instruments, USA). RIPA buffer was used as blank. The quantity of protein in test samples were obtained from the standard curve.

2.2.18 Statistical method

All data represent mean $\pm$ standard error of mean (SEM). All data were statistically analysed using Prism 7.0c (GraphPad, USA) and were deemed to be parametric in nature (through frequency histograms). Statistical analysis was determined using Student's t-test for comparison between two groups, and one-way ANOVA for multiple comparisons between different groups. A p-value of <0.05 is taken to be statistically significant.

RESULTS-I

DEVELOPMENT OF PRE-DIABETIC MOUSE MODEL

3.1 Introduction

C57BL/6J mice fed with HFD is a well-established model of T2DM and has been shown to recapitulate the diabetic phenotype with rapid impaired glucose tolerance and compensatory hyperinsulinemia [151, 152]. Chronic HFD feeding for 16 weeks to C57BL/6 mice is known to cause full-fledged obesity, T2DM and hypertension [153]. Hence, in this study, we chose 8-9 week old C57BL/6 male mice fed with 60% HFD for a relatively acute period of 21 days as a model to study the changes occurring in the pancreatic islets in very early stages of T2DM i.e. during pre-diabetes. We used 10% LFD (calorifically compensated using starch) as control. The significant advantage of using LFD control over normal chow is that the 10% LFD used in our study was calorifically higher than normal chow and closely matched to the 60% HFD (Table 1.1), thus offering a more controlled milieu for the study.

(DIO) Formulas		60 kcal % fat		10 kcal % fat Matched diet	
Product #		D12492		D12450J	
		gm%	kcal%	gm%	kcal%
Protein		26	20	19	20
Carbohydrate		26	20	67	70
Fat		35	60	4	10
Total			100		100
kcal/gm		5.24		3.85	
Ingredient		gm	kcal	gm	kcal
Casein, 80 Mesh		200	800	200	800
L-Cystine		3	12	3	12
Corn Starch		0	0	506.2	2025
Maltodextrin 10		125	500	125	500
Sucrose		68.8	275	68.8	275
Cellulose		50	0	50	0
Soybean Oil		25	225	25	225
Lard		245	2205	20	180
Mineral Mix S10026		10	0	10	0
DiCalcium Phosphate		13	0	13	0
Calcium Carbonate		5.5	0	5.5	0
Potassium Citrate, 1 H ₂ O		16.5	0	16.5	0
Vitamin Mix V10001		10	40	10	40
Choline Bitartrate		2	0	2	0
FD&C Yellow Dye #5		0	0	0.04	0
FD&C Red Dye #40		0	0	0	0
FD&C Blue Dye #1		0.05	0	0.01	0
Total		773.85	4057	1055.05	4057

Table 3.1. Comparison of diet formula between HFD and LFD. This table highlights the similarities and differences of the HFD and LFD composition and how these diets are calorifically matched. The (DIO) formula diets, 60% HFD (D12492) and calorifically matched 10% LFD (D12450J) are compared. Table adapted from [154].

Several studies have shown the mice body weight changes and β -cell function in C57BL/6J mice fed with HFD, but due to high variations in the initial parameters and design of the experiments, the results are not comparable [155]. Some studies have observed significant weight gain and hyperglycaemia just after 1-week of HFD feeding [156, 157]. In this chapter, we describe the body weight change and the metabolic changes that occur in the mice fed with HFD, with calorifically matched LFD control, during the span of 21 days i.e. on day 0, 10 and 20, to discern the effect of the acute term treatment. In order to determine whether dietary fat drives compensatory hyperinsulinemia often seen in human pre-diabetes [158], we measured FBG, fasting plasma insulin, IPGTT and glucose stimulated plasma insulin at 0, 7.5 and 15 min in both LFD-fed and HFD-fed mice on day 20. As islet remodelling has been described as critical step for compensatory hyperinsulinemia in T2DM [159], we sought to determine the effect of acute term HFD feeding on islet remodelling parameters such as islet area, number of pancreatic β -cells per islet, and islet capillary area were compared and contrasted with the control LFD group [160].

In this chapter, we summarize our findings from the aforementioned studies and the results gathered in this section first show that exposure of mice exposed to an acute HFD treatment results in compensatory hyperinsulinemia with concomitant islet remodelling.

3.2 Methods

3.2.1 Animal studies: high-fat/ low-fat diet feeding

A total of 64 male C57BL/6 mice aged 8-9 weeks were randomized into 8 groups with 8 mice each (Figure 3.1), such that the average weight of mice amongst the groups are similar. 4 groups (32 mice) were fed with 60% HFD and the remaining 4 (32 mice) were

fed with 10% LFD (diet details are mentioned in chapter 2 section 2.1.2). On days 0, 10 and 20, the body weight of the mice was measured and IPGTT was performed after a 6h fast (as elaborated in chapter 2 Section 2.2.1). The FBG, fasting plasma insulin levels and GSIS (as described in chapter 2 section 2.2.2 and 2.2.3) were also measured on day 0, 10 and 20 in mice from all groups.

One group of each of LFD and HFD mice were subsequently culled across each of the four different time-points; day 3, 7, 14 and 21, and the pancreas was removed for IF staining (Figure 3.1). The pancreas was fixed in 4% PFA for IF studies (as described in section 2.2.5).

Figure 3.1. Experimental design to determine islet changes in mice fed with HFD. Each mouse in the figure represents a group of 8 mice (8-9 week old) that were fed with HFD or LFD (control). One group of each LFD and HFD fed mice were culled on day 3, 7, 14 and 21. Body weight, IPGTT, FBG and fasting plasma insulin levels were measured on day 0, 10 and 20.

3.2.2 Immunofluorescence staining: Islet vascularization

The pancreatic tissues of low-fat and HFD fed mice were fixed, mounted and sectioned at 10 μm thickness (as elaborated in chapter 2 section 2.2.6) and observed under Leica DM 1000 LED microscope (Leica Microsystems, Germany) without any staining (Figure 3.2) to first ensure that at least 3 islets were visible per pancreatic tissue obtained from each mouse. The pancreatic sections from mice of all 4 groups were stored at -80°C until further use. The sections were thawed and subjected to IF staining (elaborated in section 2.2.7). The pancreatic tissue sections were then stained with guinea pig anti-mouse insulin (1:400) and goat anti-mouse CD31 (1:200) primary antibodies (details of the antibodies are described in chapter 2 section 2.1.4). The secondary antibody cocktail was prepared using Alexa Fluor 647 Donkey Anti-Guinea Pig antibody (1:400), Alexa Fluor 568 Donkey Anti-Goat antibody (1:400) and DAPI (1:3000).

Figure 3.2. Unstained mouse pancreatic islet. The mice pancreatic tissues were sectioned at 10 μm thickness and observed under Leica DM1000 LED microscope to confirm the presence of islets before IF staining. Scale bar represents 200 pixels equivalent to 20 μm .

3.3 Results

3.3.1 Effect of acute-term high-fat and low-fat diet feeding on metabolic profiles of C57BL/6 mice

The first aim is to establish a mouse model that recapitulates the early stages of T2DM pathogenesis where β -cell insulin secretion is heightened to compensate for increasing peripheral insulin resistance. We sought to determine whether an acute period of HFD feeding will mimic the metabolic changes seen during this compensatory phase and pre-diabetes. To evaluate this, it is essential that changes in body weight and glucose excursion profiles be observed for the HFD fed and the LFD fed (control) mice. Hence, the body weight and IPGTT were measured on days 0, 10 and 20 to determine the effect of acute

term (21 days) HFD feeding on C57BL/6 mice. Though there is a trend of increasing body weight in both LFD and HFD groups of mice across the study period, we found a significant increase in HFD fed mice weight but not in the LFD fed mice weight on day 20 compared to day 0 (Figure 3.3). Though slightly higher, the weight of HFD-fed mice was not statistically different from the LFD-fed control at day 20.

Figure 3.3. Body weight changes upon acute-term of HFD feeding. C57BL/6J mice were fed with 60% HFD for on acute period of 21 days. The body weights were measured on day 0, 10 and 20. The control mice were fed with 10% LFD. $n=8$, data represents mean $\pm$ SEM. ##p-value was calculated using one-way ANOVA with Tukey test as post hoc analysis. ## $p \leq 0.01$ vs day 0.

In order to determine the impact of short term high-fat feeding on glucose homeostasis, mice were subjected to an IPGTT. We found no difference in glucose excursion profile of HFD and LFD fed mice on day 0 (Figure 3.4-A), but at day 10 (Figure 3.4-B) and at day 20 (Figure 3.4-C) the HFD fed mice profiles were significantly higher than that of LFD fed mice. The FBG was measured after 6h of fasting on days 0, 10 and 20, and we noticed a

significantly higher value in HFD fed mice when compared to LFD fed mice (Figure 3.4-D). The FBG levels showed a decreasing trend in LFD fed mice and an increasing trend in HFD fed mice across the 20 days but this difference was not significant (day 20 vs day 0).

Figure 3.4. Glucose excursion profiles and FBG of HFD/LFD fed mice. C57BL/6 mice were fed with 60% HFD for on acute period of 21 days. IPGTT was carried out on days (A) 0, (B) 10 and (C) 20. (D) FBG levels on days 0, 10 and 20 are shown. The control mice were fed with 10% LFD. n=8, data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch's correction * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

The plasma insulin levels were measured in the HFD/LFD fed mice on day 0, 10 and 20 (Figure 3.5). Though there was an increasing trend of plasma insulin from day 10 to day 20 in both HFD and LFD fed mice, we found no statistical significance between the two groups.

Together the metabolic phenotype data obtained here suggests that feeding mice with HFD for a short period (acute - 21 days) induces significant weight differences, an increased glucose excursion as early as 10 days after HFD feeding, with slightly elevated FBG levels, and a trend of increasing fasting insulin levels although the latter is not statistically significant. The *in vivo* glucose stimulated insulin release was moderately but not significantly higher in the HFD mice compared to the LFD controls.

Figure 3.5. Fasting plasma insulin and GSIS of acute-term HFD/LFD fed mice. C57BL/6J mice were fed with 60% HFD for an acute term of 21 days. (A) Plasma insulin after 6h fasting was measured on days 0, 10 and 20. (B) Glucose-stimulated insulin secretion was measured before and after IP loading with 2 g D-glucose/kg on day 20. The control mice were fed with 10% LFD. n=8, data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test.

3.3.2 Increased islet area in pancreatic islets of mice fed with HFD for 21 days

As described in chapter 2 section 1.2, the pancreatic islets try to compensate for the insulin demand due to insulin resistance in peripheral tissues, by islet hyperplasia and proliferation of pancreatic β -cells. In order to observe whether such changes occur in our acute term HFD fed mice model, we measured the islet size and calculated β -cell population in at least 3 islets per animal used in the study. Interestingly, we found that the size of the pancreatic islets was increased on day 21 in HFD fed mice compared to the control LFD fed mice (Figure 3.6-A). Also, there was a significant increase in pancreatic β -cell population on day 21 of HFD fed mice than in the LFD fed mice control group (Figure 3.6-B). This could imply that upon acute term HFD feeding (21 days), the islets enlarge and β -cells proliferate to compensate for the insulin demand.

Figure 3.6. Islet size and pancreatic β -cell population changes in acute-term HFD fed mice. C57BL/6J mice were fed with 60% HFD and 10% LFD control for on acute term of 21 days. HFD and LFD mice were culled on days 3, 7, 14 and 21 and the pancreatic sections stained for insulin and DAPI. (A) The area of the islet and (B) number of pancreatic β -cells per islet (10 μ m thick section) is shown. Quantification of the data was carried out using Fiji 2.0 software. $n=8$ mice, and each data set constitutes 26-43 islets. Data represents mean $\pm$ SEM. p -values were calculated using unpaired t-test with Welch's correction * $p \leq 0.05$.

3.3.3 Effect of HFD on islet vascularization

During β -cell compensation the islets are remodelled to enhance the insulin production by islet enlargement and increased β -cell mass [161]. Vasculogenesis has been known to be a key driver of tissue remodelling [162] and there has been several reports of increased islet vasculature in obese mice [163, 164]. With this in mind, we stained the HFD and LFD fed mice pancreatic tissues against a well-established endothelial cell marker, CD31 [165, 166]. We found significant increase in the islet vasculature area of mice fed with HFD just for a span of 3 days. However, by day 7, mice fed with low fat- but high starch diet caught up with the area of islet vascularization. These results suggest that the changes in islet vascularization perhaps occur before the increase in islet area, islet macrophage numbers and β -cell proliferation. Also, there is a modest but not significant reduction in CD31 area per islet indicating that vascularization has probably decreased on day 14. However, the

increase seen on day 21 is of similar levels as seen on day 7, suggesting that this decrease in area of islet vascularization (on day 14) could be an experimental artefact or technical anomaly. To address this discrepancy, more islets could be analysed on day14.

Figure 3.7. Islet vascularization changes in acute-term HFD fed mice. C57BL/6J mice were fed with 60% HFD and 10% LFD control for an acute term of 21 days. One batch each of HFD and LFD mice were culled on days 3, 7, 14 and 21 and the pancreatic sections stained for insulin (green), CD31 endothelial cell surface marker (red) and DAPI (blue). (A) The representative images for day 3 and day 21 and (B) area of CD31+ regions is shown. $n=4$ mice, and each bar is a representative of 13-28 islets. Data represents mean $\pm$ SEM. p -values were calculated using unpaired t-test with Welch's correction $*p \leq 0.05$. Scale bars represent $20\mu\text{m}$.

3.4 Discussion

In this chapter, we have summarized our findings on the changes of mice islet microenvironment during a very short term HFD treatment of 3 weeks. We used the C57BL/6J mice fed with 60% HFD for 21 days to recapitulate human pre-diabetes. Mice fed with 10% LFD were used as control.

As predicted, we found that HFD feeding diet over a period of 21 days caused a significant weight gain, but the LFD consumption did not have a significant effect. The increased weight gain of HFD fed mice confirms that we have a functioning model. Also, we found impaired glucose tolerance in HFD fed mice on day 10 and 20 and increased fasting glucose in HFD fed mice on day 20 itself, indicating that HFD feeding has a negative effect on glucose metabolism in a very short duration. Also, we observed an increasing trend in fasting plasma insulin of both HFD and LFD mice from day 10 to day 20, although we did not observe a significant difference in GSIS during acute-term HFD feeding. As the *in vivo* GSIS is very sensitive to stress response, it is possible that statistical significance was not achieved due to the stress experienced by the mice during the course of the experiment. As it is technically difficult to measure insulin levels *in vivo*, isolating pancreatic islets and performing GSIS *ex vivo* could have been a better method to evaluate insulin secretion. Overall, these results suggest that metabolic changes start occurring at a very early stage during the progression of T2DM and targeting this stage for therapeutics may help preventing the disease.

We also observed a significant increase in islet area in HFD fed mice on day 21, which may indicate that the islets are beginning to compensate for insulin resistance by enlarging. Also, the β -cell population in HFD fed mice showed a significant increase on day 21 and

there is a distinct increase in the number of islets with higher β -cell population in HFD compared to LFD on day 21. In fact, a distinct number of islets with increased area can also be observed on day 21 corresponding to β -cell population. This may suggest that the islet size enlargement and increase in β -cell population during compensation are not uniform in all islets and some islets are more prone to remodelling than others. If HFD feeding were continued for more than 3 weeks, we probably might have seen an additional number of islets with enlarged islet area and increased β -cell population.

Our results suggest that C57BL/6 mice fed with HFD for an acute term of 21 days can be used as a model for pre-diabetes, as there is significant islet enlargement and increase in β -cell population indicating compensatory phase of T2DM. We also observed impaired glucose tolerance as early as 10 days and slightly elevated FBG. Though there was moderate increase in fasting plasma insulin and GSIS, we could not detect significant increase in insulin secretion, which could be due stressed condition of the mice. We might be able to detect the insulin upon increasing the duration of fasting from 4h to overnight. We next aim to use this pre-diabetic mouse model to study the role of macrophages in β -cell compensation.

RESULTS-II

EFFECT OF ACUTE TERM HIGH-FAT DIET FEEDING ON PANCREATIC ISLET MACROPHAGES

4.1 Introduction

As described in chapter 1 section 1.7, islet macrophages are known to play a detrimental role in pancreatic β -cell function in fully developed T2DM [71], but studies on the role of these macrophages in pre-diabetes and early T2DM is scarce. Hence, in this study we aim to elucidate the role of macrophages in islet remodelling during β -cell compensation and pre-diabetes. Once we established a model for pre-diabetes by feeding C57BL/6 mice with HFD for 21 days, we proceeded to measure the changes in macrophage population within pancreatic islets after 3, 7, 14 and 21 days of HFD feeding. In this chapter, we show the changes in islet macrophage population during islet remodelling in the β -cell compensatory phase of T2DM.

Next, we advanced to elucidate the role of macrophages during islet remodelling by ablating the macrophages using a mouse model that is genetically modified to express DTR specifically on macrophages. The DTR mouse model is a well-established model that achieves *in vivo* targeted ablation of a specific cell subset that are modified in such a way that they express human DTR on their cell surface. Once diphtheria toxin (DT) is injected into these mice, the targeted subset of cells is rapidly ablated from their system. CD169 is a surface marker present on tissue-resident macrophages that include F4/80⁺ cells [167]. CD169 marker was originally reported to be found in macrophages of bone-marrow, lymph, liver and spleen [168]. Apart from F4/80, CD169 is also known to co-express with

CD68, MHC-II, CD11b and CD11c [168]. CD169⁺ macrophages were reported to be accumulated around the periphery of pancreatic islets of pre-diabetic NOD mice [169, 170]. Due to experimental availability of CD169-DTR mice model and aforementioned literary evidences suggesting co-expression of CD169 in F4/80⁺ and CD68⁺ macrophages (present in islet), we hypothesized that islet macrophage population could be CD169⁺. Hence, we targeted the CD169⁺ macrophages for ablation using CD169-DTR mice (Figure 4.1), to study whether the ablation of islet macrophages affect islet remodelling and insulin secretion in early compensatory phase of β -cells. Based on our earlier obtained results of islet remodelling during acute HFD treatment (21 days), we chose the same diet-induced model for the macrophage depletion study. We fed the CD169-DTR mice with HFD for 21 days and injected the mice with DT every 3-4 days to maintain them in a macrophage ablated state over the duration of the experiment. We first verified the macrophage ablation in the CD169-DTR mice and control by IF staining of the pancreatic tissues against insulin, F4/80 and DAPI. Interestingly, islet macrophages present in the periphery and within the CD169-DTR⁺ mice islets were depleted when compared to control CD169-DTR⁻ mice islets. These results confirmed our hypothesis that pancreatic islet macrophages could be positive for CD169, which has not been reported thus far. We then observed whether the absence of macrophages had any effect on the islet size, β -cell population and islet vascularization. We also show the body weight changes and metabolic profiling, similar to our HFD studies using C57BL/6J mice, on days 0, 10 and 20.

Figure 4.1. Targeted Macrophage ablation using the CD169 expressing DT receptor (DTR) mice model. The DTR mice are genetically modified in such a way that a specific subset of cells is present in the human DTR on their cell surface. Upon injection of DT, this subset of cells that express CD169 are ablated. Here, targeted ablation of mouse macrophages (CD169⁺) is attained by injection of DT in CD169-DTR⁺ mouse model.

In this chapter, we summarize our findings from the aforementioned studies and we present a holistic view of the role of islet macrophages in islet remodelling during β -cell compensation. The results gathered in the previous chapter show that mice exposed to an acute HFD treatment can be used as a model for islet compensation during pre-diabetes. In this chapter we show that a part of the islet remodelling involves a significant increase in islet macrophages. Second, the results gathered here suggest that when islet macrophages are ablated, there is reduced islet remodelling as determined by measuring gross islet area and islet capillary area. Taken together, these results for the very first time show a beneficial role of islet macrophages in islet remodelling during very early stages of T2DM pathogenesis.

4.2 Methods

4.2.1 Immunofluorescence staining: Islet macrophages

The pancreatic sections obtained from the HFD and LFD fed C57BL/6 mice on days 3, 7, 14 and 21 described in chapter 3 section 3.2.1, were used to study the islet macrophage populations during pre-diabetes. The sections were thawed and subjected to immunofluorescent staining (elaborated in chapter 2 section 2.2.7). The pancreatic tissue sections were stained with guinea pig anti-mouse insulin (1:400) and rat anti-mouse F4/80 (1:400) primary antibodies (details of antibodies provided in chapter 2 section 2.1.4). Also, another set of pancreatic sections were stained with anti-insulin (1:400) and anti-CD68 (1:400) primary antibodies. For both F4/80 and CD68 macrophage staining, the secondary antibody cocktail was prepared using Alexa Fluor 568 Goat Anti-Guinea Pig antibody (1:400), Alexa Fluor 488 Goat Anti-Rat (1:400) and DAPI (1:3000).

4.2.2 Animal studies: DTR mice model

A total of 23 female C57BL/6 mice aged 8-9 weeks were used in this study, out of which 12 mice were CD169-DTR positive (DTR⁺) and 11 were DTR negative (DTR⁻) control mice. Female mice were used instead of male because of experimental availability of the mice. For metabolic studies, there are contradicting reports of gender variations affecting metabolic phenotype [171, 172]. Experiments using female mice will help elucidate whether islet macrophage dynamics during high-fat feeding is different from that of male mice. All mice were fed with 60% HFD for 21 days. A total of 20 µg/kg of DT was injected intraperitoneally every 3 days and subsequently the mice were culled to harvest pancreas and liver. The tissues were fixed in 4% PFA for IF studies.

On days 0, 10 and 20, the body weight of all the mice were measured and IPGTT was performed after a 6h fast for 6 DTR⁻ and 7 DTR⁺ mice (as described in chapter 2 section 2.2.1). The rest of the mice (5 DTR⁺ and 5 DTR⁻) were subjected to insulin tolerance tests (ITT) (as elaborated in chapter 2 section 2.2.4), which were carried out on day 0, 10 and 20 after a 4h fast, instead of IPGTT. During IPGTT, the tail vein blood was collected (as elaborated in chapter 2 section 2.2.2) for plasma insulin measurement at 0, 7.5 and 15 min. The pancreatic tissue sections were stained for insulin, F4/80 and DAPI to determine number of islet macrophages (as mentioned in section 4.2.1). The liver tissues were also obtained and stained for F4/80 and DAPI to verify macrophage ablation.

Figure 4.2. Experimental design for determining role of macrophages in islet dynamics in response to HFD. In this model, DT was injected into CD169-DTR⁺ mice fed with HFD. The control group (CD169 without DTR) was also fed with HFD and injected with DT. Each mouse in the figure represents a group of 12 DTR⁺ or 11 DTR⁻ mice that were fed with HFD for 21 days. All mice were injected with 20 µg/kg of DT every 3-4 days and were culled on day 21.

4.2.3 Immunofluorescence staining: Islet vascularization

The pancreatic tissue sections were stained with guinea pig anti-mouse insulin (1:400) and goat anti-mouse CD31 (1:200) primary antibodies (as described in section 2.2.7). The secondary antibody cocktail was prepared using Alexa Fluor 647 Donkey Anti-Guinea Pig antibody (1:400), Alexa Fluor 568 Donkey Anti-Goat antibody (1:400) and DAPI (1:3000).

4.3 Results

4.3.1 Changes in islet macrophage population in HFD fed mice

After establishing that an acute 21-day period of HFD feeding induces a form of glucose dysregulation (islet compensation) that mimics early human T2DM pathogenesis, we next proceeded to determine whether pancreatic islet macrophages were altered during the islet compensation phase. In order to observe this, we culled HFD and LFD fed mice on days 3, 7, 14 and 21 (as described in chapter 3 section 3.2.1) and stained the pancreatic sections with antibodies against macrophage surface markers (F4/80 and CD68), along with insulin. We found a significant increase in F4/80⁺ islet macrophages in HFD fed mice on day 21 when compared to day 3 and day 7 (Figure 4.3 and Figure 4.4). We also found that the islet macrophage population was significantly higher (approximately 3-fold) in HFD fed mice than in LFD fed mice on day 21. The islet macrophages in HFD fed mice on day 3 and day 7 showed no difference compared to LFD, whereas by day 14 there was a moderate (approximately 40%) but not a significant increase in macrophage population of HFD fed mice.

In order to verify the changes in islet macrophage percentages, we stained pancreata from both HFD- and LFD- fed mice with another established pan-macrophage marker CD68, which is known to be found in pancreatic islets [173]. Similar to F4/80 staining, we co-

stained with insulin antibody to locate islets, and DAPI to count number of islet cells based on nuclei stain. We found a significant increase in CD68⁺ macrophage population on day 21 HFD fed mice islets compared to LFD control (Figure 4.5). When quantified, we found a 2-fold increase in CD68⁺ macrophages in HFD islets, which was significant when compared to LFD on day 21 (Figure 4.6). We also found an increase of CD68⁺ macrophages on day 3 which was not significant. The CD68⁺ HFD mice macrophage population data corroborates with our F4/80⁺ macrophage data on day 21, thus corroborating the F4/80⁺ islet cell results obtained above.

Figure 4.3. Representative images showing increased F4/80⁺ macrophage population in HFD fed mice islets over time. C57BL/6J mice were fed with 60% HFD and 10% LFD control for on acute term of 21 days. One batch each of HFD and LFD mice were culled on days 3, 7, 14 and 21 and the pancreatic sections were stained for insulin (red), F4/80⁺ macrophages (green), DAPI (blue). n=4 mice, and each image is a representative of 10-12 islets. Scale bars represent 20μm.

Figure 4.4. Increased F4/80⁺ macrophage population in HFD fed mice islets over time. C57BL/6J mice were fed with 60% HFD and 10% LFD control for on acute term of 21 days. One batch each of HFD and LFD mice were culled on days 3, 7, 14 and 21 and the pancreatic sections were stained for insulin, F4/80⁺ macrophages and DAPI. The number of F4/80⁺ cells were manually counted using cell counter plug-in in Fiji 2.0 software. The percentage of F4/80⁺ macrophages per islet is shown. n=4 mice, and each bar is a representative of 10-12 islets. Data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch's correction ***p $\leq$ 0.001. ##p-value was calculated using one-way ANOVA using Tukey test as post hoc analysis. ##p $\leq$ 0.01 vs day 3 and day 7.

Figure 4.5. Representative images showing increased CD68⁺ macrophage population in HFD fed mice islets on day 21. C57BL/6J mice were fed with 60% HFD and 10% LFD (control) for an acute term of 21 days. One batch each of HFD and LFD mice were culled on days 3, 7, 14 and 21 and the pancreatic sections were stained for insulin (red), CD68⁺ macrophage (green), DAPI (blue). n=4 mice, and each image is a representative of 9-12 islets. Scale bars represent 20 μm.

Figure 4.6. Increased CD68⁺ macrophage population in HFD fed mice islets on day 21. C57BL/6J mice were fed with 60% HFD and 10% LFD control for on acute term of 21 days. One batch each of HFD and LFD mice were culled on days 3, 7, 14 and 21 and the pancreatic sections were stained for insulin, CD68⁺ macrophages and DAPI. The number of CD68⁺ cells were manually counted using cell counter plug-in in Fiji 2.0 software. The percentage of CD68⁺ macrophages per islet is shown. n=4 mice, and each bar is a representative of 9-12 islets. Data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch's correction *p $\leq$ 0.05.

4.3.2 Confirming the ablation of macrophages in DTR⁺ mice

As we found increased macrophage population in pancreatic islets of our pre-diabetic mouse model after 21 days of HFD feeding when compared to control, we then proceeded to examine the process of islet remodelling in pre-diabetes without these macrophages, to elucidate their role in islet compensation. For this purpose, we used CD169-DTR⁺ transgenic mice to ablate macrophages by IP injection of DT. Both DTR⁺ and DTR⁻ control mice were fed with HFD for 21 days, as macrophage population was significantly increased upon 21-days of HFD feeding in macrophage-present mice. Firstly, to verify whether the F4/80⁺ macrophages have been depleted in CD169-DTR⁺ mice, the liver sections were IF stained for insulin, F4/80 (macrophages) and DAPI (Figure 5.10). We found that more than 90% of the macrophages in liver were depleted, indicating that a majority of the macrophages present in HFD fed mice liver were positive for both F4/80 and CD169, and macrophage depletion has occurred in CD169-DTR⁺. Hence, we used the F4/80 marker to verify macrophage depletion in pancreatic islets of 21-days HFD fed CD169-DTR mice.

In the islets of CD169-DTR⁺ mice, we found a significant reduction in F4/80⁺ macrophages (approximately 5-fold lower) in DTR⁺ mice compared to DTR⁻ control mice (Figure 4.8 and Figure 4.9) and the results were statistically significant. These results suggest that 80% of the islet macrophage population in the islets in 21 days HFD fed mice are positive for both F4/80 and CD169.

Figure 4.7. Representative images of macrophage ablation in HFD fed CD169-DTR⁺ mice liver. CD169-DTR⁺ mice were fed with HFD for 21 days and injected with DT every 3-4 days to ablate macrophages. DTR⁻ mice fed with HFD were used as control. All mice were culled after 21 days and the liver sections were stained for F4/80⁺ macrophages (green) and DAPI (blue). The representative images are shown (n=4). Scale bars represent 20 μ m.

Figure 4.8. Representative images of macrophage ablation in HFD fed CD169-DTR⁺ mice islets. CD169-DTR⁺ mice were fed with HFD for 21 days and injected with DT every 3-4 days to ablate macrophages. DTR⁻ mice fed with HFD were used as control. All mice were culled after 21 days and the pancreatic sections stained for insulin (red), F4/80⁺ macrophages (green), DAPI (blue). n=5-6 mice, and each image is a representative of 26-32 islets. Scale bars represent 20 μ m.

Figure 4.9. Quantification of macrophage ablation in HFD fed CD169-DTR⁺ mice islets. CD169-DTR⁺ mice were fed with HFD for 21 days and injected with DT every 3-4 days to continuously ablate macrophages. DTR⁻ mice fed with HFD were used as control. All mice were culled after 21 days and the pancreatic sections were stained for insulin, F4/80⁺ macrophages and DAPI. The number of F4/80⁺ cells were manually counted using cell counter plug-in in Fiji 2.0 software. The percentage of F4/80⁺ macrophages per islet is shown. n=5-6 mice, and each bar is a representative of 26-32 islets. Data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch's correction ***p $\leq$ 0.001.

4.3.3 Reduced islet area in islets of macrophage depleted mice

As we found that islet size enlargement occurs just after 21 days of HFD feeding (as described in chapter 3 section 3.3.2) in C57BL/6J mice, we proceeded to explore whether macrophages play a role in it. Hence, we stained the pancreatic sections of 21 days HFD fed DTR⁺ and DTR⁻ mice with insulin, F4/80 and DAPI to verify the ablation of macrophages in DTR⁺ mice, to measure the area of the islets. With this data we observed a significant reduction in islet area of the macrophage depleted DTR⁺ mice, compared to macrophage-present mice (Figure 4.10-A). We observed a reduction in pancreatic β -cell population in macrophage depleted mice, but the data was found to be statistically not significant (Figure 4.10-B). However, the reduction in islet area corresponds to the

decrease in β -cell population per islet, and this could possibly mean that inclusion of more replicates is required reach statistical significance in β -cell population data. These results suggest that macrophages could play a key role in morphological and physiological plasticity of the islet during early β -cell compensation.

Figure 4.10. Reduced islet area and β -cell population in macrophage depleted mice. CD169-DTR⁺ mice were fed with HFD for 21 days and injected with DT every 3-4 days to ablate macrophages. DTR⁻ mice fed with HFD were used as control. All mice were culled after 21 days and the pancreatic sections were stained for insulin and DAPI. The (A) area of islets and (B) number of pancreatic β -cells per islet (10 μm thick section) were quantified. n=5-6 mice, each data set constitutes 26-32 islets. Data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch's correction **p $\leq$ 0.01.

4.3.4 Reduced islet vascularization in macrophage ablated mice

C57BL/6J mice fed with HFD showed a significant increase in CD31 area within an acute treatment span of 3 days, and also an unstable trend in islet vascularization. This suggested that the islets initially expand the endothelial cell (CD31⁺ cells) coverage within the islet, however whether this is due to hypertrophy or hyperplasia is not known (Figure 3.7). The results also suggest that increase in islet vascularization precedes increase in islet area (Figure 3.6). To understand whether islet macrophages play a role in the increase in CD31⁺ islet area, we studied the impact of macrophage ablation on 21-day HFD fed mice. In order to carry this out, the DTR⁺ macrophage ablated mice pancreatic tissues and the DTR⁻ macrophage-present mice pancreatic tissues were stained against insulin, CD31 and DAPI. We observed significant reduction in islet vascularization in macrophage depleted DTR⁺ mice, both by visualization (Figure 4.11) and quantification (Figure 4.12) of the obtained data. These results emphasize the beneficial role the macrophages may play in islet remodelling during the very early stages of T2DM.

Figure 4.11. Representative images of islet vascularization in macrophage ablated mice. CD169-DTR⁺ mice were fed with HFD for 21 days and injected with DT every 3-4 days to ablate macrophages. DTR⁻ mice fed with HFD were used as control. All mice were culled after 21 days and the pancreatic sections stained for insulin (green), CD31 endothelial cell surface marker (red) and DAPI (blue). $n=5-6$ mice, and each image is a representative of 15-25 islets. Scale bars represent 20 μm .

Figure 4.12. Reduced islet vascularization in macrophage ablated mice. CD169-DTR⁺ mice were fed with HFD for 21 days and injected with DT every 3-4 days to ablate macrophages. DTR⁻ mice fed with HFD were used as control. All mice were culled after 21 days and the pancreatic sections stained for insulin, CD31 endothelial cell surface marker and DAPI. The area of CD31⁺ cells were quantified using cell counter plug-in in Fiji 2.0 software. The area of F4/80⁺ macrophages per islet is shown. n=5-6 mice, and each data set constitutes 15-25 islets. Data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch's correction *p $\leq$ 0.05.

4.3.5 Effect of acute-term high-fat feeding on metabolic profiles of macrophage ablated mice

Next, we measured the body weights of the DTR⁺ and DTR⁻ mice fed with HFD for 21 days duration on days 0, 10 and 20. We found a significant difference in weight between DTR⁺ and DTR⁻ mice on day 20 (Figure 4.13). We observed a significant weight gain in DTR⁻ mice over time, whereas there was a significant weight reduction in DTR⁺ mice on day 20 compared to day 10. The reduction in weight could be due to depletion of macrophages in peripheral organs and adipose tissue, which is discussed later in this chapter (section 4.4).

Figure 4.13. Body weight changes of macrophage depleted mice upon acute-term of HFD feeding. CD169-DTR⁺ mice were fed with HFD for 21 days and injected with DT every 3-4 days to ablate macrophages. The body weights were measured on day 0, 10 and 20. DTR⁻ mice fed with HFD were used as control. n=9-11, data represents mean $\pm$ SEM. p-values for DTR⁻ vs DTR⁺ were calculated using unpaired t-test with Welch's correction * $p \leq 0.05$. p-values for different time point comparisons were calculated using ratio paired t-test * $p \leq 0.05$ vs day 0, *** $p \leq 0.001$ vs day 0, # $p \leq 0.05$ vs day 10.

We then measured the effect of macrophage depletion on peripheral insulin resistance by performing the insulin tolerance test (Figure 4.14). The test was carried out on days 0, 10 and 20. The glucose levels at 15 min after an insulin stress were significantly reduced in macrophage ablated mice when compared to control on both day 10 and 20. Overall, the ability of the peripheral tissues to respond to the insulin load is enhanced in the macrophage-present mice than the macrophage ablated DTR⁺ mice.

Figure 4.14. Insulin tolerance test of acute-term of HFD fed macrophage depleted mice. CD169-DTR⁺ mice were fed with HFD for 21 days and injected with DT every 3-4 days to ablate macrophages. ITT was carried out on days (A) 0, (B) 10 and (C) 20. DTR- mice fed with HFD were used as control. n=4, data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch's correction *p $\leq$ 0.05.

We then progressed to measure the fasting plasma in the macrophage ablated mice on days 0, 10 and 20. We found a significant increase in fasting plasma insulin on day 10 and 20 when compared to day 0, in both macrophage-present and absent mice fed with HFD (Figure 4.15). Upon measuring the GSIS at 7.5 and 15 min after an IP injection of 20 g/Kg body weight of D-glucose, we found a significant reduction in plasma insulin at 7.5 min in macrophage ablated mice.

Figure 4.15. Fasting plasma insulin and GSIS of acute-term HFD fed macrophage ablated mice. CD169-DTR⁺ mice were fed with HFD for 21 days and injected with DT every 3-4 days to ablate macrophages. DTR- mice fed with HFD were used as control. (A) Plasma insulin after 6h fasting was measured on days 0, 10 and 20. Each bar represents data from 5-6 mice. (B) Glucose-stimulated insulin secretion was measured before and after IP loading with 2 g D-glucose/kg on day 20. Each bar represents data from 9-10 mice. Data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch's correction * $p \leq 0.05$ and # $p < 0.05$.

We then measured the IPGTT and FBG in the DTR⁺ and DTR⁻ HFD fed mice on days 0, 10 and 20 (Figure 4.16-A to C). As the body weight profile of the DTR⁻ and DTR⁺ mice were diverging and there was significant difference in body weights on day 21, and the IP glucose injection is based on the body weight, here we normalized the blood glucose values to the body weights. Without the influence of mice body weight, we found an increased glucose excursion profile in DTR⁺ mice as early as 10 days, though the increase was not significant. On day 20, we observed significant increase in glucose excursion profile of macrophage depleted mice at 30 min after negating the influence of body weight. We then observed that even though moderate changes in FBG of HFD fed DTR⁺ mice were observed when compared to control (Figure 4.16-D), there was no statistical significance. These results suggest that impaired glucose tolerance may occur upon depletion of macrophages in pre-diabetic mice. Whether this impaired glucose tolerance is due to islet macrophage depletion or due to whole body macrophage depletion remains to be explored.

To summarize, our metabolic phenotyping of 21 days HFD fed macrophage-ablated mice showed a significant reduction in body weight compared to macrophage-present control. We found insulin intolerance in macrophage depleted mice suggesting that the presence of macrophages in pre-diabetes may reduce insulin resistance in peripheral tissues. We also observed a significant increase in fasting insulin in both macrophage- present and absent mice with a significant reduction in GSIS at 7.5 min after 20 days on HFD, indicating that presence of macrophages is essential to ensure insulin secretion during pre-diabetes.

Figure 4.16. Glucose excursion profile with respect to body weight and FBG of acute-term HFD fed macrophage depleted mice. CD169-DTR⁺ mice were fed with HFD for 21 days and injected with DT every 3-4 days to ablate macrophages. DTR⁻ mice fed with HFD were used as control. IPGTT was carried out on days (A) 0, (B) 10 and (C) 20. (D) FBG was measured on days 0, 10 and 20. DTR⁻ mice fed with HFD were used as control. $n=5-6$, data represents mean $\pm$ SEM. p -values were calculated using unpaired t -test with Welch's correction * $p \leq 0.05$.

4.4 Discussion

Macrophages have been known to have an inflammatory and detrimental effect on pancreatic β -cells during the late stages of T2DM progression [86, 174]. On the other hand, resident macrophages are known to play a protective role during normal physiological conditions to maintain islet homeostasis [174]. In our study, we wanted to explore whether islet macrophage population is altered in very early stages of T2DM, specifically during the compensatory phase of β -cells and pre-diabetes. Also, we wanted to elucidate whether macrophages played a role in islet remodelling during very early stages of T2DM. In this chapter, we have summarized our findings on the changes of mice islet microenvironment

during a very short term HFD treatment of 3 weeks. C57BL/6J mice fed with 60% HFD for 21 days served as a model to study islet remodelling during early stages of T2DM progression, and 10% LFD fed mice as control. Our results suggest that macrophages may play a key role in islet remodelling during very early stages of T2DM.

In the article published by Ehse and colleagues, it was reported that increased islet macrophage population can be observed in T2DM human subjects, 8-week HFD fed C57BL/6J mice, as well as in 8- to 9-week old db/db (diabetic) mice [88]. In our study, we observed increased macrophage population upon HFD feeding of C57BL/6J mice for an acute term of 21 days. These results suggest that recruitment of macrophages to pancreatic islets may occur in early stages of impaired glucose tolerance. During the course of our research, a study published by Woodland and colleagues suggested that the islet macrophage population may increase significantly after 7 days of HFD feeding to C57BL/6J male mice, whereas the control group used in this study was normal chow fed mice [175]. The fat content of the HFD and normal chow in their study was 21% and 5.5% respectively, whereas the diets were not calorifically matched. As we have used a better-suited calorifically matched 10% LFD control to the 60% HFD (by adjusting the carbohydrate in diet), our findings may be a better reflection of the pathophysiological state.

Next, we wanted to study the role of these macrophages during the early stages of T2DM progression. Though the role of inflammation in T2DM is widely explored, whether macrophages play a role in early stages is not clear. In order to determine the effect of macrophages on pancreatic islet remodelling, we used CD169-DTR mice model for the

macrophage ablation while feeding the mice with HFD for 21 days. We confirmed that the macrophages have been ablated by IF staining of liver tissues and pancreas.

We found a significant reduction in islet area in macrophage ablated HFD fed mice after 21 days of treatment. Though not significant, we also observed a decrease in β -cell population of macrophage ablated mice corresponding to the islet area. This indeed suggests that macrophages could play a role in islet compensation by aiding in β -cell proliferation. Also, we found a significant reduction in islet vasculature in macrophage ablated mice. Further, we observed reduced plasma insulin and impaired glucose tolerance with respect to body weight in macrophage ablated mice on day 21. Interestingly, our ITT results showed that macrophage ablation reduced the response of peripheral tissues to the supplied insulin load. Overall, these data suggest that all the classical mechanisms of islet remodelling to compensate for insulin resistance are hindered by the ablation of macrophages. Our novel findings strongly suggest that macrophages play a beneficial role in preparing the islets to resist the progression of T2DM.

A study by Bu and team have shown a significant decrease in body weight in macrophage depleted C57BL/6J mice fed with 60% HFD. The macrophage depletion in their experiment was carried out by IP injection of clodronate liposomes into these mice, with a focus on depletion of visceral adipose tissue macrophages. Their finding suggests that the weight gain due to HFD is curtailed upon macrophage depletion in the visceral adipose tissues [176]. We see a similar effect i.e. significant reduction in weight gain of the HFD fed macrophage ablated mice compared to the macrophage-present mice, which may indicate that systemic ablation of macrophages has a protective effect from HFD treatment. This in turn might imply that macrophages could play a beneficial or detrimental role in

T2DM depending on the tissues they are present in and their polarization. Targeted depletion of macrophages within the islets could shed more light on the role of macrophages specifically on islet function.

As anti-inflammatory or M2 macrophages have been shown to aid in β -cell replication and stimulating angiogenesis [86], we speculate that the increased macrophage population found in islets during acute term HFD feeding could largely represent M2 phenotype rather than M1. Thus, specific modulation of macrophages to M2 phenotype in islets in very early stages of T2DM could preserve islet function and avoid the progression of the disease. This change in phenotype, if it occurs, would be an important finding that would strengthen the work presented. However, it is beyond the scope of this thesis to test this phenotype change. In the next chapter, we explore the modulation of macrophages to anti-inflammatory phenotype and the effect of the cytokines produced on pancreatic β -cells.

RESULTS-III

MODULATION OF MACROPHAGES TO ANTI-INFLAMMATORY PHENOTYPE AND THEIR EFFECT ON PANCREATIC β -CELL FUNCTION

5.1 Introduction

Macrophages are heterogenous cells that exist in a variety of phenotypes, ranging from the classically activated M1 (pro-inflammatory) to alternatively activated M2 phenotype (anti-inflammatory) [177]. Macrophages originate either from circulatory monocytes that are recruited to sites of inflammation (monocyte-derived macrophages) [178] or originate from embryonic stage known as tissue resident macrophages that perform homeostatic functions, immune surveillance and clearance of cell debris [179]. They are highly plastic cells and their phenotypic changes depend on their micro-environment. Hence, by controlling the micro-environment of macrophages and exposing them to specific stimulants, the macrophages can be modulated to attain distinct phenotypic subsets that have unique functions [180, 181]. In the previous chapter, we have shown that islet macrophages may aid in pancreatic islet remodelling in very early stages of T2DM to help β -cells compensate for the increased insulin demand. To translate the novel finding that islet macrophages expand in early T2DM and that they are essential for islet compensation, there is a need to explore whether modulation of macrophages to anti-inflammatory phenotype can have a protective effect on the pancreatic β -cell function.

Small molecule BET protein inhibitors have recently gained wide attention in cancer research as they are proving to be effective tumour inhibitors [124, 125] and I-BET151 is

one such BET protein inhibitor that is also implicated in subduing inflammatory response in LPS stimulated BMDMs [126]. In a study by Fu and colleagues, it has been suggested that I-BET151 could play dual beneficial roles in protecting mice from developing T1DM [123]. Fu and co-workers show that I-BET151 may enable the macrophages to adopt anti-inflammatory phenotype and could facilitate proliferation of pancreatic β -cells in NOD mice, a mouse model of T1DM [123]. Hence, it is possible that if the macrophages can be modulated to a phenotype that is more anti-inflammatory, rather than inflammatory, using I-BET151 during very early stages of T2DM, detrimental effects on β -cells due to inflammatory stimuli leading to the onset of T2DM could be contained. Hence, we first decided to explore the effect of macrophage cytokine response after treatment with I-BET151 *in vitro*, and to study the effect of macrophage conditioned media on pancreatic β -cell lines.

We treated the mouse macrophage cell line RAW 264.7 with 1 μ M I-BET151, washed the cells and replaced with fresh media for up to 24h to remove the drug, and then used the collected media (rich in macrophage-releasing factors [182, 183]), labelled as conditioned media (CM), on pancreatic β -cell lines for up to 8h (Figure 4.1). We aimed to study the changes in expression of β -cell signature genes and T2DM susceptibility genes.

We used lipid A as a positive control to verify that the conditioned media of activated macrophages is able to influence the expression of genes critical for pancreatic β -cell function. Lipid A is a lipid component of the gram-negative bacterial cell wall and it is a endotoxin moiety of LPS, a well-known stimulant for macrophage pro-inflammatory activation [184]. The influence of activated macrophages by lipid A and LPS have been

well described [185-187]. After validating the utility of macrophage conditioned media on β -cell function using lipid A, we then analysed the effect of IBET-151 treated macrophages on β -cell activity. We found that I-BET151 induces a rapid increase in anti-inflammatory gene expression but that this increase is rapidly abrogated upon IBET-151 removal. As our experimental plan included a drug washout period (Lipid A or IBET-151), we had then to modify our protocol to ensure that I-BET151 is present throughout the course of the experiment.

Figure 5.1. Overall Experimental plan to study the effect of I-BET151 on macrophages and the CM effect on pancreatic β -cells. We aimed to treat mouse macrophage cell line, RAW 264.7 cells with I-BET151, wash and replace with fresh media to remove I-BET151 and use the CM to treat murine β -cells. The expression levels of β -cell signature genes and T2DM susceptibility genes were to be quantified. Due to our observation that the washing step abrogated the effect of I-BET151 on RAW 264.7 cells, few experimental changes were carried out, which are later explained in this chapter.

In our hands, the I-BET151 conditioned macrophage cell media did not induce a significant change over and above what was observed when IBET-151 was added to the β -cells directly. IBET-151 treatment to β -cells, at concentrations reported in literature to reduce inflammatory response in macrophages [188], resulted in a significant downregulation of genes in β -cells. We found significant reduction in genes associated with T2DM-linked risk alleles [34, 189] suggesting that I-BET151 can have adverse effects on β -cell function. This is the first report showing adverse effects of I-BET151 on β -cells *in vitro*. In this chapter, we describe in detail, how I-BET151, a modulator of immune response, can adversely affect β -cell function. We further investigated whether these findings are extended to other known BET protein inhibitors.

5.2 Methods

5.2.1 Activation of mouse macrophages by lipid A

RAW 264.7 cells were seeded in T-75 flask (Corning, USA) for 24h to reach 70% confluence and treated with 0.1 ng/ μ l of lipid A (Sigma, USA), overnight, to activate the macrophages. The treated medium was discarded, and cells were washed with 1x PBS to wash off lipid A. The flasks were replaced with fresh medium and incubated at 37°C for 24h. The CM was collected and centrifuged at 2000 rpm for 10 min at RT. The supernatant was collected, filtered using 0.45 μ m syringe filter to remove cells if present, and this lipid A CM was used for further experiments. The activated RAW 264.7 cells were trypsinised, washed once with 1x PBS and used for further experiments. RAW 264.7 cells without lipid A activation were used as control.

5.2.2 Verification of macrophage activation by lipid A

The RAW 264.7 cells collected after lipid A activation were subjected to cell lysis followed by RNA isolation and cDNA synthesis (As elaborated in chapter 2 Section 2.2.10 and 2.2.11). qPCR was then carried out using primers specific for genes *Tnf- α* , *Il-1 β* , *Il-6* and *Nf- κ B* (primer list is available in chapter 2 Table 2.2).

5.2.3 Treatment of β -cells with macrophage CM

MIN6 and INS1E cells were cultured in 6 well plates for 24 hr (70% confluence) followed by treatment with non-activated and lipid A activated RAW 264.7 CM. The CM was freshly obtained from the RAW 264.7 cells and directly added to β -cells without storage. Fresh RAW 264.7 media (without conditioning) was used as control. The treatment was carried out for 8h and 24h time points after which the cells were lysed. RNA isolation and cDNA synthesis were carried out. Gene expression levels were then measured by qPCR using *Gck1*, *Ins1*, *Ins2*, *MafA*, *NeuroD1*, *Pdx1* and *Glut2* gene specific mouse/rat primers (primer list available in chapter 2 Table 2.2 and Table 2.3), according to the protocol in chapter 2 section 2.2.12. Level of gene expression was normalized to that of β -actin. A schematic workflow of CM preparation from RAW 264.7 cells and treatment of β -cells is shown in Figure 5.2.

Figure 5.2. Schematic workflow of macrophage activation using lipid A and CM treatment on β-cells. Mouse macrophage cell line (RAW 264.7) when activated with lipid A releases cytokines and many other factors. The CM was then used to expose the murine β -cells, MIN6 (mouse) and INS1E (rat), to study the changes in β-cell signature genes related to glucose metabolism and insulin production.

5.2.4 Treatment of macrophages with I-BET151

RAW 264.7 cells were seeded in T-75 flask (Corning, USA) for 24h to reach 70% confluence and were treated with 1 μ M and 3 μ M I-BET151 for 6h. The treated medium was discarded, and cells were washed with 1x PBS. RNA isolation and cDNA synthesis were carried out according to (As elaborated in chapter 2 Section 2.2.10 and 2.2.11). qPCR was then carried out using *Tnf- α* , *Il-1 β* , *Nf- κ B*, *Ccl2* and *Vegf-b* genes specific primers (primer list is available in chapter 2 Table 2.2). β -actin was used as housekeeping control to normalise for expression levels of the measured genes. The housekeeping control is used because its expression level does not change across cells or conditions, and hence can be used to normalise the levels of the other genes under study.

5.2.5 Macrophage wash after treatment with I-BET151

RAW 264.7 cells were seeded in 6 well plates for 24h to reach 70% confluence and were treated with 1 μ M I-BET151 for 6h. The treated medium was discarded, and cells were washed with 1x PBS to wash off I-BET151. The wells were replaced with fresh medium and incubated at 37°C for 18-24h. The treated RAW 264.7 cells were lysed with lysis buffer and was used for RNA isolation followed by cDNA synthesis. qPCR was then carried out using *Tnf- α* , *Il-1 β* , *Nf- κ B*, *Ccl2*, *Arg1* and *Vegf-b* genes specific primers (primer list is available in chapter 2 Table 2.2). RAW 264.7 cells treated with DMSO were used as control.

5.2.6 β -cell treatment with I-BET151 treated RAW 264.7 CM

INS1E and MIN6 cells were treated with I-BET151 treated RAW 264.7 CM for 4h and 8h. Parallely, both cell lines were treated with 1 μ M I-BET151 for the same time points. The cells were washed with 1x PBS and lysed for RNA isolation followed by cDNA synthesis.

Gene expression levels were then measured by qPCR for β -cell signature genes, namely, *Gck1*, *Ins1*, *Ins2*, *MafA*, *NeuroD1*, *Pdx1* and *Glut2*, and T2DM susceptibility genes, namely, *Tcf7l2*, *Grb14*, *Hnf4a*, *Glis3*, *Xrcc4* and *Manf*. The list of mouse/rat specific primers chapter 2 Table 2.2 and Table 2.3, and qPCR was carried out according to the protocol in chapter 2 section 2.2.12. INS1E and MIN6 cells treated with DMSO treated RAW 264.7 CM or DMSO were used as respective controls. schematic workflow of CM preparation from RAW 264.7 cells and treatment of β -cells is shown in Figure 5.3.

5.2.7 INS1E cells wash after treatment with I-BET151

INS1E cells were seeded in 6 well plates for 24h to reach 70% confluence and were treated with 1 μ M I-BET151 for 4h. The treated medium was discarded, and cells were washed with 1x PBS to wash off I-BET151. The wells were replaced with fresh medium and incubated at 37°C for 6h. The treated INS1E cells were lysed with lysis buffer and was used for RNA isolation followed by cDNA synthesis. Gene expression levels were then measured by qPCR for β -cell signature genes, namely, *Gck1*, *Ins*, *MafA*, *NeuroD1*, *Pdx1* and *Glut2*, and T2DM susceptibility genes, namely, *Tcf7l2*, *Grb14*, *Hnf4a*, *Xrcc4* and *Manf*. INS1E cells treated with DMSO were used as control.

5.2.8 Human β -cell treatment with I-BET151

7×10^5 Endoc- β H1 cells (human pancreatic β -cells) were seeded per well in a 6-well plate coated with Matrigel (as described in chapter 2 section 2.1.5) and incubated for 4 days and treated with 1 μ M I-BET151 for 4h. The cells were washed with 1x PBS and lysed for RNA isolation using buffer RLT, followed by cDNA synthesis. Gene expression levels were then measured by qPCR for β -cell signature genes, namely, *Gck1*, *Ins*, *MafA*, *NeuroD1*, *Pdx1*

and *Glut2*, and T2DM susceptibility genes, namely, *Tcf7l2*, *Grb14*, *Hnf4a*, *Xrcc4* and *Manf*. Endoc-βH1 cells treated with DMSO were used as control.

5.2.9 β-cell treatment with BET protein inhibitors JQ1 and I-BET762

INS1E cells were cultured in 6 well plate for 36h (70% confluence) prior to drug treatment. The cells were treated with 1 μM JQ1 or I-BET762 for 4h. The cells were washed with 1x PBS and lysed for RNA isolation followed by cDNA synthesis. Gene expression levels were then measured by qPCR for β-cell signature genes, namely, *Gck1*, *Ins1*, *Ins2*, *MafA*, *NeuroD1*, *Pdx1* and *Glut2*, and T2DM susceptibility genes, namely, *Tcf7l2*, *Grb14*, *Hnf4a*, *Glis3*, *Xrcc4* and *Manf*. INS1E cells treated with DMSO were used as control.

5.2.10 INS1E pre-treatment with actinomycin D

INS1E cells were cultured in 6 well plate for 36h (70% confluence) and pre-treated with 5 μg/ml Actinomycin D (Sigma, USA). The cells were washed with 1x PBS and then treated with 1 μM I-BET151 for 4h. The cells were washed with 1x PBS and lysed for RNA isolation followed by cDNA synthesis. Gene expression levels were then measured by qPCR for β-cell signature genes, namely, *Gck1*, *Ins1*, *Ins2*, *MafA*, *NeuroD1*, *Pdx1* and *Glut2*, and T2DM susceptibility genes, namely, *Tcf7l2*, *Grb14*, *Hnf4a*, *Glis3*, *Xrcc4* and *Manf*. Actinomycin D pre-treated INS1E cells treated with DMSO were used as control.

Figure 5.3. Schematic workflow of macrophage exposure to I-BET151 and I-BET151 CM treatment on β-cells. Mouse macrophage cell line (RAW 264.7) was treated with I-BET151 and the CM containing released cytokines and other factors was then used to expose the murine β -cells, MIN6 (mouse) and INS1E (rat). These experiments were done to study the changes in β-cell signature genes related to glucose metabolism and insulin production, as well as T2DM susceptibility genes. As I-BET151 was present in the CM, β-cells treated directly with DMSO/I-BET151 were used as controls.

5.3 Results

5.3.1 Increased pro-inflammatory gene expression levels in RAW 264.7 cells upon exposure to lipid A

Lipid A (constituent of the interior region of LPS) being a gram negative bacterial cell wall component, is a potent stimulator of inflammatory response in macrophages [190]. Endotoxicity of LPS is enabled by the lipid A moiety [191]. Activation of macrophages by lipid A in the body causes highly elevated inflammatory cytokine production which could in turn induce organ failure due to septic shock [191]. In order to verify the response of macrophages to an external stimulus in our laboratory setting, we subjected mouse macrophage cell line RAW 264.7 cells to the well-established inducer of inflammation, lipid A. For this study, RAW 264.7 cells were treated with 0.1 ng/ μ l lipid A, overnight. We then aspirated the media and the cells were washed with once with 1x PBS and replaced with fresh RAW 264.7 cell media for 24h to subsequently deplete lipid A from the culture media following treatment. This cell culture media labelled as lipid A CM was used for further experiments.

Gene expression levels of pro-inflammatory cytokines *Tnf- α* , *Il-1 β* , *Il-6* and transcription factor potentially involved in inflammation, *Nf- κ B*, in the cells treated with lipid A were quantified by qPCR. We observed a significant increase in the pro-inflammatory cytokines, *Il-1 β* and *Il-6*, expression 24 hours following lipid A treatment (Figure 3.3). Although *Tnf- α* and *Nf- κ B* expression is observed to increase by 20- and 3-fold, respectively, the variations between experiments, perhaps due to the washout period of Lipid A, renders these changes not to be significant. The increased expression of *Tnf- α* , *Nf- κ B*, *Il-1 β* and *Il-6* after lipid A treatment corroborates past observations of THP-1 (human monocyte cell line) and RAW 264.7 cells adopting a pro-inflammatory gene

signature upon treatment with lipid A and LPS [191, 192]. Overall, the results suggest that lipid A treatment results in a gene expression signature that resembles that of activated macrophages.

Figure 5.4. Pro-inflammatory genes and transcription factor, *Nf- κ B*, expression of RAW 264.7 cells upon activation by lipid A. RAW 264.7 cells were treated with lipid A to a final concentration of 0.1 ng/ μ l, overnight. The cells were washed with 1x PBS and incubated in fresh media for 24h. The cells were then collected, and RNA extraction was carried out followed by cDNA conversion and qPCR for pro-inflammatory genes. Significantly increased expression of pro-inflammatory cytokines in treated cells ensured activation by lipid A. Data presented are representative of 3 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using 2-tailed paired t-test with *p<0.05 and ***p<0.001.

5.3.2 Reduced expression of key β -cells genes upon treatment with activated macrophage pre-conditioned media

We next used the lipid A CM to treat two widely used pancreatic β -cell lines. We used pancreatic β -cell lines MIN6 and INS1E of mouse and rat origin respectively, because of ease of culture, well-established function and because the β -cell gene expression signature remains stable over several passages. The β -cell lines were exposed to conditioned media for 8h and 24h time periods to study the β -cell signature gene expressions. The β -cell signature genes whose expression levels were quantified, were selected based on their importance of function (Table 1.1).

In both treatment time points, we found significant decrease in expression (approximately 5-fold reduction) of *MafA*, *Pdx1* and *Glut2* transcripts in MIN6 cells (Figure 3.4). *MafA* and *Pdx1* are transcription factors that activate the insulin gene by binding to the C1 element (control element in the enhancer region of the gene) [193, 194] and A3 element [194], respectively. They are also responsible for functional maintenance of islets by regulation of glucagon, somatostatin, *Glut2*, β -glucokinase and many other genes essential for islet function [194]. *Glut2* is the trans-membrane glucose transporter protein that enables β -cells to detect the blood glucose level, and hence confers functionality to β -cells [195]. The reduced expression levels of *MafA* and *Pdx1* may perhaps account for the lower expression of *Ins1* and *Ins2* genes observed in the 8h treatment, as both *MafA* and *Pdx1* act as transcription factors for the insulin gene. More importantly, this is the first report showing that conditioned media from activated macrophages have a negative impact on insulin gene expression through perhaps the downregulation of *Pdx1* and *MafA*.

Gene	Description	Alias	Function
Gck1	Glucokinase isoform 1	Hexokinase 4 (HK4), MODY2, Hexokinase D	Tissue specific expression: Pancreatic β -cells (GSIS) and liver (glucose to glycogen), catalyses first step in glucose metabolism (converts to Glu-6-P), highly specific to glucose, high glucose levels do not affect the functionality [196, 197].
Ins1	Insulin isoform 1 (only murine)	Insulin dependent diabetes mellitus (IDDM1)	Produces insulin to control blood glucose level, found only in murine cells (retrogene) [198, 199].
Ins2	Insulin isoform 2	Insulin dependent diabetes mellitus (IDDM2)	Produces insulin to control blood glucose level, found both in murine and human cells [198, 200].
MafA	V-Maf avian musculoaponeurotic Fibrosarcoma oncogene homolog A	Pancreatic β -cell specific transcriptional activator, MAF BZIP transcription factor A	Transcription factor, involved in mature β -cell phenotype, binds to insulin enhancer element RIPE3b and activates insulin gene, functions together with NeuroD1 and Pdx1, phosphorylation increases transcriptional activity (required for oncogenic activity). Acts as both oncogene and tumour suppressor: depends on cell type [201].
NeuroD1	Neuronal Differentiation isoform 1	Neurogenic differentiation factor 1, BETA2, MODY6	Transcriptional activator, involved in development of pancreas and regulation of ins gene and β -cell differentiation, mutations result in T2DM [202, 203].
Pdx1	Pancreatic and duodenal homeobox 1	Insulin Promoter factor 1 (IPF1), somatostatin-transactivating factor 1 (STF1)	“Transcriptional activator of insulin, somatostatin, glucokinase, islet amyloid polypeptide and GLUT2”. Essential for β -cell function and survival. Defects in this gene leads to IDDM [204].
Glut2	Glucose transporter isoform 2	Solute carrier family 2 member 2 (SLC2A2)	Glucose transporter protein. Transmembrane protein (low sensitivity to glucose) present in pancreatic β -cells that senses glucose the blood and aids in insulin secretion. Also present in liver [205, 206].

Table 5.1. β -cell signature gene description and functions. The details and functions of the β -cell signature genes used in this study are listed.

Similarly, we observed significant reduction in expression levels of *MafA* and *Glut2* in INSIE cell line treated with activated RAW 264.7 conditioned media for 8h (Figure 5.6-A). We also observed significant decline in expression of *Ins1* and *Ins2* in 24h treatment (Figure 5.6-B). Overall, our study suggests that lipid A activation of RAW 264.7 cells induces the release of factors (cytokines and chemokines) that in turn promote β -cell dysfunction [71, 207, 208]. The observed data also suggest that the presence of pro-inflammatory macrophages within pancreatic islets may be detrimental for β -cells, which could be due to the secreted factors from macrophages that reduce the expression of vital genes like *MafA*, *Pdx1* and *Glut2*.

Figure 5.5. β -cell signature gene expression upon treatment with lipid A activated macrophage CM in MIN6 cells. MIN6 cell line was treated with lipid A activated or non-activated macrophage conditioned media for (A) 8h and (B) 24h. Cells treated with fresh MIN6 medium were used as control. The cells were then subjected to RNA extraction followed by cDNA conversion and qPCR. Mouse β -actin was used as housekeeping control. Ct values were first normalized to β -actin and then to control. Data presented are representative of 4 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using 2-tailed paired t-test with $*p < 0.05$.

Figure 5.6. β -cell signature gene expression upon treatment with lipid A activated macrophage CM in INS1E cells. INS1E cell line was treated with lipid A activated or non-activated macrophage conditioned media for (A) 8h and (B) 24h. Cells treated with fresh INS1E medium were used as control. The cells were then subjected to RNA extraction followed by cDNA conversion and qPCR, rat β -actin was used as housekeeping control. Ct values were first normalized to β -actin and then to control. Data presented are representative of 3 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using 2 tailed paired t-test with * $p < 0.05$.

To summarize, our data agrees with the available literature that pro-inflammatory cytokines are detrimental to β -cell function [209, 210] and with these results we verify that our pre-conditioned experimental model can be used to study the indirect effect of I-BET151 on β -cells. Also, we did not find any significant gene expression changes in *Gck1*, *Ins1* and *Ins2* in MIN6 cells, and *NeuroD1* and *Pdx1* in INS1E cells, at both 8h and 24h. The changes in gene expression might have been undetectable as the gene expression changes could have reverted back due to the long exposure time to the CM. Hence, we decided to treat the β -cells with RAW 264.7 CM for a shorter exposure time of 4h and 8h for the future experiments.

5.3.3 Reduced expression of *Il-1 β* and *Ccl2* in RAW 264.7 cells treated with I-BET151

Upon verification using lipid A, we then proceeded to treat the RAW 264.7 cells with I-BET151 to study the cytokine release profile of macrophages. We treated RAW 264.7 cell line with two different concentrations of I-BET151: 1 μ M and 3 μ M. In a study by Barrett and colleagues, RAW 264.7 cells were treated with different concentrations of I-BET151 (1-60 μ M) followed by LPS treatment, and ELISA was performed on the secretome to detect cytokines. They observed that 1 μ M I-BET151 significantly reduced the amount of IL-6 in the cell supernatant at 6h and 24h without any effect on TNF- α and IL-1 β . Whereas, a higher concentration of I-BET151, at least 3 μ M, was required to significantly reduce TNF- α in the culture supernatant at the same treatment time points [188]. Therefore, in our set of experiments we used both 1 μ M and 3 μ M concentrations of I-BET151 to ensure its effects on macrophages and observe cytokine gene expression changes in RAW 264.7 cells.

The treatment of I-BET151 on RAW 264.7 macrophages caused a significant reduction in *Il-1 β* and *Ccl2* expression levels at both concentrations of I-BET151. We found the cytokine gene expression profiles were similar for both 1 μ M and 3 μ M I-BET151 treatment of RAW 264.7 cells, however *Ccl2* expression level was found to be significant only in 3 μ M I-BET151 treatment. Out of the panel of genes analysed (which included *Il-6*, *Il-10*, mannose receptor (MR) and *Arg1*), only *Tnf- α* , *Il-1 β* , *Nf-kB*, *Ccl2* and *Vegf-b* were expressed. In this study we found significant reduction in gene expression levels of *Il-1 β* and *Ccl2* (Figure 4.3) in RAW 264.7 cells directly treated with I-BET151, which has not been reported thus far. Interestingly, the reduction in gene expression levels were not observed if I-BET151 was washed off the RAW cells and the cells were incubated in fresh media for 18-24h (Figure 5.8). These results suggest that constant presence of I-BET151 on RAW 264.7 cells is required for I-BET151 to impart its effects on the cells.

Figure 5.7. Reduced IL-1 β and CCL2 gene expressions in mouse macrophages treated with I-BET151. RAW 264.7 cells were treated with 1 μ M or 3 μ M I-BET151 for 6h. The cells were then collected, and RNA extraction was carried out followed by cDNA conversion and qPCR for pro-inflammatory genes. Data presented are representative of 4 independent experiments. Data represents means $\pm$ SEM. p-values were calculated using ratio-paired t-test with * $p < 0.05$ and ** $p < 0.01$.

Figure 5.8. Gene expression changes in I-BET151 treated macrophage cells abrogated following a wash to remove the drug. RAW 264.7 cells were treated with 1 μ M for 6h. The cells were washed with 1x PBS and replaced with fresh media for 18-24h. The cells were then collected, and RNA extraction was carried out followed by cDNA conversion and qPCR for pro-inflammatory cytokines and anti-inflammatory macrophage markers. Significant reduction in IL-1 β and CCL2 gene expressions in mouse macrophages treated with I-BET151 were abrogated following the wash step. Data presented are representative of 3 independent experiments. Data represents means $\pm$ SEM. p-values were ratio-paired t-test with *p<0.05.

Overall, we observed reduced inflammatory effects in the constant presence of I-BET151 with mouse macrophages, which may indicate that I-BET151 could induce anti-inflammatory effects on macrophages coaxing them to an M2-like phenotype. This indicates that I-BET151 could be a potential drug to target inflammation in T2DM. Administration of I-BET151 in early T2DM could play a significant role in preventing the progression of the disease by protecting pancreatic β -cells.

5.3.4 Marked reduction in specific T2DM susceptibility genes upon I-BET151 CM treatment

The observed reduction in the expression of *Il-1 β* and *Ccl2* in I-BET151 treated RAW 264.7 cells is perhaps indicative that these macrophages were more anti-inflammatory, M2-like which was what was proposed in prevailing literature [123]. We expected that the CM of I-BET151 treated RAW 264.7 cells will produce results opposite of lipid A CM when exposed to β -cells. In order to determine the effect of I-BET151 CM on β -cells we proceeded to treat the I-BET151 CM on INS1E and MIN6 cells, but with a few modifications to the original experiment design described in Figure 4.1. As the washing of I-BET151 from the RAW 264.7 cells abrogated the changes in gene expression levels, it was essential to retain the drug in the CM. Hence, we used the CM containing I-BET151 to treat INS1E and MIN6 cells. In order to verify the effect of I-BET151 CM alone on β -cell gene expression levels, we treated INS1E and MIN6 cells directly with I-BET151 to be used as appropriate controls. The modifications to the experiment design are depicted in Figure 5.3.

We treated both INS1E and MIN6 cells (i) directly with I-BET151 and (ii) indirectly with I-BET151 treated RAW CM for 4h and 8h and observed the changes in expression levels of both β -cell signature genes involved in insulin production and signalling and T2DM susceptibility genes associated with β -cell function. The description and functions of the T2DM susceptibility genes, namely *Tcf7l2*, *Grb14*, *Hnf4 α* , *Glis3*, *Xrcc4* and *Manf*, whose expression levels were quantified in this study are listed in Table 1.1.

We observed moderate changes in β -cell signature gene expression levels in INS1E cells upon both direct and indirect treatment of I-BET151, though the results were not significant

(Figure 5.9). A modest but not significant reduction in *Glut2* expression was observed at 4h in both direct and in indirect treatment. Also, a decreased expression of *NeuroD1* was observed at 8h in indirect treatment of I-BET151, but again the results obtained were not significant.

The lack of effect of I-BET151 CM and I-BET151 directly on β -cells was unexpected especially since levels of *Il-1 β* and *Ccl2* were reduced in the macrophages treated with I-BET151. Further analysis of cytokines secreted by RAW 264.7 macrophages however showed that there were no significant changes, and this is probably why there was no impact of I-BET151 CM on β -cells. Intriguingly, the direct and indirect treatment of I-BET151 on INS1E cells revealed marked reductions in the expression levels of all of the chosen T2DM susceptibility genes at both 4h and 8h, with an exception of *Manf* at 4h treatment. These genes were specifically chosen in our study as polymorphisms in these genes have been associated with genetic predisposition to T2DM and they played a major role in β -cell function (Table 5.2). We observed significantly reduced gene expression levels of *Tcf7l2*, *Hnf4a* and *Xrcc4* in INS1E cells at both 4h (Figure 5.10-A) and 8h (Figure 5.10-B). Interestingly, we found that the pattern of reduced gene expression levels of T2DM susceptibility genes at both time points are similar in both direct treatment of I-BET151 and indirect treatment of I-BET151 CM, in INS1E cells. Also, there was no significant difference in gene expression between DMSO treated and DMSO CM treated INS1E cells. These results suggested that the effect of I-BET151 on T2DM susceptibility genes in INS1E cells are largely due to the direct exposure of the cells to I-BET151 than due to the effect of I-BET151 treated RAW 264.7 cell CM. Importantly, these results indicated that epigenetic changes incurred by I-BET151 on pancreatic β -cells could be detrimental to the β -cell function.

The finding that I-BET151 negatively impacts a myriad of genes associated with T2DM in INS1E cells needed to be corroborated with a different β -cell line. Using MIN6 cells, we found significantly reduced expression of *Glut2* gene at both 4h (Figure 4.10-A) and 8h (Figure 4.10-B) treatment durations. No other measured β -cells genes were significantly altered at either 4h or 8h after I-BET151 treatment. We also found a significant reduction in expression of *Hnf4 α* in MIN6 cells at 4h treatment (Figure 5.12). Also, we observed a reducing trend in *Xrcc4* and *Grb14* in MIN6 cells at after 4h treatment, though the gene expression changes were not significant. Of all the genes studied, *Hnf4 α* gene expression is consistently affected by I-BET151. The effect on *Hnf4 α* in both β -cell lines tested is irrespective of long-lived factors present in the CM of RAW 264.7 macrophages and more directly an effect of I-BET151 acting on β -cell itself. This finding is significant especially since *Hnf4 α* is also the first discovered MODY (MODY1) and loss-of-function mutations are strongly implicated in diabetes. This is a first report showing a possible detrimental role of I-BET151 on β -cells through a mechanism that involves *Hnf4 α* .

Gene	Description	Alias	Function
Tcf7l2	Transcription factor 7 like 2	T-cell specific transcription factor 4 (TCF4), HMG box transcription factor 4	“Transcription factor, plays a key role in Wnt signalling pathway, genetic variants are associated with high risk of T2DM” [211].
Grb14	Growth factor receptor bound protein	GRB14 adapter protein	Adapter protein, interacts with insulin receptor (tyrosine kinase receptor family), may inhibit insulin signalling [212, 213].
Hnf4a	Hepatocyte nuclear factor 4 alpha	Transcription factor 14 (TCF14), MODY1	Nuclear transcription factor, mutation leads to MODY1, Hnf4a loss of function may cause impaired insulin secretion, may regulate genes encoding Glut2 [214].
Glis3	GLI similar zinc finger protein family 3	Zinc finger protein 515 (ZNF515)	Transcriptional activator and repressor, contributes to pancreatic development, may regulate Ins2 gene expression [215], reduced expression in pancreatic cells of T2DM patients [216].
Xrcc4	X-Ray repair cross complementing protein 4	X-Ray Repair Complementing Defective Repair in Chinese Hamster Cells 4, SSMED	Double strand DNA break repair, missense polymorphism may lead to faulty expression of Glis3 gene [217].
Manf	Mesencephalic astrocyte- derived neurotrophic factor 4	Arginine-rich, mutated in early stage tumours (ARMET), Arginine-rich protein (ARP)	“Reducing expression of this gene increases susceptibility to ER stress-induced death” [218], may promote β -cell proliferation and regeneration [219].

Table 5.2. Functions of T2DM susceptibility genes involved in pancreatic β -cell function. The description and functions of the T2DM susceptibility genes used in this study are listed. Genetic variations in these genes have been associated with increased susceptibility to T2DM [34, 189].

Figure 5.9. Modest changes in β -cell signature gene expression levels upon treatment with I-BET151 conditioned RAW 264.7 cell media in INS1E cells. INS1E cells were treated with 1 μ M I-BET151 or DMSO treated RAW 264.7 cell CM media for (A) 4h and (B) 8h. Cells treated directly with I-BET151 or DMSO were used as respective controls. The cells were then subjected to RNA extraction followed by cDNA conversion and qPCR. Rat β -actin was used as housekeeping control. Ct values were first normalized to β -actin and then to control. Data presented are representative of 3-4 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using one-way ANOVA using Bonferroni post hoc test with * $p < 0.05$.

Figure 5.10. Reduced expression of T2DM susceptibility genes in INS1E cells upon I-BET151 CM treatment. INS1E cells were treated with 1 μ M I-BET151 or DMSO treated RAW 264.7 cell CM media for (A) 4h and (B) 8h. Cells treated directly with I-BET151 or DMSO were used as respective controls. The cells were then subjected to RNA extraction followed by cDNA conversion and qPCR. Rat β -actin was used as housekeeping control. Ct values were first normalized to β -actin and then to control. Data presented are representative of 3-4 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using one-way ANOVA using Bonferroni post hoc test with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 5.11. Reduced *Glut2* expression in MIN6 cells upon I-BET151 treated RAW 264.7 cell CM treatment and direct treatment of I-BET151. MIN6 cells were treated with 1 μ M I-BET151 or DMSO treated RAW 264.7 cell CM media for (A) 4h and (B) 8h. Cells treated directly with I-BET151 or DMSO were used as respective controls. The cells were then subjected to RNA extraction followed by cDNA conversion and qPCR. Mouse β -actin was used as housekeeping control. Ct values were first normalized to β -actin and then to control. Data presented are representative of 3 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using one-way ANOVA using Bonferroni post hoc test with * $p < 0.05$.

Figure 5.12. Significant reduction in *Glut2* and *Hnf4a* gene expression in MIN6 cells upon I-BET151 treated RAW 264.7 cell CM treatment and direct treatment of I-BET151. INS1E cells were treated with 1 μ M I-BET151 or DMSO treated RAW 264.7 cell CM media for (A) 4h and (B) 8h. Cells treated directly with I-BET151 or DMSO were used as respective controls. The cells were then subjected to RNA extraction followed by cDNA conversion and qPCR. Mouse β -actin was used as housekeeping control. Ct values were first normalized to β -actin and then to control. Data presented are representative of 3 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using one-way ANOVA using Bonferroni post hoc test with **p<0.01.

Overall, our novel finding suggests that (i) the T2DM susceptibility genes *Tcf7l2*, *Hnf4a* and *Xrcc4*, strongly implicated for β -cell function, are reduced in expression mainly due to direct effect of I-BET151 on β -cells and not due to secreted factors present in I-BET151 CM media and (ii) I-BET151 could have detrimental effects on β -cell function as the expression levels of essential genes required for β -cell function are reduced.

As the effect of direct I-BET151 treatment was predominant and the pattern of gene expressions were similar in both 4h and 8h treatment time points, we next decided to

explore the effects of direct I-BET151 treatment on β -cells (without I-BET151 CM) at 4h treatment duration.

5.3.5 IBET151 does not affect cell viability

As we observed reduced inflammatory cytokine expressions upon treatment of I-BET151 on RAW 264.7 macrophages, we decided that 1 μ M concentration was ideal to elicit a M2-like response. We used the same concentration on β -cells, as direct treatment of I-BET151 on β -cells was intended to be a control to study the macrophage CM effects on β -cells. As we observed adverse effects in β -cells at this concentration, we used the same 1 μ M concentration to study the cell viability of macrophages and β -cells. In order to confirm that the gene expression changes observed in β -cells and macrophages are not due to I-BET151-dependent cell toxicity, we treated INS1E, MIN6 and RAW 264.7 cells with 1 μ M I-BET151 and measured cell viability by colorimetrically measuring the formazan product formed due the reaction of live cells with WST-8 solution. This time, we also included a human pancreatic β -cell line, EndoC- β H1, as it is a well-established human pancreatic β -cell line [220]. We observed no significant cell death in INS1E cells (Figure 5.13-A), MIN6 cells (Figure 5.13-B), EndoC- β H1 cells (Figure 5.13-D) at a treatment time of 4h, and RAW 264.7 cells (Figure 5.13-C) at a treatment time of 6h suggesting that I-BET151 is not cytotoxic at the given concentration. The time points of treatments are as used in the previous studies.

Figure 5.13. No significant cell death in β -cells and macrophages treated with I-BET151. (A) INS1E cells, (B) MIN6 cells, (C) RAW 264.7 cells and (D) EndoC- β H1 cells were treated with 1 μ M I-BET151 for the given time durations. Cells treated with DMSO were used as controls. Cell viability assay was carried out using CCK-8 kit, Dojindo. Data presented are representative of 3-6 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch correction with * $p < 0.05$.

5.3.6 Comparison of the direct effect of I-BET151 on pancreatic β -cells of different origins

Once we found that I-BET151 is not cytotoxic to β -cells, we proceeded to investigate the direct effect of I-BET151 on 3 different β -cell lines, INS1E, MIN6 and EndoC- β H1 cells of rat, mouse and human origins, to find if the effect of I-BET151 are similar on β -cells of different origins. As we already had the gene expression changes due to direct effect of I-BET151 on INS1E and MIN6 cells, we proceeded to treat EndoC- β H1 cells with I-BET151 for 4h and quantified the gene expression levels of β -cell signature genes (Figure 5.14-C) and T2DM susceptibility genes. For the purpose of comparison with EndoC- β H1 cell line, the β -cell signature gene expression data of INS1E and MIN6 cells directly treated with I-BET151 have been separated from the CM treatment and shown in Figure 5.14. Additionally, *Gck1* gene expression in both INS1E (Figure 5.14-A) and MIN6 (Figure 5.14-B) cells was included in the panel as they were not quantified earlier due to non-availability of primers.

Interestingly, upon comparing the β -cell signature gene expression levels, we observed a pattern of reduction in gene expression levels of *Gck1* and *Glut2* in all 3 β -cell lines. Though the reduced expression of *Gck1* was significant in INS1E cells and that of *Glut2* was found to be significant in MIN6 cells, the trend was common to rat, mouse and human β -cells. These results reinforced our findings that I-BET151 treatment could be detrimental to β -cells, which has not been reported thus far.

Figure 5.14. Reducing trend in gene expression levels of *Gck1* and *Glut2* in three different β -cell lines. (A) INS1E (rat), (B) MIN6 (mouse) and (C) EndoC- β H1 (human) β -cell were treated with 1 μ M I-BET151 for 4h. β -cell signature gene expression levels were quantified using qPCR. β -actin was used as housekeeping control. Ct values were first normalized to β -actin and then to control. Data presented are representative of 3 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch correction with * $p < 0.05$.

In EndoC- β H1 cells, we expected a reduction in the expression levels of a majority of the T2DM susceptibility genes as observed in rat and mouse β -cell lines. Intriguingly, we found no significant reduction in T2DM susceptibility gene expression levels, but a significant increase in *Manf* gene expression (Figure 5.15).

Taken together, we report a novel finding that I-BET151 could have a detrimental effect on murine pancreatic β -cells mainly through T2DM susceptibility genes involved in β -cell function. The pattern of reduced expression levels of *Gck1* and *Glut2* in murine and human β -cell lines could indicate that I-BET151 can have overall adverse effects on β -cell function.

Figure 5.15. Changes in T2DM susceptibility genes in EndoC-βH1 cells upon direct treatment of I-BET151. EndoC-βH1 cells were treated with 1 μM I-BET151 for 4h. The cells were then subjected to RNA extraction followed by cDNA conversion and qPCR for (A) β-cell signature genes and (B) T2DM susceptibility genes. Human β-actin was used as housekeeping control. Ct values were first normalized to β-actin and then to control. Data presented are representative of 3 independent experiments. Data represents mean ± SEM. p-values were calculated using unpaired t-test with Welch correction with *p<0.05.

5.3.7 I-BET151 induced gene expression changes abrogated following a wash in INS1E cells

As mentioned in section 5.3.3, the effect of I-BET151 on cytokine expression levels was abrogated following an 18-24h long wash step to remove I-BET151 in RAW 264.7 cells. In order to verify whether the phenomenon occurred in β -cells as well, we used INS1E cells as a representative. We treated the INS1E cells with I-BET151 for 4h, washed the cells and replaced with fresh media for 6h. We then quantified the expression levels of β -cell signature genes and T2DM susceptibility genes. As observed with RAW 264.7 cells, we found that the significant changes in gene expression levels of INS1E cells were also abrogated even at a shorter wash duration of 6h (Figure 5.16). These results suggest that I-BET151 could be fast acting and thus needs to be present at all times to impart their effects on the cells.

Figure 5.16. Gene expression changes in I-BET151 treated INS1E cells abrogated following a wash to remove the drug. INS1E cells were treated with 1 μ M I-BET151 for 4h. The cells were washed with 1x PBS and replaced with fresh media for 6h. The cells were then collected, and RNA extraction was carried out followed by cDNA conversion and qPCR for (A) β -cell signature genes and (B) T2DM susceptibility genes. Significant reduction in T2DM susceptibility gene expressions observed in INS1E cells treated with I-BET151 were abrogated following the wash step. Data presented are representative of 3 independent experiments. Data represents means $\pm$ SEM. p-values were calculated using unpaired t-test with Welch correction with * $p < 0.05$.

5.3.8 Effect of I-BET151 on glucose stimulated insulin secretion

As I-BET151 reduced the expression of genes essential for β -cell function, we wanted to see if there was any effect of I-BET151 on GSIS in the pancreatic β -cells. For this study, we used INS1E and MIN6 cells and we treated the cells with I-BET151 for a total of 4h in 2 steps. In the first step, I-BET151 was added to the respective cell media and the cells were incubated for 2h. In the second step, the cells were incubated in GSIS buffer containing 1.5 mM glucose (1.5G buffer) with I-BET151 for 2h, to starve the cells. The starvation is to normalise the initial insulin content of the cells. As we found I-BET151 to be a fast acting drug and removal of the drug from the cells abrogated the gene expression effects, we starved the cells in the presence of I-BET151. Following starvation, GSIS was carried out using 1.5 mM basal glucose and 16.7 mM high glucose.

In both INS1E and MIN6 cells, the high glucose treatment evoked an increased insulin secretion from the cells. In INS1E cells, we found a significant increase in insulin secretion in both DMSO and I-BET151 treated cells upon 16.7 mM glucose stimulation, compared to respective controls (Figure 5.17-A). We observed reduced insulin secretion in I-BET151 treated INS1E cells upon high glucose stimulation, though the results were not significant. In contrast, we did not observe a change in insulin secretion upon I-BET151 treatment in MIN6 cells (Figure 5.17-B). These variations could be due to differences in rat and mouse cell lines. Also, we found significantly reduced expression of *Tcf7l2* in INS1E cells treated with I-BET151, but no reduction in MIN6 cells in our previous studies. As decreased expression of *Tcf7l2* is associated with decreased GSIS [221], this could be the reason for observed reduction in insulin secretion in INS1E but not in MIN6 cells.

Figure 5.17. Glucose stimulated insulin secretion in β -cells treated with I-BET151. (A) INS1E cells and (B) MIN6 cells were treated with 1 μ M I-BET151 for 4h i.e. 2h in INS1E media and 2h in Ca-10 media containing 1.5 mM glucose to starve cells. GSIS was measured at 1.5 mM glucose and 16.7 mM glucose and normalised with total protein of the cells. Data presented are representative of 3-6 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch correction (DMSO vs I-BET151) and ratio-paired t-test (1.5G vs 16.7G) with * $p < 0.05$ vs 1.5G-DMSO, ** $p < 0.01$ vs 1.5G-DMSO and # $p < 0.05$ vs 1.5G-I-BET151.

5.3.9 Reduced expression of T2DM susceptibility genes in INS1E cells is due to BET protein inhibition in general

Our previous studies show marked reduction in gene expression levels of *Gck1*, *Tcf7l2*, *Hnf4a* and *Xrcc4* in INS1E cells upon treatment with I-BET151. In order to verify whether the gene expression level changes are specifically due to I-BET151 or BET protein inhibitors in general, we treated INS1E cells with two other BET protein inhibitors namely, JQ1 and I-BET762 for 4h. We used the same 1 μ M concentration of JQ1 and I-BET762 to treat the β -cells as used in the previous I-BET151 studies. We then observed the changes in expression levels of both β -cell signature genes involved in insulin signalling (Figure 5.18-A and Figure 5.19-A) and T2DM susceptibility genes associated with β -cells (Figure 5.18-B and Figure 5.19-B).

Interestingly, our results indicated significant reduction in gene expression levels of *Gck1*, *Tcf7l2*, *Hnf4a*, *Glis3* and *Xrcc4* in both JQ1 (Figure 5.18) and I-BET762 (Figure 5.19) treated INS1E cells, similar to that of I-BET151 treatment. In addition, JQ1 treatment showed a significantly reduced expression of *Ins2* and *Glis3* and I-BET762 treatment showed a significantly reduced expression of *Glis3*. We can also see a reduction in expression of *Ins1* and *Grb14* genes in both JQ1 and I-BET762 BET protein inhibitors, although not significant statistically.

Our novel finding of reduced gene expression levels associated with β -cell function suggests that the adverse effects observed could be due to BET protein inhibition in general and not specific to I-BET151.

Figure 5.18. Reduced expression of β -cell signature genes and T2DM susceptibility genes in INS1E cells treated with BET protein inhibitor JQ1. INS1E cells were treated with $1\mu\text{M}$ JQ1 for 4h. The cells were then subjected to RNA extraction followed by cDNA conversion and qPCR for (A) β -cell signature genes and (B) T2DM susceptibility genes. Rat β -actin was used as housekeeping control. Ct values were first normalized to β -actin and then to control. Data presented are representative of 3 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch's correction with * $p \leq 0.05$ and ** $p \leq 0.01$.

Figure 5.19. Reduced expression of β -cell signature genes and T2DM susceptibility genes in INS1E cells treated with BET protein inhibitor I-BET762. INS1E cells were treated with 1 μ M I-BET762 for 4h. The cells were then subjected to RNA extraction followed by cDNA conversion and qPCR for (A) β -cell signature genes and (B) T2DM susceptibility genes. Rat β -actin was used as housekeeping control. Ct values were first normalized to β -actin and then to control. Data presented are representative of 3 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch's correction with * $p \leq 0.05$ and ** $p \leq 0.01$.

5.3.10 Effect of I-BET151 on reducing β -cell gene expression is through transcription

Next, we wanted to explore whether I-BET151 is acting on these genes at a transcriptional level. In order to find this, we pre-treated INS1E cells with actinomycin D, a well-known transcriptional inhibitor [222], and then treated these cells with I-BET151 and quantified the expression of β -cell signature genes and T2DM susceptibility genes. We found that reduced gene expression levels in INS1E cells due to I-BET151 were not observed upon transcriptional inhibition. These results suggest that I-BET151 regulates the expression of the genes essential for β -cell function at transcriptional level. *Ins1* may be an exception to this, as reduced level of *Ins1* gene is observed in transcriptionally inhibited INS1E cells treated with I-BET151, though the results are not significant.

Figure 5.20. Effect of I-BET151 on INS1E cells is through transcription. INS1E cells were pre-treated 5 μ g/ml of actinomycin D with 1 μ M I-BET762 for 4h. The cells were then subjected to RNA extraction followed by cDNA conversion and qPCR for (A) β -cell signature genes and (B) T2DM susceptibility genes. Rat β -actin was used as housekeeping control. Ct values were first normalized to β -actin and then to control. Data presented are representative of 3 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using unpaired t-test with Welch's correction with $*p \leq 0.05$.

5.4 Discussion

BET proteins knockdown and inhibition are known to be associated with reduced pro-inflammatory cytokine secretion in macrophages challenged with bacterial endotoxins [127]. Also, Inhibition of BET proteins by small molecule I-BET151 have been shown to have anti-inflammatory effects in islet macrophages of NOD mice and have been implicated in proliferation of pancreatic β -cells [123, 129]. Based on these evidences, our major aim was to modulate macrophages to anti-inflammatory phenotype with BET protein inhibitor I-BET151 and study the effect of the macrophage secreted factors on pancreatic β -cells.

After confirming that our experimental model was functioning by activating mouse macrophage cell line, RAW 264.7, with lipid A and treating β -cells with lipid A CM, we proceeded to study the effect of I-BET151 on RAW 264.7 macrophages. We observed reduced expression of pro-inflammatory cytokines in I-BET151 treated macrophages, as suggested in literature, even without any external inflammatory stimulation. The interesting observation here was that once the cell environment is free from I-BET151, the reduction in pro-inflammatory gene expression levels was no longer present. We also verified this phenomenon later in our research by washing off I-BET151 from β -cells and quantifying genes associated with β -cell function and found that the gene expression changes caused due to I-BET151 treatment were abrogated. As we quantified the pro-inflammatory genes after 18-24h wash, we wondered whether this long duration is required for the effect of I-BET151 to nullify. Hence, we reduced the wash step to 6h in β -cells and yet we found that the effects were abrogated. Also, the treatment time of I-BET151 on macrophages and β -cells were not more than 8h and nevertheless, significant changes in gene expression levels were observed at such short durations. These findings

suggest that I-BET151 is a fast-acting drug and care should be taken while administering the drug in clinical settings in future, as clearance of the drug from the proximity of the target cells may abrogate the desired effects.

The next major novel finding in this study is the adverse effect of I-BET151 on pancreatic β -cell gene expression essential for their function. We found that even at a nominal dose of 1 μ M, I-BET151 could elicit reduction in gene expression of glucokinase and other genes essential for β -cell function, whose variants have been associated with T2DM risk, such as *Tcf7l2*, *Hnf4a* and *Xrcc4*. Interestingly, the observed effects on the gene expression levels were not due to secreted factors from the macrophages exposed to I-BET151, but due to direct effect of I-BET151 on β -cells. We found that the expression levels of these genes are regulated at transcriptional level by I-BET151 (as transcriptional inhibition followed by I-BET151 did not affect the gene expression) using INS1E cells. Even though the reduced expression of T2DM susceptibility genes were not observed in the human β -cells, a pattern of reduction in expression levels of *Gck1* and *Glut2* genes were observed in human as well as murine β -cells.

After confirming that the chosen dosage of I-BET151 (1 μ M) is not cytotoxic for the treatment time periods used in this study by cell viability assay, we proceeded to explore the effect of I-BET151 on GSIS of murine β -cells. Even though our results were not statistically significant, we did find a reduction in insulin secretion in the rat β -cells, but not in the mouse cells. This could be due to the reduced expression of *Tcf7l2* gene in rat β -cells upon I-BET151 treatment, which was not observed in mouse cells. These results suggest that the effect of I-BET151 on insulin secretion from pancreatic β -cell lines is not consistent, possibly due to the variations in cell lines. *In vivo* experiments designed to study

the serum insulin levels upon glucose administration, could be a much better model to study the effect of I-BET151 on insulin secretion.

We wondered whether the gene expression changes observed in β -cells were specific to I-BET151 or to BET protein inhibitor in general. Hence, we quantified the same panel of genes used for I-BET151 studies in INS1E cells treated with other BET protein inhibitors, JQ1 or I-BET762. Interestingly, similar reduction in T2DM susceptibility gene expression levels as seen in I-BET151, were found in JQ1 and I-BET762 treated cells, indicating that BET protein inhibition in general can affect the genes required for β -cells.

The overall summary of this chapter has been consolidated in 3 sequential parts, namely, validation of experimental design (Figure 5.21), main research focus (Figure 5.22) and further exploration from obtained inference (Figure 5.23). As our results suggest that direct exposure of β -cells to I-BET151 can be detrimental to β -cell function, we conclude that BET protein inhibitors should be used with caution.

Figure 5.21. Workflow of validating experimental design using lipid A treated macrophages. First, we treated macrophage cell line with lipid A and used lipid A CM to treat β-cells to validate our experimental model. We found increased expression of pro-inflammatory cytokines in macrophages and reduced β-cell signature genes in murine β-cells, consistent with our model.

Figure 5.22. Workflow to validate CM-mediated gene expression changes in β -cells. Summary of major findings and possible inferences from the I-BET151 study. (i) Effect of I-BET151 is primarily due to direct treatment to β -cells and not through secreted factors from macrophages upon I-BET151 stimulation (ii) I-BET151 could be detrimental to pancreatic β -cell function (iii) I-BET151 is fast acting and its constant presence may be required for it to impart its effects.

Figure 5.23. Direct effect of I-BET151 on β -cells. Different experiments were performed to determine the direct effects of I-BET151 on β -cells. The list of experiments, their outcomes and inferences are as shown.

RESULTS-IV

CONTROLLED DELIVERY OF I-BET151 USING PLGA NANOPARTICLES

6.1 Introduction

In our previous chapter, we found that BET protein inhibition using small molecule inhibitor, I-BET151, resulted in the reduction in gene expression of pro-inflammatory cytokines, *IL-1 β* and *Ccl2*, indicating that I-BET151 aids in macrophage modulation to M2-like phenotype. We also observed that at the same concentration of 1 μ M, I-BET151 and other BET protein inhibitors JQ1 and I-BET762, have adverse effects on pancreatic β -cells by reducing the expression levels of most of the T2DM susceptibility genes related to β -cell function.

We have already elaborated in chapter 5 section 5.3.3 that the reason for the choice of 1 μ M concentration of I-BET151 for all our studies is based combinedly on the study by Barrett and colleagues [188] and our previous results on the reduction in inflammatory gene expressions in RAW 264.7 cells upon I-BET151 treatment. As we found that this concentration may have detrimental effects on β -cell function, we decided to explore whether the gene expression changes in β -cells were dependent on the dosage of I-BET151. If the gene expression changes due to I-BET151 are dose dependent, modulating the dosage could have the potential to reduce inflammatory response in macrophages without causing any adverse effects on β -cells.

In our earlier experiments we observed that I-BET151 could be fast-acting and constant presence of I-BET151 is required to elicit a response in both macrophages and β -cells, as washing off the drug caused abrogation of gene expression changes. Hence, sustained and controlled dose release of I-BET151 is essential to attain reduced inflammatory state without affecting β -cell function in the pancreas.

As described in chapter 2 section 1.12, polymeric nanoparticles are highly preferred for drug delivery systems due to their remarkably biodegradable nature, ability to enable controlled drug release and their ease of fabrication [223]. PLGA, is one of the commonly used polymers in the field of nanomedicine as it is an FDA approved polymer and has been rendered safe for biological applications as the degradation products do not cause adverse effects [224]. Hence, we aimed to synthesize PLGA nanoparticles suitable for controlled and sustained release of I-BET151 and study its characteristics.

6.2 Methods

6.2.1 I-BET151 dose dependent treatment on murine β -cells

INS1E or MIN6 cells were seeded in 12-well plates for 24-36h to reach 70% confluence and were treated with different concentrations of I-BET151 ranging from 25 nM to 10,000 nM, for 4h. The treated medium was discarded, and cells were washed thrice with 1x PBS to wash off I-BET151. The treated cells were lysed with lysis buffer and was used for RNA isolation followed by cDNA synthesis. Gene expression levels were then measured in INS1E cDNA samples by qPCR for β -cell signature genes, namely, *Gck1*, *Ins2* and *Glut2*, and T2DM susceptibility genes, namely, *Tcf7l2*, *Hnf4a* and *Xrcc4*. Gene expression levels of *Gck1*, *Glut2*, *Hnf4a* and *Xrcc4* were measured in MIN6 cDNA samples by qPCR. INS1E or MIN6 cells treated with DMSO were used as control.

6.2.2 Fabrication of I-BET151 loaded nanoparticles

I-BET151-loaded PLGA nanoparticles were fabricated by the solvent evaporation method. A 10 μ M concentration of I-BET151 was added in PLGA/dichloromethane (DCM) (0.2g in 1 ml) solution. The resultant solution was mixed with 2 ml of 2 % (w/v) polyvinyl alcohol (PVA) solution and ultra-sonicated for a minute with ice. The primary emulsion was then transferred into 5ml of distilled water under stirring at 400 rpm for 4h. Next, the nanoparticle solution was washed with water followed by centrifugation at 20,000 rpm for 10 min.

6.2.3 Field emission scanning electron microscopy

In order to characterize the I-BET151 loaded PLGA nanoparticles, field-emission scanning electron microscopy (FESEM 7600F, JEOL Co.) was used and the surface morphology was determined. The lyophilized nanoparticles were uniformly dispersed onto double-sided carbon tape and these samples were made conductive by sputter coating under vacuum using platinum fine coater for 45 s at 30 mA and imaged at an acceleration voltage of 5 kV.

6.2.4 Particle size and zeta potential measurement

The PLGA nanoparticles containing I-BET151 were suspended in distilled water and sonicated for 30s in order to minimize interparticle interactions. The hydrodynamic diameter, polydispersity index and zeta potential of the nanoparticles were quantified using a dynamic light scattering analyser (Malvern Zetasizer-Nano ZS).

6.2.5 Drug release study

In vitro release studies of different formulations were evaluated using a dialysis bag with a molecular weight cut-off of 7000 (Membra-Cel; Viskase, Inc., USA) filled with 10 mg of PLGA nanoparticles. The dialysis bag was then immersed in 20 ml of 1x PBS at pH 7.4 at 37°C. A total of 5 ml of release medium was collected and replaced with the same volume of fresh medium at pre-determined time points up to 48h. The amount of the released drug was measured by HPLC system.

6.2.6 High-performance liquid chromatography (HPLC)

An Agilent 1200 liquid chromatography system with an autosampler and UV detector were used for HPLC. A C18 column (Supelco™; MetaChem, USA) of 4×250 mm dimension and 5 µm particle size. The flow rate of the mobile phase was 1 ml/min, and the detection wavelength was set to 250 nm. The mobile phase was a mixture of 0.1% formic acid solution and 0.1% formic acid in acetonitrile (30:70, v/v). Encapsulation efficiency was calculated using indirect method by calculating the free unencapsulated drug in the supernatant from which the particles were collected. The encapsulation efficiency was calculated using the following equation:

$$\% \text{ Encapsulation Efficiency} = \frac{\text{drug added} - \text{Free unencapsulated drug}}{\text{drug added}} \times 100$$

6.3 Results

6.3.1 Dose dependent effects of I-BET151 on INS1E cells

As we observed markedly reduced gene expression levels of *Gck1*, *Glut2*, *Tcf7l2*, *Hnf4a* and *Xrcc4* in INS1E cells treated with 1 µM I-BET151, we chose this set of genes to study

the dose dependent effects of I-BET151. *Ins2* gene was used a control, as there was no significant reduction in *Ins2* expression upon 1 μ M I-BET151 treatment of INS1E cells. A concentration range of 25-10,000 nM of I-BET151 was chosen for this study. Henceforth, the 1 μ M concentration is referred to as 1000 nM for the sake of convenience.

Though we found moderate changes in expression of *Ins2* upon treatment with different concentrations of I-BET151, there was no significant change at any of the concentrations used in study (Figure 3.3) when compared to control. This suggests that I-BET151 does not have an effect on insulin gene expression in INS1E cells at concentrations below 10000 nM. In all the other 5 genes whose expression levels were quantified, we observed a remarkable dose dependent effect due to I-BET151 treatment. In *Gck1* and *Hnf4 α* , the gene expression levels reduced gradually and uniformly with the increase in drug dosage, though the reduction in *Gck1* expression was significant at a dosage of 100 nM and that of *Hnf4 α* was at 500 nM (Figure 3.3-B and E). For both genes, the reduction in expression level was more than 50% at a dose of 100nM. *Glut2*, *Tcf7l2* and *Xrcc4* expression levels were also found to be reducing with increasing concentration of I-BET151 (Figure 3.3-C, D and F). Significant reduction of *Glut2* in INS1E was at 500 nM, and that of *Tcf7l2* was at 100 nM concentration of I-BET151.

Figure 6.1. Dose dependent effects of I-BET151 on INS1E cells. INS1E cells were treated with I-BET151 at a concentration range of 25-10000 nM. A concentration of 1000nM was used in all the previous studies. The gene expression levels of (A) *Ins2*, (B) *Gck1*, (C) *Glut2*, (D) *Tcf7l2*, (E) *Hnf4a* and (F) *Xrcc4* were measured by qPCR. Data presented are representative of 3 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using one-way ANOVA followed by Bonferroni's post hoc analysis with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Overall, our results suggest that the effect of I-BET151 on the β -cell signature genes and T2DM susceptibility genes necessary for β -cell function observed earlier is dependent on the dosage of I-BET151 in INS1E cells. From this study, we observe that an I-BET151 dosage of 100 nM or above of could cause detrimental effects on *Gck1*, *Tcf7l2* and probably on *Hnf4a* as well. Whereas, a dosage of 500 nM or above is required to affect the *Glut2* and *Xrcc4* genes. Hence, delivery of 100 nM or lesser dosage of I-BET151 could diminish the adverse effects of I-BET151 on INS1E cells.

6.3.2 Verification of dose dependent effects of I-BET151 on MIN6 cells

Next, we verified whether the reduced β -cell gene expression corresponded to increasing dosage of I-BET151 using MIN6 cell line. The genes whose expression was reduced at a 1000 nM concentration in our previous studies were *Gck1*, *Glut2*, *Hnf4a* and *Xrcc4*. Hence, we chose the same genes to study the dose dependent effects of I-BET151 on MIN6 cells.

Our results confirmed that the changes in β -cell gene expression levels in MIN6 are also dependent on I-BET151 dosage. *Gck1* and *Hnf4a* expressions were reduced by 50% (approximately) at a concentration of 100 nM of I-BET151 (Figure 6.2-A and C), which is similar to the effect seen in INS1E cells. Interestingly, *Glut2* expression was slightly elevated at a concentration of 25 nM, though not statistically significant (Figure 6.2-B). Both *Glut2* and *Xrcc4* (Figure 6.2-D) were reduced by about 50% at an I-BET151 concentration of 500 nM. An increasing dosage of I-BET151 up to 10000 nM drastically decreased the expression of all of the chosen genes.

Our novel finding suggests that I-BET151 has adverse effects on pancreatic β -cells gene expressions and these adverse effects can be diminished by delivering low concentration (<100 nM) of I-BET151. Delivering lower dosages of I-BET151 could diminish the detrimental effects on β -cells significantly and using such a dosage could elicit desired effects of BET protein inhibition in the body without challenging the functionality of pancreatic β -cells.

Figure 6.2. Dose dependent effects of I-BET151 on MIN6 cells. MIN6 cells were treated with I-BET151 at a concentration range of 25-10000 nM. The gene expression levels of (A) *Gck1*, (B) *Glut2*, (C) *Hnf4a* and (D) *Xrcc4* were measured by qPCR. Data presented are representative of 3 independent experiments. Data represents mean $\pm$ SEM. p-values were calculated using one-way ANOVA followed by Bonferroni's post hoc analysis with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

6.3.3 Synthesis and characterization of I-BET151 encapsulated PLGA nanoparticles

Next, we fabricated I-BET151 loaded PLGA nanoparticles for controlled dose release and sustained delivery of I-BET151. We used the solvent evaporation technique to prepare nanoparticles and 10 μ M I-BET151 was loaded into the particles with a theoretical loading of 5% (weight of drug to polymer ratio). The free unencapsulated drug in the solution was measured by HPLC and the encapsulation efficiency was calculated using the equation in section 6.2.6. We achieved a good encapsulation efficiency of 72% and this implied that the actual loading of I-BET151 in the PLGA nanoparticles was 3.6% (weight of drug to polymer ratio).

The I-BET151 loaded PLGA nanoparticles were imaged by scanning electron microscopy (SEM) to observe the morphological characteristics and uniformity of the particles (Figure 6.3). The PLGA nanoparticles appeared as individual and non-fused spheres with a smooth surface morphology. We then measured the size of the nanoparticles using dynamic light scattering (DLS) technique and found the hydrodynamic diameter to be 115.4 ± 17.2 nm (Figure 6.4). We also measured the polydispersity index and found it to be 0.214 ± 0.012 indicating that the I-BET151 loaded PLGA nanoparticles are nearly uniform in size distribution.

Figure 6.3. Representative SEM image of I-BET151 encapsulated PLGA nanoparticles. I-BET151 loaded PLGA nanoparticles were prepared by solvent evaporation method and the nanoparticles were imaged using SEM. Individual smooth and spherical nanoparticles of PLGA were observed.

Figure 6.4. Dynamic laser scattering of I-BET151 encapsulated PLGA nanoparticles. The I-BET151-loaded PLGA nanoparticles were subjected to dynamic light scattering technique to measure the particle size distribution using Malvern Zetasizer-Nano ZS. The hydrodynamic diameter was found to be 115.4 ± 17.2 nm.

6.3.4 Zeta potential

We then quantified the zeta potential of the PLGA nanoparticles and found it to be -19.8 ± 5.4 mV (Figure 6.5). The sharp peak and short band of apparent zeta potential indicated that the particles are uniformly charged. The obtained zeta potential indicates that the I-BET151 loaded PLGA nanoparticles have a good stability and that they remain in suspension. The surface charge of PLGA nanoparticles are negative, similar to the cell surface, and hence are safer compared to positively charged nanoparticles that are cytotoxic.

Figure 6.5. Zeta potential of I-BET151-loaded PLGA nanoparticles. The zeta potential of the I-BET151-loaded PLGA nanoparticles was measured using Malvern Zetasizer-Nano ZS. The zeta potential was found to be -19.8 ± 5.4 mV.

6.3.5 I-BET151 release study from PLGA nanoparticles

The release of I-BET151 from PLGA nanoparticles were measured by HPLC at 2, 4, 8, 12, 24 and 48h (Figure 6.6). The absolute release at the mentioned time points were found to be 17.2%, 8.9%, 17.4%, 13.4%, 18.7% and 14%, respectively. From the drug release profile, we observe that maximum release of I-BET151 can be obtained from the particles after 48h. By adjusting the initial amount of I-BET151 loaded into these nanoparticles, the desired dosage of I-BET151 can be released based on the percentage release obtained.

Figure 6.6. The release profiles of I-BET151 from PLGA nanoparticles in 1x PBS. The release of I-BET151 from the I-BET151 loaded PLGA particles was determined by dialysis at pH 7.4 for 48h at 37°C using 1x PBS as release medium. Data presented are representative of 5 independent experiments. Data represents mean $\pm$ SD.

6.4 Discussion

In this chapter, we show that the reduced expression of genes required for pancreatic β -cell function due to I-BET151 is dose dependent. Increase in concentration of the drug from 25 nM to 10000 nM resulted in increased deterioration in the expression of β -cell signature and T2DM susceptibility genes involved in β -cell function. We also suggest that a dosage of up to 100 nM might not adversely affect the β -cell function. We have already shown that I-BET151 reduces the expression of pro-inflammatory cytokines (M2-like phenotype) in mouse macrophage cell line at a concentration of 1000 nM. We speculate that a dosage of 100 nM or lower may still elicit reduced inflammatory responses in macrophages.

In a study by Chan and team, it has been shown that I-BET151 could reduce the expression of inflammatory cytokines CXCL10 and CXCL11 in LPS- or TNF α - stimulated human macrophages, at a concentration ranging from 50-500 nM [225]. These results suggest that suppression of inflammation in macrophages could be facilitated by I-BET151 up to a low concentration of 50 nM. Concentrations lower than 1000 nM have also been used to study the effect of BET protein inhibitor, JQ1, on macrophages; and these doses have been implicated in the suppression of inflammatory cytokine expression in macrophages induced with LPS or other inducers of inflammation [226-228]. In a study by Belkina and colleagues, it is reported that LPS induced gene expression levels of *Tnf- α* , *Il-6* and *MCP-1* were significantly reduced by JQ1 in RAW 264.7 cells. The concentration of JQ1 used in this study was 400 nM. It was also shown that LPS induced expression of the aforementioned genes were significantly reduced by JQ1 in mice BMDMs, at different JQ1 concentrations of 100, 200 and 400 nM [226]. These studies endorse our hypothesis that a low dosage of I-BET151 i.e. less than 100 nM could render the macrophages to adopt a M2-like phenotype.

Delivering low doses of drug to the required tissues in the body can be quite challenging as the bioavailability of a drug greatly diminishes by the time it reaches the target cells. As I-BET151 requires a low-dose sustained release, we decided to fabricate a nanoparticle delivery system to deliver I-BET151 to the β -cells. We achieved 72% encapsulation efficiency of I-BET151 and an approximate particle size of 115 nm that is suitable for detection by macrophages. The polydispersity index was measured to be 0.214 (5% error approximately) indicating that the particles were uniformly distributed in size. This ensures that the drug release is uniform and there is better control over release kinetics. The release profile of I-BET151 from the PLGA nanoparticles suggest that low dose drug release is possible for these nanoparticles for over a period of 48h, and by tailoring the amount of initial drug loaded into the nanoparticles, low dose sustained release of I-BET151 could be enabled. Hence, delivering I-BET151 using PLGA nanoparticles can enable sustained delivery over a period of 48h and the amount of drug release can be controlled to reach the desired dosage of less than 100 nM.

DISCUSSION AND FUTURE PERSPECTIVES

7.1 Discussion

The global burden and burgeoning healthcare cost associated with T2DM and its associated complications has fuelled research into its pathophysiology and underlying mechanisms so as to identify new therapeutic targets for the treatment of T2DM. One such major pathophysiological mechanism identified in T2DM progression is the dynamic change in pancreatic islet morphology and the corresponding impairment of insulin secretion from pancreatic β -cells [86]. During pre-diabetes, the pancreatic islets undergo hyperplasia and enhanced β -cell function to secrete more insulin to compensate for insulin resistance, whereas fully developed T2DM is characterized by β -cell dysfunction and islet degeneration [229]. If the progression of the disease can be stalled at the pre-diabetes stage by enhancing β -cell function to maintain a normoglycemic state, the development of T2DM and complications could be mitigated and prevented.

It has been reported that 60% of the β -cell mass is lost due to increased apoptosis and the functioning of the remaining pancreatic islet β -cells reduces by 50% by the time T2DM is diagnosed [230]. In mammals, the capacity of β -cells to regenerate is low and their replication rate in adulthood only contributes to the maintenance of β -cell mass [231]. The presence of adult endocrine multipotent stem cells that are capable of differentiating to β -cells is still in debate [232]. These evidences suggest that once lost the β -cells cannot be adequately restored. In rodents, only upon extreme β -cell loss do α - and δ -cells transdifferentiate to β -cells [233]. Such massive β -cell loss is not seen in T2DM. In addition, the delivery of transcription factors Pdx1, Ngn3 and MafA to mouse pancreas through viral vectors have been shown to induce transdifferentiation of acinar cells to β -

cells [234]. Transdifferentiation of other pancreatic cells to β -cells though observed in mice, has not been shown to occur in humans. Once β -cells are lost, there is no biological capacity for them to return. Therefore, a prophylactic strategy is imperative to preserve β -cell function and mass in very early stages of diabetes to prevent the development of T2DM. The pre-diabetes stage provides the most favourable opportunity to protect the β -cells as increased functioning of β -cells causing hyperinsulinemia is observed during compensation in pre-diabetes.

The current major drugs used in treatment of T2DM include metformin, sulphonyl urea, thiazolidinediones (TZD), glucagon-like peptide (GLP-1) receptor antagonists [235, 236] sodium-glucose cotransporter 2 (SGLT2) inhibitors [237] and α -glucosidase inhibitors [236]. Metformin reduces the blood glucose levels by increasing the sensitivity of the peripheral tissues to insulin and also by inhibition of hepatic glucose production, whereas it has no role in stimulating insulin secretion or preservation of β -cell function [236, 238]. TZD also acts as insulin sensitizer in liver, muscle and adipose tissues. Sulphonylurea drugs enhance the responsiveness of β -cells to glucose thus stimulating insulin secretion. GLP-1 antagonists are also known to increase insulin secretion and they have a beneficial effect on weight loss [235]. SGLT2 inhibitors reduce the absorption of glucose in kidneys so that the excess glucose is secreted in the urine [237]. The mechanism of α -glucosidase inhibitors is by inhibition of carbohydrate absorption from the intestine [236]. Intensive insulin therapy has been implicated in preservation of β -cell function later on in diabetes. Apart from therapeutic interventions, lifestyle modifications such as diet and exercise are known to improve β -cell function [239]. Despite these interventions, the incidence of T2DM is increasing. Hence, therapeutic interventions targeting the very early stages of pre-diabetes could help prevent the progression of T2DM.

A well-established key driver of this complex and heterogenous disease is chronic inflammation [240, 241], that leads to a relentless decline in pancreatic β -cell function [208] in fully developed T2DM. Infiltration of macrophages into pancreatic islets due to chronic hyperglycaemia and insulin resistance have been identified to cause β -cell dysfunction [86, 208]. Though many studies have reported the role of inflammation and islet macrophage infiltration in fully developed T2DM, macrophages and their role in islet remodelling during the pre-diabetes stage is scarcely explored [86]. As resident M2-like macrophages have been associated with development of pancreas in the embryonic stage [72], we hypothesised that macrophages could be recruited to pancreatic islets and they may play a beneficial role in protecting β -cell function during pre-diabetes before the β -cells start to lose their function.

In order to validate our hypothesis, we first needed to establish a pre-diabetic animal model and subsequently determine whether islet macrophage is altered during pre-diabetes. We successfully developed a pre-diabetic mouse model by feeding HFD (60% fat) to male C57BL/6 mice for an acute term of 21 days. The mice developed insulin resistance with signs of compensatory hyperinsulinemia, an effect not seen previously with the calorifically matched LFD (10% fat) control mice. We found a significant increase in islet area and also β -cell population, with greater number of islets found to have increased β -cell population, after 21 days of HFD feeding, both of which are the key mechanisms supporting β -cell compensation during pre-diabetes. Increased islet area and β -cell numbers per islet suggest that more insulin is being secreted. However, the *in vivo* GSIS was only modestly (but not significantly) higher, perhaps due to other stimuli (e.g. epinephrine) that are released when blood is being withdrawn from fully conscious mice.

It is not possible to conduct such experiments under anaesthesia as such drugs directly affect the insulin secretion pathway.

Using this mouse model, we then proceeded to study the macrophage population within the islets of the pre-diabetes induced mice. We found a significant increase in F4/80⁺ and CD68⁺ macrophage population after 21 days of HFD feeding compared to 21 days of LFD feeding. To further determine whether macrophages play a direct role in islet dysfunction during compensatory phase, we used 21 days HFD fed CD169-DTR mice to deplete macrophages. We found that both the islet area and islet vascularization were significantly reduced in macrophage depleted mice along with markedly reduced β -cell population, suggesting that islet macrophages contribute to β -cell compensation during pre-diabetes. Our results suggest that macrophage numbers increase to facilitate islet remodelling in HFD fed mice as early as 21 days, and that the islet macrophages could help the β -cells to compensate for insulin resistance in pre-diabetes.

As M1 macrophages cause inflammation in the pancreatic islet and lead to β -cell dysfunction in T2DM [80], modulating the islet macrophages to M2-like anti-inflammatory phenotype during pre-diabetes could protect β -cells. M2-like modulation of macrophages have been shown to be attained in T1DM using the BET protein inhibitor I-BET151, and this drug was also implicated in pancreatic β -cell proliferation in a T1DM mouse model [123]. Hence, we used an equivalent dose of I-BET151 (used in the above study) to determine whether macrophage modulation to reduce inflammation can be attained *in vitro*, and whether the secreted factors from these macrophages can affect β -cell function. Our results suggested that I-BET151 can suppress inflammatory cytokine gene expressions such as *Il-1 β* and *Ccl2* in non-activated macrophages, whereas there could be

adverse effects on β -cells as we found reduced expression of genes essential for β -cell function, especially the genes whose polymorphisms are linked to T2DM susceptibility. These adverse effects are not just a reflection of I-BET151 treatment but are due to BET protein inhibition in general as other BET inhibitors similarly inhibit the expression of genes associated with T2DM. Though BET protein inhibition has been implicated in modulating macrophages, and with clinical trials on the horizon, care should be taken when the drug is used because our data suggests that it could adversely affect β -cell function.

We then suggest that upon reducing the dosage of I-BET151, β -cell function could be preserved whilst modulating macrophages to anti-inflammatory phenotype. In other words, by decreasing the concentration, one could maximize the immune benefit but yet not incur the metabolic cost. In order to overcome the challenge that low dose sustained release of I-BET151 is required to elicit the desired response, we devised a PLGA based nano-delivery system capable of controlled and sustained release of I-BET151. More studies on the effectiveness of the delivery system on macrophages and β -cells could shed light on the methods to tailor the dosage released. The overview of this thesis is depicted in Figure 7.1.

Figure 7.1. Overview of research. The flow of research in this thesis is depicted.

7.2 Clinical significance

The therapeutic interventions for T2DM are mainly targeted at the full-fledged stage of T2DM when β -cell mass is low and beyond recovery. Targeting the pre-diabetic stage, when compensatory changes in β -cell mass aids with overcoming peripheral insulin resistance, could help in prevention of T2DM and its associated complications. As obesity and genetic predisposition are key risk factors in the development of T2DM, a person's body weight and family history, together with regular blood glucose monitoring can be used to diagnose pre-diabetes. The challenge for an individual in the pre-diabetes stage is to stop (and possibly) reverse the pathogenesis of diabetes. If left unchecked, β -cell function will wane and β -cell mass will decline and from this point onwards, there is no remission to T2DM. An overt T2DM patient with low β -cell capacity can only manage the disease with no remission [64]. In order to develop therapeutic interventions targeting pre-diabetes, it is essential to understand the underlying mechanisms that occur to islets during

stage. The evidences presented in our study suggest that targeting islet macrophages and perhaps modulating them to adopt an anti-inflammatory, tissue-remodelling, phenotype during pre-diabetes could save the β -cells from losing their function and I-BET151 could be one such drug for this role. Adverse effects of I-BET151 observed on the pancreatic β -cells can be curtailed by adjusting the drug dosage or targeted delivery to islet macrophages, so as to coax islet macrophages to the anti-inflammatory phenotype without negatively affecting β -cell function.

7.3 Novelty of research

Though the role of islet macrophages during early T2DM has been questioned before [86], a comprehensive analysis of islet remodelling and role of islet macrophages during pre-diabetes has not been reported thus far. Short term HFD feeding of C57BL/6J mice for 7 days and increased islet macrophages has been shown by Woodland and colleagues [175], but the study was done at a single time point and the test and control diets were not calorifically matched which means that the HFD treated mice ingested more calories/ g of food compared to the controls. To circumvent this, we have used the calorifically matched test and control diets which offer a more controlled milieu, and studied the metabolic phenotypes, islet remodelling and islet macrophages at 4 different time points within a 3-week window of HFD feeding.

Also, I-BET151 has been shown to have anti-inflammatory effect on macrophages and proliferative effect on β -cells in T1DM [123], whereas our novel finding suggests that I-BET151 could have a detrimental effect on β -cell function. We advise to use BET protein inhibitors with caution as they significantly reduce the expression of genes essential for

pancreatic β -cell function and insulin secretion. Utilizing PLGA nanoparticles to deliver I-BET151 and to modulate drug dosage has also not been reported thus far.

7.4 Future perspectives

We have shown in our studies that the macrophage population is increased in the pre-diabetic stage and that the depletion of macrophages is causing detrimental effect to islet compensation. Finding the exact phenotype of the islet macrophages in pre-diabetes by studying the surface markers and cytokines secreted, a clearer approach to modulation of the macrophages can be attained.

We have seen the reduced β -cell gene expression levels upon exposure to I-BET151 and that these effects are dose dependent. Though reducing the dosage is one approach, elucidating the molecular mechanism behind these effects can shed light on controlling these detrimental effects. Also, studying how these gene expression changes affect the downstream functions and whether these genes are translated to protein expression could be imperative for the safe usage of BET inhibition as a therapeutic approach to diabetes and other diseases. Administering I-BET151 to C57BL/6 mice and feeding with HFD for acute term could reveal the role of BET protein inhibition during pre-diabetes and whether the adverse effects on β -cells observed *in vitro* affect glucose metabolism and islet remodelling *in vivo*.

In this thesis, we have shown a potential nanoparticle system for the delivery of I-BET151 in a controlled manner and for sustained drug release. These findings warrant further investigations to verify the ability of the I-BET151 loaded PLGA nanoparticles on macrophages and pancreatic β -cells *in vitro*. Surface modification of these PLGA

nanoparticles can be tailored to target islet macrophages *in vivo* and deliver I-BET151 specifically to macrophages and not to β -cells.

7.5 Conclusion

Initially shown to be important for overt T2DM, this thesis has expanded the role of islet macrophages to the pre-diabetic stage; this stage is important because it offers a narrow therapeutic window to prevent β -cell dysfunction and death. It is the vital stage in the progression of diabetes with high clinical significance as it provides an opportunity to prevent development of T2DM. Modulating macrophages to perhaps a less inflammatory phenotype during pre-diabetes in the pancreatic islets could save pancreatic β -cells from hyperglycaemia- and insulin resistance- induced β -cell dysfunction, thus preventing development of T2DM. By using BET protein inhibitor, I-BET151, our studies identified reduced inflammatory cytokines in non-activated macrophages *in vitro* (which has not been reported thus far), and hence, this drug could potentially be used for macrophage modulation during pre-diabetes. However, our novel findings also show that I-BET151 should be used with caution as it could be detrimental to β -cells. Attuning the dosage could be a key strategy to elicit a reduced inflammatory response from macrophages without affecting β -cell function. Our studies also suggest that I-BET151 is fast-acting and its presence is constantly required to influence the cells. Both regulation of I-BET151 dosage and sustained delivery can be achieved by PLGA based nanoparticle system designed to deliver the drug. Overall, modulation of macrophages to reduce inflammatory cytokine production in pancreatic islets during pre-diabetes could prevent development of T2DM and this could be achieved by controlled and sustained delivery of BET protein inhibitor, I-BET151.

REFERENCES

1. International Diabetes Federation, *IDF DIABETES ATLAS*. 2015. **7th edition**.
2. *Diabetes*. 2018; Available from: <http://www.who.int/news-room/fact-sheets/detail/diabetes>.
3. World Health Organisation, *10 facts about diabetes*. 2014.
4. *WHO / Diabetes mellitus*. WHO, 2010.
5. Diabetes4cast, *Complications*. 2018.
6. *Diabetes: The War Continues / Ministry of Health*. 2018; Available from: https://www.moh.gov.sg/content/moh_web/home/pressRoom/pressRoomItemRelease/2017/diabetes--the-war-continues.html.
7. Boh, S. *Why are Singaporeans so prone to diabetes? They can't produce enough insulin*, *Singapore News & Top Stories - The Straits Times*. 2017 2017-10-16T14:31+08:00; Available from: <https://www.straitstimes.com/singapore/why-are-singaporeans-so-prone-to-diabetes-they-cant-produce-enough-insulin>.
8. Kahn, S.E., M.E. Cooper, and S. Del Prato, *PATHOPHYSIOLOGY AND TREATMENT OF TYPE 2 DIABETES: PERSPECTIVES ON THE PAST, PRESENT AND FUTURE*. *Lancet*, 2014. **383**(9922): p. 1068-1083.
9. Kahn, S.E., et al., *Quantification of the relationship between insulin sensitivity and beta-cell function in human subjects. Evidence for a hyperbolic function*. *Diabetes*, 1993. **42**(11): p. 1663-72.
10. Barbara A. Ramlo-Halsted, S.V.E., *The Natural History of Type 2 Diabetes: Practical Points to Consider in Developing Prevention and Treatment Strategies*. *Clinical Diabetes*, 2000. **18**(2): p. 80-84.
11. Mazze, R.S., *Staged diabetes management. [electronic resource]*. 2011: Oxford : Wiley-Blackwell, 2011.
3rd ed.
12. Rao, S.S., P. Disraeli, and T. McGregor, *Impaired glucose tolerance and impaired fasting glucose*. *Am Fam Physician*, 2004. **69**(8): p. 1961-8.
13. Gregg Simonson, R.C., Diane Reader and Richard Bergenstal, *International diabetes center treatment of type 2 diabetes glucose algorithm*. *Diabetes management*, 2011. **1**(2): p. 175-189.

-
14. mayoclinic. *Type 2 diabetes - Symptoms and causes*. 2018; Available from: <http://www.mayoclinic.org/diseases-conditions/type-2-diabetes/symptoms-causes/syc-20351193>.
 15. *Polydipsia - Excessive Thirst Causes and Symptoms*. 2018; Available from: <https://www.diabetes.co.uk/symptoms/polydipsia.html>.
 16. *Type 2 Diabetes Symptoms*. 2018; Available from: <https://www.endocrineweb.com/conditions/type-2-diabetes/type-2-diabetes-symptoms>.
 17. *HBA1C - Clinical: Hemoglobin A1c, Blood*. 2018; Available from: <https://www.mayomedicallaboratories.com/test-catalog/Clinical+and+Interpretive/82080>.
 18. *Type 2 diabetes - Diagnosis and treatment - Mayo Clinic*. 2018; Available from: <https://www.mayoclinic.org/diseases-conditions/type-2-diabetes/diagnosis-treatment/drc-20351199>.
 19. Mannarino, M., M. Tonelli, and G.M. Allan, *Screening and diagnosis of type 2 diabetes with HbA(1c)*. Canadian Family Physician, 2013. **59**(1): p. 42-42.
 20. Nathan, D.M., et al., *Impaired Fasting Glucose and Impaired Glucose Tolerance*. Diabetes Care, 2007. **30**(3): p. 753.
 21. t2d_Hu. *Prediabetes - Type2Diabetes.com*. 2018; Available from: <https://type2diabetes.com/what-is-t2d/types/prediabetes/>.
 22. Al-Goblan, A.S., M.A. Al-Alfi, and M.Z. Khan, *Mechanism linking diabetes mellitus and obesity*. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy, 2014. **7**: p. 587-591.
 23. Il'yasova, D., et al., *Prospective Association between Fasting NEFA and Type 2 Diabetes: Impact of Post-load Glucose*. Diabetologia, 2010. **53**(5): p. 866-874.
 24. Roden, M., et al., *Mechanism of free fatty acid-induced insulin resistance in humans*. Journal of Clinical Investigation, 1996. **97**(12): p. 2859-2865.
 25. Lee, S.K., et al., *Defective Glucose-Stimulated Insulin Release from Perfused Islets of C57BL/6J Mice*. Pancreas, 1995. **11**(2): p. 206-211.
 26. Kaiyala, K.J., et al., *Reduced beta-cell function contributes to impaired glucose tolerance in dogs made obese by high-fat feeding*. Am J Physiol, 1999. **277**(4 Pt 1): p. E659-67.

-
27. Mittelman, S.D., et al., *Longitudinal compensation for fat-induced insulin resistance includes reduced insulin clearance and enhanced beta-cell response*. Diabetes, 2000. **49**(12): p. 2116-25.
 28. Kahn, S.E., *The Importance of β -Cell Failure in the Development and Progression of Type 2 Diabetes*. The Journal of Clinical Endocrinology & Metabolism, 2001. **86**(9): p. 4047-4058.
 29. Ali, O., *Genetics of type 2 diabetes*. World Journal of Diabetes, 2013. **4**(4): p. 114-123.
 30. McCarthy, M.I. and E. Zeggini, *Genome-wide association studies in type 2 diabetes*. Current diabetes reports, 2009. **9**(2): p. 164-171.
 31. Billings, L.K. and J.C. Florez, *The genetics of type 2 diabetes: what have we learned from GWAS?* Annals of the New York Academy of Sciences, 2010. **1212**: p. 59-77.
 32. Yamagata, K., et al., *Mutations in the hepatocyte nuclear factor-4 α gene in maturity-onset diabetes of the young (MODY1)*. Nature, 1996. **384**: p. 458.
 33. Dufresne, A.M. and R.J. Smith, *The adapter protein GRB10 is an endogenous negative regulator of insulin-like growth factor signaling*. Endocrinology, 2005. **146**(10): p. 4399-409.
 34. Dooley, J., et al., *Genetic predisposition for beta cell fragility underlies type 1 and type 2 diabetes*. Nat Genet, 2016. **48**(5): p. 519-527.
 35. Park, K.S., *The Search for Genetic Risk Factors of Type 2 Diabetes Mellitus*. Diabetes & Metabolism Journal, 2011. **35**(1): p. 12-22.
 36. Gujral, U.P., et al., *Type 2 diabetes in South Asians: similarities and differences with white Caucasian and other populations*. Annals of the New York Academy of Sciences, 2013. **1281**(1): p. 51-63.
 37. Toyoda, K., et al., *Factors responsible for age-related elevation in fasting plasma glucose: a cross-sectional study in Japanese men*. Metabolism - Clinical and Experimental, 2008. **57**(2): p. 299-303.
 38. Fletcher, B., M. Gulanick, and C. Lamendola, *Risk factors for type 2 diabetes mellitus*. J Cardiovasc Nurs, 2002. **16**(2): p. 17-23.
 39. Calderon, B., et al., *The pancreas anatomy conditions the origin and properties of resident macrophages*. The Journal of Experimental Medicine, 2015. **212**(10): p. 1497.

-
40. Mastracci, T.L. and L. Sussel, *The Endocrine Pancreas: insights into development, differentiation and diabetes*. Wiley interdisciplinary reviews. Membrane transport and signaling, 2012. **1**(5): p. 609-628.
 41. Arrojo e Drigo, R., et al., *New insights into the architecture of the islet of Langerhans: a focused cross-species assessment*. Diabetologia, 2015. **58**(10): p. 2218-2228.
 42. Dolenšek, J., M.S. Rupnik, and A. Stožer, *Structural similarities and differences between the human and the mouse pancreas*. Islets, 2015. **7**(1): p. e1024405.
 43. Goke, B., *Islet cell function: alpha and beta cells--partners towards normoglycaemia*. Int J Clin Pract Suppl, 2008(159): p. 2-7.
 44. Kahn, B.B., *Type 2 Diabetes: When Insulin Secretion Fails to Compensate for Insulin Resistance*. Cell, 1998. **92**(5): p. 593-596.
 45. *17.9 The Endocrine Pancreas* â€“ *Anatomy and Physiology*. 2018; Available from: <https://opentextbc.ca/anatomyandphysiology/chapter/17-9-the-endocrine-pancreas/>.
 46. *Hormones secreted by the pancreas the pancreas - digikalla.info*. 2018.
 47. Reference, G.H. *Type 2 diabetes*. 2018; Available from: <https://www.ncbi.nlm.nih.gov/pubmed/>.
 48. Cefalu, W.T., *Inflammation, Insulin Resistance, and Type 2 Diabetes: Back to the Future?* Diabetes, 2009. **58**(2): p. 307-308.
 49. Luft, V.C., et al., *Chronic inflammation role in the obesity-diabetes association: a case-cohort study*. Diabetology & Metabolic Syndrome, 2013. **5**: p. 31-31.
 50. Imai, Y., et al., *Interaction between cytokines and inflammatory cells in islet dysfunction, insulin resistance, and vascular disease*. Diabetes, obesity & metabolism, 2013. **15**(0 3): p. 117-129.
 51. León-Pedroza, J.I., et al., *Low-grade systemic inflammation and the development of metabolic diseases: From the molecular evidence to the clinical practice*. Cirugía y Cirujanos (English Edition), 2015. **83**(6): p. 543-551.
 52. Mirza, S., et al., *Type 2-Diabetes is Associated With Elevated Levels of TNF-alpha, IL-6 and Adiponectin and Low Levels of Leptin in a Population of Mexican American: A Cross-Sectional Study*. Cytokine, 2012. **57**(1): p. 136-142.

-
53. Greenfield, J.R. and L.V. Campbell, *Relationship between inflammation, insulin resistance and type 2 diabetes: 'cause or effect'?* Curr Diabetes Rev, 2006. **2**(2): p. 195-211.
 54. Zhao, G., et al., *Possible Role of Interleukin-1 β in Type 2 Diabetes Onset and Implications for Anti-inflammatory Therapy Strategies.* PLoS Computational Biology, 2014. **10**(8): p. e1003798.
 55. O'Neill, C.M., et al., *Circulating Levels of IL-1 β +IL-6 Cause ER Stress and Dysfunction in Islets From Prediabetic Male Mice.* Endocrinology, 2013. **154**(9): p. 3077-3088.
 56. Hotamisligil, G.S., N.S. Shargill, and B.M. Spiegelman, *Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance.* Science, 1993. **259**(5091): p. 87-91.
 57. Feinstein, R., et al., *Tumor necrosis factor-alpha suppresses insulin-induced tyrosine phosphorylation of insulin receptor and its substrates.* J Biol Chem, 1993. **268**(35): p. 26055-8.
 58. Donath, M.Y. and S.E. Shoelson, *Type 2 diabetes as an inflammatory disease.* Nature Reviews Immunology, 2011. **11**: p. 98.
 59. Francisco, C.O., et al., *Cytokine profile and lymphocyte subsets in type 2 diabetes.* Brazilian Journal of Medical and Biological Research, 2016. **49**(4): p. e5062.
 60. Shoelson, S.E., J. Lee, and A.B. Goldfine, *Inflammation and insulin resistance.* Journal of Clinical Investigation, 2006. **116**(7): p. 1793-1801.
 61. Sartipy, P. and D.J. Loskutoff, *Monocyte chemoattractant protein 1 in obesity and insulin resistance.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(12): p. 7265-7270.
 62. Weisberg, S.P., et al., *CCR2 modulates inflammatory and metabolic effects of high-fat feeding.* Journal of Clinical Investigation, 2006. **116**(1): p. 115-124.
 63. Cai, D., et al., *Local and systemic insulin resistance resulting from hepatic activation of IKK- β and NF- κ B.* Nature medicine, 2005. **11**(2): p. 183-190.
 64. Taylor, R., et al., *Remission of Human Type 2 Diabetes Requires Decrease in Liver and Pancreas Fat Content but Is Dependent upon Capacity for β Cell Recovery.* Cell Metabolism.

-
65. Donath, M.Y., et al., *Hyperglycemia-induced beta-cell apoptosis in pancreatic islets of *Psammomys obesus* during development of diabetes*. Diabetes, 1999. **48**(4): p. 738-44.
66. Maedler, K., et al., *Glucose induces beta-cell apoptosis via upregulation of the Fas receptor in human islets*. Diabetes, 2001. **50**(8): p. 1683-90.
67. Maedler, K., et al., *Glucose-induced β cell production of IL-1 β contributes to glucotoxicity in human pancreatic islets*. The Journal of Clinical Investigation, 2002. **110**(6): p. 851-860.
68. Collier, J.J., et al., *Pancreatic β -Cell Death in Response to Pro-Inflammatory Cytokines Is Distinct from Genuine Apoptosis*. PLoS ONE, 2011. **6**(7): p. e22485.
69. Donath, M.Y., et al., *Islet Inflammation Impairs the Pancreatic β -Cell in Type 2 Diabetes*. Physiology, 2009. **24**(6): p. 325-331.
70. Donath, M.Y., *Targeting inflammation in the treatment of type 2 diabetes: time to start*. Nat Rev Drug Discov, 2014. **13**(6): p. 465-476.
71. Donath, M.Y. and S.E. Shoelson, *Type 2 diabetes as an inflammatory disease*. Nat Rev Immunol, 2011. **11**(2): p. 98-107.
72. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation*. Nature Reviews Immunology, 2008. **8**: p. 958.
73. Hume, D.A., *The mononuclear phagocyte system*. Curr Opin Immunol, 2006. **18**(1): p. 49-53.
74. Epelman, S., K.J. Lavine, and G.J. Randolph, *Origin and Functions of Tissue Macrophages*. Immunity, 2014. **41**(1): p. 21-35.
75. Honold, L. and M. Nahrendorf, *Resident and Monocyte-Derived Macrophages in Cardiovascular Disease*. Circ Res, 2018. **122**(1): p. 113-127.
76. Italiani, P. and D. Boraschi, *Development and Functional Differentiation of Tissue-Resident Versus Monocyte-Derived Macrophages in Inflammatory Reactions*. Results Probl Cell Differ, 2017. **62**: p. 23-43.
77. Samokhvalov, I.M., *Deconvoluting the ontogeny of hematopoietic stem cells*. Cell Mol Life Sci, 2014. **71**(6): p. 957-78.
78. Orkin, S.H. and L.I. Zon, *Hematopoiesis: an evolving paradigm for stem cell biology*. Cell, 2008. **132**(4): p. 631-44.
79. Mosser, D.M., *The many faces of macrophage activation*. Journal of Leukocyte Biology, 2003. **73**(2): p. 209-212.

-
80. Kraakman, M.J., et al., *Macrophage Polarization in Obesity and Type 2 Diabetes: Weighing Down our Understanding of Macrophage Function?* *Frontiers in Immunology*, 2014. **5**.
 81. Cucak, H., et al., *Macrophage contact dependent and independent TLR4 mechanisms induce beta-cell dysfunction and apoptosis in a mouse model of type 2 diabetes*. *PLoS One*, 2014. **9**(3): p. e90685.
 82. Eguchi, K., et al., *Saturated fatty acid and TLR signaling link beta cell dysfunction and islet inflammation*. *Cell Metab*, 2012. **15**(4): p. 518-33.
 83. Xiao, X., et al., *M2 macrophages promote beta-cell proliferation by up-regulation of SMAD7*. *Proceedings of the National Academy of Sciences*, 2014. **111**(13): p. E1211-E1220.
 84. Tessem, J.S., et al., *Critical roles for macrophages in islet angiogenesis and maintenance during pancreatic degeneration*. *Diabetes*, 2008. **57**(6): p. 1605-17.
 85. Odegaard, J.I. and A. Chawla, *Connecting Type 1 and Type 2 Diabetes through Innate Immunity*. *Cold Spring Harbor Perspectives in Medicine*, 2012. **2**(3): p. a007724.
 86. Morris, D.L., *Minireview: Emerging Concepts in Islet Macrophage Biology in Type 2 Diabetes*. *Mol Endocrinol*, 2015. **29**(7): p. 946-62.
 87. Kamata, K., et al., *Islet amyloid with macrophage migration correlates with augmented β -cell deficits in type 2 diabetic patients*. *Amyloid*, 2014. **21**(3): p. 191-201.
 88. Ehses, J.A., et al., *Increased number of islet-associated macrophages in type 2 diabetes*. *Diabetes*, 2007. **56**(9): p. 2356-70.
 89. Homo-Delarche, F., et al., *Islet inflammation and fibrosis in a spontaneous model of type 2 diabetes, the GK rat*. *Diabetes*, 2006. **55**(6): p. 1625-33.
 90. Nackiewicz, D., et al., *TLR2/6 and TLR4-activated macrophages contribute to islet inflammation and impair beta cell insulin gene expression via IL-1 and IL-6*. *Diabetologia*, 2014. **57**(8): p. 1645-54.
 91. Westwell-Roper, C.Y., J.A. Ehses, and C.B. Verchere, *Resident macrophages mediate islet amyloid polypeptide-induced islet IL-1 β production and beta-cell dysfunction*. *Diabetes*, 2014. **63**(5): p. 1698-711.

-
92. Butcher, M.J., et al., *Association of proinflammatory cytokines and islet resident leucocytes with islet dysfunction in type 2 diabetes*. *Diabetologia*, 2014. **57**(3): p. 491-501.
 93. Cucak, H., L.G. Grunnet, and A. Rosendahl, *Accumulation of M1-like macrophages in type 2 diabetic islets is followed by a systemic shift in macrophage polarization*. *J Leukoc Biol*, 2014. **95**(1): p. 149-60.
 94. Murray, P.J. and T.A. Wynn, *Protective and pathogenic functions of macrophage subsets*. *Nat Rev Immunol*, 2011. **11**(11): p. 723-737.
 95. Sorenson, R.L. and T.C. Brelje, *Adaptation of islets of Langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones*. *Horm Metab Res*, 1997. **29**(6): p. 301-7.
 96. Togashi, Y., et al., *β -Cell Proliferation After a Partial Pancreatectomy Is Independent of IRS-2 in Mice*. *Endocrinology*, 2014. **155**(5): p. 1643-1652.
 97. Dai, C., et al., *Pancreatic Islet Vasculature Adapts to Insulin Resistance Through Dilation and Not Angiogenesis*. *Diabetes*, 2013. **62**(12): p. 4144-4153.
 98. Mantovani, A., et al., *The chemokine system in diverse forms of macrophage activation and polarization*. *Trends Immunol*, 2004. **25**(12): p. 677-86.
 99. Sica, A. and A. Mantovani, *Macrophage plasticity and polarization: in vivo veritas*. *The Journal of Clinical Investigation*, 2012. **122**(3): p. 787-795.
 100. Giorgio, S., *Macrophages: plastic solutions to environmental heterogeneity*. *Inflamm Res*, 2013. **62**(9): p. 835-43.
 101. Liu, Y.C., et al., *Macrophage polarization in inflammatory diseases*. *Int J Biol Sci*, 2014. **10**(5): p. 520-9.
 102. Gordon, S. and P.R. Taylor, *Monocyte and macrophage heterogeneity*. *Nat Rev Immunol*, 2005. **5**(12): p. 953-64.
 103. Vasiliadou, I. and I. Holen, *The role of macrophages in bone metastasis*. *Journal of Bone Oncology*, 2013. **2**(4): p. 158-166.
 104. Zhu, L., et al., *Cellular metabolism and macrophage functional polarization*. *Int Rev Immunol*, 2015. **34**(1): p. 82-100.
 105. Saccani, A., et al., *p50 nuclear factor-kappaB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance*. *Cancer Res*, 2006. **66**(23): p. 11432-40.

-
106. Turner, B.M., *Epigenetic responses to environmental change and their evolutionary implications*. Philosophical Transactions of the Royal Society B: Biological Sciences, 2009. **364**(1534): p. 3403-3418.
 107. de Groot, A.E. and K.J. Pienta, *Epigenetic control of macrophage polarization: implications for targeting tumor-associated macrophages*. Oncotarget, 2018. **9**(29): p. 20908-20927.
 108. *histone / histones*. 2018; Available from: <https://www.nature.com/scitable/definition/histone-histones-57>.
 109. Lu, D., *Epigenetic modification enzymes: catalytic mechanisms and inhibitors*. Acta Pharmaceutica Sinica B, 2013. **3**(3): p. 141-149.
 110. Van den Bossche, J., et al., *Macrophage polarization: the epigenetic point of view*. Curr Opin Lipidol, 2014. **25**(5): p. 367-73.
 111. Ivashkiv, L.B., *Epigenetic regulation of macrophage polarization and function*. Trends Immunol, 2013. **34**(5): p. 216-23.
 112. Khan, S.N. and A.U. Khan, *Role of histone acetylation in cell physiology and diseases: An update*. Clinica Chimica Acta, 2010. **411**(19): p. 1401-1411.
 113. Iyer, A., D.P. Fairlie, and L. Brown, *Lysine acetylation in obesity, diabetes and metabolic disease*. Immunol Cell Biol, 2012. **90**(1): p. 39-46.
 114. Virani, S., et al., *Cancer epigenetics: a brief review*. Ilar j, 2012. **53**(3-4): p. 359-69.
 115. Taniguchi, Y., *The Bromodomain and Extra-Terminal Domain (BET) Family: Functional Anatomy of BET Paralogous Proteins*. International Journal of Molecular Sciences, 2016. **17**(11): p. 1849.
 116. Ntranos, A. and P. Casaccia, *Bromodomains: Translating the words of lysine acetylation into myelin injury and repair*. Neurosci Lett, 2016. **625**: p. 4-10.
 117. Wyce, A., et al., *Inhibition of BET bromodomain proteins as a therapeutic approach in prostate cancer*. Oncotarget, 2013. **4**(12): p. 2419-2429.
 118. Jiang, Y.W., et al., *Mammalian mediator of transcriptional regulation and its possible role as an end-point of signal transduction pathways*. Proc Natl Acad Sci U S A, 1998. **95**(15): p. 8538-43.
 119. Doroshow, D.B., J.P. Eder, and P.M. LoRusso, *BET inhibitors: a novel epigenetic approach*. Annals of Oncology, 2017. **28**(8): p. 1776-1787.

-
120. Devaiah, B.N., et al., *BRD4 is a Histone Acetyltransferase that Evicts Nucleosomes from Chromatin*. Nature structural & molecular biology, 2016. **23**(6): p. 540-548.
 121. Klein, K., et al., *The bromodomain protein inhibitor I-BET151 suppresses expression of inflammatory genes and matrix degrading enzymes in rheumatoid arthritis synovial fibroblasts*. Annals of the Rheumatic Diseases, 2016. **75**(2): p. 422.
 122. Filippakopoulos, P. and S. Knapp, *Targeting bromodomains: epigenetic readers of lysine acetylation*. Nature Reviews Drug Discovery, 2014. **13**: p. 337.
 123. Fu, W., et al., *Epigenetic modulation of type-1 diabetes via a dual effect on pancreatic macrophages and β cells*. eLife, 2014. **3**: p. e04631.
 124. Dawson, M.A., et al., *Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia*. Nature, 2011. **478**: p. 529.
 125. Filippakopoulos, P., et al., *Selective inhibition of BET bromodomains*. Nature, 2010. **468**(7327): p. 1067-1073.
 126. Nicodeme, E., et al., *Suppression of inflammation by a synthetic histone mimic*. Nature, 2010. **468**: p. 1119.
 127. Andrieu, G., A.C. Belkina, and G.V. Denis, *Clinical trials for BET inhibitors run ahead of the science*. Drug Discov Today Technol, 2016. **19**: p. 45-50.
 128. Chaidos, A., V. Caputo, and A. Karadimitris, *Inhibition of bromodomain and extra-terminal proteins (BET) as a potential therapeutic approach in haematological malignancies: emerging preclinical and clinical evidence*. Therapeutic Advances in Hematology, 2015. **6**(3): p. 128-141.
 129. Lehuen, A., *A double-edged sword against type 1 diabetes*. N Engl J Med, 2015. **372**(8): p. 778-80.
 130. Falkenberg, K.J. and R.W. Johnstone, *Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders*. Nat Rev Drug Discov, 2014. **13**(9): p. 673-91.
 131. Kitagawa, Y. and N. Ohkura, *Treating type-1 diabetes with an epigenetic drug*. eLife, 2014. **3**: p. e05720.
 132. Jain, N.K., V. Mishra, and N.K. Mehra, *Targeted drug delivery to macrophages*. Expert Opin Drug Deliv, 2013. **10**(3): p. 353-67.

-
133. Sharma, A., S.V. Madhunapantula, and G.P. Robertson, *Toxicological considerations when creating nanoparticle based drugs and drug delivery systems? Expert opinion on drug metabolism & toxicology*, 2012. **8**(1): p. 47-69.
 134. Shi, J., et al., *Nanotechnology in Drug Delivery and Tissue Engineering: From Discovery to Applications*. Nano letters, 2010. **10**(9): p. 3223-3230.
 135. Kumari, A., S.K. Yadav, and S.C. Yadav, *Biodegradable polymeric nanoparticles based drug delivery systems*. Colloids and Surfaces B: Biointerfaces, 2010. **75**(1): p. 1-18.
 136. Soppimath, K.S., et al., *Biodegradable polymeric nanoparticles as drug delivery devices*. Journal of Controlled Release, 2001. **70**(1-2): p. 1-20.
 137. Imbuluzqueta, E., et al., *Hydrophobic gentamicin-loaded nanoparticles are effective against Brucella melitensis infection in mice*. Antimicrob Agents Chemother, 2013. **57**(7): p. 3326-33.
 138. Imbuluzqueta, E., et al., *Cellular pharmacokinetics and intracellular activity against Listeria monocytogenes and Staphylococcus aureus of chemically modified and nanoencapsulated gentamicin*. Journal of Antimicrobial Chemotherapy, 2012. **67**(9): p. 2158-2164.
 139. Rollett, A., et al., *HSA nanocapsules functionalized with monoclonal antibodies for targeted drug delivery*. International Journal of Pharmaceutics, 2013. **458**(1): p. 1-8.
 140. Bilthariya, U., et al., *Folate-conjugated albumin nanoparticles for rheumatoid arthritis-targeted delivery of etoricoxib*. Drug Development and Industrial Pharmacy, 2015. **41**(1): p. 95-104.
 141. Duan, J., et al., *Polyethyleneimine-functionalized iron oxide nanoparticles for systemic siRNA delivery in experimental arthritis*. Nanomedicine, 2014. **9**(6): p. 789-801.
 142. Pei, Y. and Y. Yeo, *Drug delivery to macrophages: Challenges and opportunities*. Journal of Controlled Release, 2016. **240**: p. 202-211.
 143. Anselmo, A.C. and S. Mitragotri, *Cell-mediated delivery of nanoparticles: Taking advantage of circulatory cells to target nanoparticles*. Journal of Controlled Release, 2014. **190**: p. 531-541.
 144. Batrakova, E.V., H.E. Gendelman, and A.V. Kabanov, *Cell-mediated drug delivery*. Expert Opinion on Drug Delivery, 2011. **8**(4): p. 415-433.

-
145. Tran, T.-H., et al., *Modulation of Macrophage Functional Polarity towards Anti-Inflammatory Phenotype with Plasmid DNA Delivery in CD44 Targeting Hyaluronic Acid Nanoparticles*. Scientific Reports, 2015. **5**: p. 16632.
 146. Purnama, C., et al., *Transient ablation of alveolar macrophages leads to massive pathology of influenza infection without affecting cellular adaptive immunity*. European Journal of Immunology, 2014. **44**(7): p. 2003-2012.
 147. Merglen, A., et al., *Glucose Sensitivity and Metabolism-Secretion Coupling Studied during Two-Year Continuous Culture in INS-1E Insulinoma Cells*. Endocrinology, 2004. **145**(2): p. 667-678.
 148. Åvall, K., et al., *Apolipoprotein CIII links islet insulin resistance to β -cell failure in diabetes*. Proceedings of the National Academy of Sciences, 2015. **112**(20): p. E2611.
 149. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative C(T) method*. Nat Protoc, 2008. **3**(6): p. 1101-8.
 150. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative CT method*. Nature Protocols, 2008. **3**: p. 1101.
 151. Surwit, R.S., et al., *Diet-Induced Type II Diabetes in C57BL/6J Mice*. Diabetes, 1988. **37**(9): p. 1163.
 152. Winzell, M.S. and B. Ahren, *The High-Fat Diet-Fed Mouse*. Diabetes, 2004. **53**(suppl 3): p. S215.
 153. Wang, C.-Y. and J.K. Liao, *A Mouse Model of Diet-Induced Obesity and Insulin Resistance*. Methods in molecular biology (Clifton, N.J.), 2012. **821**: p. 421-433.
 154. Ricci, M., *The Importance of a Proper Control Diet*. Research Diets, Inc. , 2013. **IOPCD-1500-10-14**.
 155. Mosser, R.E., et al., *High-fat diet-induced β -cell proliferation occurs prior to insulin resistance in C57BL/6J male mice*. American Journal of Physiology - Endocrinology and Metabolism, 2015. **308**(7): p. E573-E582.
 156. Ahren, B. and G. Pacini, *Insufficient islet compensation to insulin resistance vs. reduced glucose effectiveness in glucose-intolerant mice*. Am J Physiol Endocrinol Metab, 2002. **283**(4): p. E738-44.
 157. Reimer, M.K. and B. Ahren, *Altered beta-cell distribution of pdx-1 and GLUT-2 after a short-term challenge with a high-fat diet in C57BL/6J mice*. Diabetes, 2002. **51 Suppl 1**: p. S138-43.

-
158. Yang, G., et al., *Assessment of Insulin Resistance in Subjects with Normal Glucose Tolerance, Hyperinsulinemia with Normal Blood Glucose Tolerance, Impaired Glucose Tolerance, and Newly Diagnosed Type 2 Diabetes (Prediabetes Insulin Resistance Research)*. Journal of Diabetes Research, 2016. **2016**: p. 9270768.
159. Reaven, G.M., *Insulin resistance: from bit player to centre stage*. CMAJ : Canadian Medical Association Journal, 2011. **183**(5): p. 536-537.
160. Linnemann, A.K., M. Baan, and D.B. Davis, *Pancreatic β -Cell Proliferation in Obesity*. Advances in Nutrition, 2014. **5**(3): p. 278-288.
161. Assmann, A., C. Hinault, and R.N. Kulkarni, *Growth factor control of pancreatic islet regeneration and function*. Pediatric diabetes, 2009. **10**(1): p. 14-32.
162. Patan, S., *Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling*. J Neurooncol, 2000. **50**(1-2): p. 1-15.
163. Carlsson, P.O., A. Andersson, and L. Jansson, *Pancreatic islet blood flow in normal and obese-hyperglycemic (ob/ob) mice*. Am J Physiol, 1996. **271**(6 Pt 1): p. E990-5.
164. Carlsson, P.O., A. Andersson, and L. Jansson, *Influence of age, hyperglycemia, leptin, and NPY on islet blood flow in obese-hyperglycemic mice*. Am J Physiol, 1998. **275**(4 Pt 1): p. E594-601.
165. Pisacane, A.M., F. Picciotto, and M. Risio, *CD31 and CD34 expression as immunohistochemical markers of endothelial transdifferentiation in human cutaneous melanoma*. Cell Oncol, 2007. **29**(1): p. 59-66.
166. Liu, L. and G.-P. Shi, *CD31: beyond a marker for endothelial cells*. Cardiovascular Research, 2012. **94**(1): p. 3-5.
167. Gupta, P., et al., *Tissue-Resident CD169+Macrophages Form a Crucial Front Line against Plasmodium Infection*. Cell Reports, 2016. **16**(6): p. 1749-1761.
168. Chávez-Galán, L., et al., *Much More than M1 and M2 Macrophages, There are also CD169(+) and TCR(+) Macrophages*. Frontiers in Immunology, 2015. **6**: p. 263.
169. Jansen, A., et al., *Immunohistochemical Characterization of Monocytes-Macrophages and Dendritic Cells Involved in the Initiation of the Insulitis and β -Cell Destruction in NOD Mice*. Diabetes, 1994. **43**(5): p. 667.

-
170. Thornley, T.B., et al., *Contrasting Roles of Islet Resident Immunoregulatory Macrophages and Dendritic Cells in Experimental Autoimmune Type 1 Diabetes*. PLoS ONE, 2016. **11**(3): p. e0150792.
171. Hwang, L.L., et al., *Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice*. Obesity (Silver Spring), 2010. **18**(3): p. 463-9.
172. Samuelsson, A.M., et al., *Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming*. Hypertension, 2008. **51**(2): p. 383-92.
173. Murray, P.J. and T.A. Wynn, *Protective and pathogenic functions of macrophage subsets*. Nat Rev Immunol, 2011. **11**(11): p. 723-37.
174. Eguchi, K. and I. Manabe, *Macrophages and islet inflammation in type 2 diabetes*. Diabetes Obes Metab, 2013. **15 Suppl 3**: p. 152-8.
175. Woodland, D.C., et al., *Short-term high-fat feeding induces islet macrophage infiltration and β -cell replication independent of insulin resistance in mice*. American Journal of Physiology - Endocrinology And Metabolism, 2016.
176. Bu, L., et al., *Intraperitoneal Injection of Clodronate Liposomes Eliminates Visceral Adipose Macrophages and Blocks High-fat Diet-induced Weight Gain and Development of Insulin Resistance*. The AAPS Journal, 2013. **15**(4): p. 1001-1011.
177. Martinez, F.O. and S. Gordon, *The M1 and M2 paradigm of macrophage activation: time for reassessment*. F1000Prime Reports, 2014. **6**: p. 13.
178. Ohradanova-Repic, A., et al., *Differentiation of human monocytes and derived subsets of macrophages and dendritic cells by the HLDA10 monoclonal antibody panel*. Clin Trans Immunol, 2016. **5**: p. e55.
179. Davies, L.C., et al., *Tissue-resident macrophages*. Nature Immunology, 2013. **14**: p. 986.
180. Shapouri-Moghaddam, A., et al., *Macrophage plasticity, polarization, and function in health and disease*. Journal of Cellular Physiology, 2018. **233**(9): p. 6425-6440.
181. Liu, G. and H. Yang, *Modulation of macrophage activation and programming in immunity*. Journal of Cellular Physiology, 2012. **228**(3): p. 502-512.
182. Amber, I.J., et al., *Activated macrophage conditioned medium: identification of the soluble factors inducing cytotoxicity and the L-arginine dependent effector mechanism*. J Leukoc Biol, 1991. **49**(6): p. 610-20.

-
183. Constant, V.A., et al., *Macrophage-conditioned medium inhibits the differentiation of 3T3-L1 and human abdominal preadipocytes*. Diabetologia, 2006. **49**(6): p. 1402-11.
184. Hampton, R.Y. and C.R. Raetz, *Macrophage catabolism of lipid A is regulated by endotoxin stimulation*. Journal of Biological Chemistry, 1991. **266**(29): p. 19499-19509.
185. Meng, F. and C.A. Lowell, *Lipopolysaccharide (LPS)-induced Macrophage Activation and Signal Transduction in the Absence of Src-Family Kinases Hck, Fgr, and Lyn*. The Journal of Experimental Medicine, 1997. **185**(9): p. 1661-1670.
186. Mosser, D.M. and X. Zhang, *Activation of Murine Macrophages*. Current protocols in immunology / edited by John E. Coligan ... [et al.], 2008. **CHAPTER**: p. Unit.
187. Langston, P.K., M. Shibata, and T. Horng, *Metabolism Supports Macrophage Activation*. Frontiers in Immunology, 2017. **8**: p. 61.
188. Barrett, E., et al., *I-BET151 selectively regulates IL-6 production*. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2014. **1842**(9): p. 1549-1555.
189. Kooner, J.S., et al., *Genome-wide association study in individuals of South Asian ancestry identifies six new type 2 diabetes susceptibility loci*. Nat Genet, 2011. **43**(10): p. 984-989.
190. Steimle, A., I.B. Autenrieth, and J.-S. Frick, *Structure and function: Lipid A modifications in commensals and pathogens*. International Journal of Medical Microbiology, 2016. **306**(5): p. 290-301.
191. Yamamoto, M. and S. Akira, *Lipid A receptor TLR4-mediated signaling pathways*. Adv Exp Med Biol, 2010. **667**: p. 59-68.
192. Rossol, M., et al., *LPS-induced cytokine production in human monocytes and macrophages*. Crit Rev Immunol, 2011. **31**(5): p. 379-446.
193. Matsuoka, T.-a., et al., *Members of the Large Maf Transcription Family Regulate Insulin Gene Transcription in Islet β Cells*. Molecular and Cellular Biology, 2003. **23**(17): p. 6049-6062.
194. Matsuoka, T.A., et al., *The MafA transcription factor appears to be responsible for tissue-specific expression of insulin*. Proc Natl Acad Sci U S A, 2004. **101**(9): p. 2930-3.
195. Stolarczyk, E., et al., *Loss of sugar detection by GLUT2 affects glucose homeostasis in mice*. PLoS One, 2007. **2**(12): p. e1288.

-
196. Database, G.H.G., *GCK Gene - GeneCards / HXK4 Protein / HXK4 Antibody*. 2018.
197. *GCK glucokinase [Homo sapiens (human)] - Gene - NCBI*. 2018; Available from: <https://www.ncbi.nlm.nih.gov/pubmed/>.
198. Shiao, M.-S., et al., *Adaptive Evolution of the Insulin Two-Gene System in Mouse*. Genetics, 2008. **178**(3): p. 1683-1691.
199. *Ins1 insulin I [Mus musculus (house mouse)] - Gene - NCBI*. 2018; Available from: <https://www.ncbi.nlm.nih.gov/pubmed/>.
200. *Ins2 insulin II [Mus musculus (house mouse)] - Gene - NCBI*. 2018; Available from: <https://www.ncbi.nlm.nih.gov/pubmed/>.
201. Database, G.H.G., *MAFA Gene - GeneCards / MAFA Protein / MAFA Antibody*. 2018.
202. *NEUROD1 - Neurogenic differentiation factor 1 - Homo sapiens (Human) - NEUROD1 gene & protein*. 2018; Available from: <https://www.uniprot.org/uniprot/Q13562>.
203. *NEUROD1 neuronal differentiation 1 [Homo sapiens (human)] - Gene - NCBI*. 2018; Available from: <https://www.ncbi.nlm.nih.gov/pubmed/>.
204. Database, G.H.G., *PDX1 Gene - GeneCards / PDX1 Protein / PDX1 Antibody*. 2018.
205. Database, G.H.G., *SLC2A2 Gene - GeneCards / GTR2 Protein / GTR2 Antibody*. 2018.
206. Efrat, S., *Making sense of glucose sensing*. Nature Genetics, 1997. **17**: p. 249.
207. Wang, C., Y. Guan, and J. Yang, *Cytokines in the Progression of Pancreatic β -Cell Dysfunction*. International Journal of Endocrinology, 2010. **2010**: p. 10.
208. Halban, P.A., et al., *β -Cell Failure in Type 2 Diabetes: Postulated Mechanisms and Prospects for Prevention and Treatment*. Diabetes Care, 2014. **37**(6): p. 1751.
209. Wachlin, G., et al., *IL-1 β , IFN- γ and TNF- α increase vulnerability of pancreatic beta cells to autoimmune destruction*. Journal of Autoimmunity, 2003. **20**(4): p. 303-312.
210. Cerf, M.E., *Beta Cell Dysfunction and Insulin Resistance*. Frontiers in Endocrinology, 2013. **4**: p. 37.
211. *TCF7L2 transcription factor 7 like 2 [Homo sapiens (human)] - Gene - NCBI*. 2018; Available from: <https://www.ncbi.nlm.nih.gov/pubmed/>.

-
212. Gondoin, A., et al., *Identification of insulin-sensitizing molecules acting by disrupting the interaction between the Insulin Receptor and Grb14*. Scientific Reports, 2017. **7**(1): p. 16901.
213. *GRB14 growth factor receptor bound protein 14 [Homo sapiens (human)]* - Gene - NCBI. 2018; Available from: <https://www.ncbi.nlm.nih.gov/pubmed/>.
214. Wang, H., et al., *Hepatocyte nuclear factor 4alpha regulates the expression of pancreatic beta -cell genes implicated in glucose metabolism and nutrient-induced insulin secretion*. J Biol Chem, 2000. **275**(46): p. 35953-9.
215. Kang, H.S., et al., *Transcription factor Glis3, a novel critical player in the regulation of pancreatic beta-cell development and insulin gene expression*. Mol Cell Biol, 2009. **29**(24): p. 6366-79.
216. *Genes linked to diabetes risk*. Nature, 2016. **531**: p. 552.
217. Dooley, J., et al., *Genetic predisposition for beta cell fragility underlies type 1 and type 2 diabetes*. Nature Genetics, 2016. **48**: p. 519.
218. *MANF mesencephalic astrocyte derived neurotrophic factor [Homo sapiens (human)]* - Gene - NCBI. 2018; Available from: <https://www.ncbi.nlm.nih.gov/pubmed/>.
219. Lindahl, M., et al., *MANF Is Indispensable for the Proliferation and Survival of Pancreatic β Cells*. Cell Reports, 2014. **7**(2): p. 366-375.
220. Tsonkova, V.G., et al., *The EndoC- β H1 cell line is a valid model of human beta cells and applicable for screenings to identify novel drug target candidates*. Molecular Metabolism, 2018. **8**: p. 144-157.
221. Zhou, Y., et al., *TCF7L2 is a master regulator of insulin production and processing*. Human Molecular Genetics, 2014. **23**(24): p. 6419-6431.
222. Bensaude, O., *Inhibiting eukaryotic transcription: Which compound to choose? How to evaluate its activity?* Transcription, 2011. **2**(3): p. 103-108.
223. Jawahar, N. and S. Meyyanathan, *Polymeric nanoparticles for drug delivery and targeting: A comprehensive review*. International Journal of Health & Allied Sciences, 2012. **1**(4): p. 217-223.
224. Xu, J., et al., *Controllable Microfluidic Production of Drug-Loaded PLGA Nanoparticles Using Partially Water-Miscible Mixed Solvent Microdroplets as a Precursor*. Scientific Reports, 2017. **7**: p. 4794.

-
225. Chan, C.H., et al., *BET Bromodomain Inhibition Suppresses Transcriptional Responses to Cytokine-Jak-STAT Signaling in a Gene-specific Manner in Human Monocytes*. European journal of immunology, 2015. **45**(1): p. 287-297.
226. Belkina, A.C., B.S. Nikolajczyk, and G.V. Denis, *BET protein function is required for inflammation: Brd2 genetic disruption and BET inhibitor JQ1 impair mouse macrophage inflammatory responses*. J Immunol, 2013. **190**(7): p. 3670-8.
227. Wienerroither, S., et al., *Regulation of NO Synthesis, Local Inflammation, and Innate Immunity to Pathogens by BET Family Proteins*. Molecular and Cellular Biology, 2014. **34**(3): p. 415-427.
228. Meng, S., et al., *BET Inhibitor JQ1 Blocks Inflammation and Bone Destruction*. Journal of Dental Research, 2014. **93**(7): p. 657-662.
229. Prentki, M. and C.J. Nolan, *Islet β cell failure in type 2 diabetes*. Journal of Clinical Investigation, 2006. **116**(7): p. 1802-1812.
230. Wajchenberg, B.L., *Clinical Approaches to Preserve β -Cell Function in Diabetes*, in *The Islets of Langerhans*, M.S. Islam, Editor. 2010, Springer Netherlands: Dordrecht. p. 515-535.
231. Dor, Y., et al., *Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation*. Nature, 2004. **429**(6987): p. 41-6.
232. Bonner-Weir, S. and G.C. Weir, *Chapter 33 - Adult Progenitor Cells as a Potential Treatment for Diabetes*, in *Essentials of Stem Cell Biology (Third Edition)*, R. Lanza and A. Atala, Editors. 2014, Academic Press: Boston. p. 491-500.
233. Kim, H.S. and M.K. Lee, *β -Cell regeneration through the transdifferentiation of pancreatic cells: Pancreatic progenitor cells in the pancreas*. Journal of Diabetes Investigation, 2016. **7**(3): p. 286-296.
234. *Beta-cell Transdifferentiation*. 2010 2010-07-19; Available from: <https://www.broadinstitute.org/project-beta-cell-biology-regenerative-medicine/beta-cell-transdifferentiation>.
235. Chaudhury, A., et al., *Clinical Review of Antidiabetic Drugs: Implications for Type 2 Diabetes Mellitus Management*. Frontiers in Endocrinology, 2017. **8**: p. 6.
236. DeFronzo, R.A. and M.A. Abdul-Ghani, *Preservation of β -Cell Function: The Key to Diabetes Prevention*. The Journal of Clinical Endocrinology & Metabolism, 2011. **96**(8): p. 2354-2366.

-
237. van Baar, M.J.B., et al., *SGLT2 Inhibitors in Combination Therapy: From Mechanisms to Clinical Considerations in Type 2 Diabetes Management*. Diabetes Care, 2018. **41**(8): p. 1543.
 238. Wu, M.S., et al., *Effect of metformin on carbohydrate and lipoprotein metabolism in NIDDM patients*. Diabetes Care, 1990. **13**(1): p. 1-8.
 239. Harrison, L.B., et al., *β -Cell Function Preservation After 3.5 Years of Intensive Diabetes Therapy*. Diabetes Care, 2012. **35**(7): p. 1406-1412.
 240. van Greevenbroek, M.M., C.G. Schalkwijk, and C.D. Stehouwer, *Obesity-associated low-grade inflammation in type 2 diabetes mellitus: causes and consequences*. Neth J Med, 2013. **71**(4): p. 174-87.
 241. Duncan, B.B. and M.I. Schmidt, *The epidemiology of low-grade chronic systemic inflammation and type 2 diabetes*. Diabetes Technol Ther, 2006. **8**(1): p. 7-17.