CELL- AND MATRIX-BASED APPROACHES FOR IMPROVEMENT OF ISLET
β-CELL SURVIVAL AND FUNCTION IN VITRO AND IN VIVO

by

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Abstract

Islet transplantation provides a feasible approach for treatment of type 1 diabetes (T1D), in which islet β-cells are destroyed by autoimmune attack. However, its efficacy is currently limited by poor long-term islet graft survival. Loss of islet extracellular matrix (ECM) and formation of human islet amyloid polypeptide (hIAPP) aggregates are two important non-immunological factors contributing to β-cell destruction. In this study, we developed novel matrix- and cell-based approaches under 3 different objectives. We hypothesized that these new strategies can protect islet β-cells from the toxicity induced by non-immune or autoimmune factors thereby enhancing the viability and function of islet β-cells in both in vitro and in vivo diabetes models.

To provide surrogate ECM materials for islets, three-dimensional scaffolds, collagen matrix (CM) alone and human fibroblasts-populated collagen matrix (FPCM) were developed in objective 1. Isolated human islets were then either embedded within the scaffolds or cultured in two-dimensional free-floating condition (control) for 7 days. The findings showed markedly lower formation of hIAPP aggregates in the scaffold-embedded islets as compared to control islets. The morphology, viability, and functionality of ECM-populated islets were significantly improved as compared to control islets.

In objective 2, we evaluated the signaling pathways involved in the cytotoxicity induced by hIAPP aggregation. Protein kinase B (PKB), a key effector of the pro-survival phosphoinositide 3 (PI3)-kinase signaling pathway was studied. Our findings demonstrated that both exogenously applied and endogenously formed hIAPP aggregates reduce PKB activation in islet β-cells, likely via IL-1β signaling.
Autoimmunity is another causative factor of β-cell destruction during islet transplantation and T1D. In objective 3, we aimed to prevent the progression of T1D in non-obese diabetic (NOD) mice. We used dermal fibroblasts expressing indoleamine 2,3-dioxygenase (IDO), which is an immuno-modulating enzyme. We found that intraperitoneal injection of $15 \times 10^6$ dermal fibroblasts into NOD mice successfully reversed the progression of T1D through inhibiting the β-cell specific autoreactive T cells and Th17 cells, as well as inducing regulatory T cells.

Collectively, the approaches developed in this study can reduce the destruction of islet β-cells by non-immune and autoimmune factors thereby enhancing viability and function of islet β-cells in diabetes.
Preface

Human studies were reviewed and approved by the Research Ethics Board of the University of British Columbia (Protocol numbers: H05-70537 and H14-00442). Animal studies were reviewed and approved by the University of British Columbia Committee on Animal Care (Protocol numbers: A10-0372 and A13-0042). Y. Zhang was trained for Animal Ethics online course (Certificate number: 4417-10) and practical course of Rodent Biology and Husbandry (Certificate number: RBH-146-11).

In Chapter 2, Y. Zhang, Dr. R. B. Jalili, Dr. L. Marzban and Dr. A. Ghahary generated the hypothesis and designed the study. Y. Zhang carried out the majority of the experiments, analyzed the data and wrote the manuscript. Dr. R. B. Jalili contributed to the experimental design and performed a part of experiments. Dr. Z. Ao from Ike Barber Human Islet Transplant Laboratory headed by Dr. G. L. Warnock isolated human islets for this study. Dr. A. Ghahary and Dr. L. Marzban edited the manuscript. A version of this chapter has been published as Zhang, Y. et al., (2012) Three-dimensional scaffolds reduce islet amyloid formation and enhance survival and function of cultured human islets. Am J Pathol, 181: 1296-1305. The journal granted permission for the author to include the published materials in this thesis.

In Chapter 3, Y. Zhang and Dr. L. Marzban generated the hypothesis and designed the study. Y. Zhang performed the experiments and wrote the manuscript. Dr. L. Marzban reviewed and edited the manuscript. N. Safikhan took the responsibility of care and maintenance of transgenic (hIAPP+/−) mice and conducted mouse islet isolation. Y. J. Park assisted with Western blot analysis. Dr. Z. Ao from Ike Barber Human Islet Transplant Laboratory headed by Dr. G. L.
Warnock isolated human islets for this study. Dr. A. Ghahary contributed to the data and manuscript review. A version of this chapter has been submitted to a peer-reviewed journal.

In Chapter 4, Y. Zhang, Dr. R. B. Jalili and Dr. A. Ghahary conceived and designed the study. Y. Zhang performed the majority of the experiments and wrote the manuscript. Dr. A. Ghahary reviewed and edited the manuscript. Dr. R. B. Jalili helped me with a part of experiments and data analysis. Dr. R. T. Kilani prepared the lenti-viral vector transduced fibroblasts. S. Salimi assisted me with kynurenine analysis. A. Farokhi measured the c-peptide levels. Dr. M. Khosravi contributed to mouse islet isolation. Dr. A. Hosseini-Tabatabaei helped to do a part of animal monitoring work. Dr. Z. Ao from Ike Barber Human Islet Transplant Laboratory headed by Dr. G. L. Warnock isolated human islets for this study. Dr. L. Marzban contributed to the data and manuscript review. A version of this chapter has been submitted to a peer-reviewed journal.
Table of Contents

Abstract.............................................................................................................................................. ii
Preface................................................................................................................................................... iv
Table of Contents ................................................................................................................................. vi
List of Tables ......................................................................................................................................... xii
List of Figures ....................................................................................................................................... xiii
List of Abbreviations .......................................................................................................................... xvi
Acknowledgements ............................................................................................................................... xxiv
Dedication .............................................................................................................................................. xxvi

Chapter 1: Introduction .......................................................................................................................... 1

1.1 Type 1 diabetes ................................................................................................................................ 1

1.1.1 Epidemiology of T1D .................................................................................................................. 2
1.1.2 Etiology of T1D .......................................................................................................................... 2
1.1.3 Pathogenesis of T1D .................................................................................................................. 4

1.1.3.1 Initiation of immune responses in T1D ................................................................................. 5
1.1.3.2 The role of different cells in immune responses in T1D ....................................................... 5

1.1.4 Complications and comorbidities ............................................................................................... 8
1.1.5 Diagnosis .................................................................................................................................... 9

1.1.6 Treatment of T1D .................................................................................................................... 10

1.1.6.1 Insulin therapy ....................................................................................................................... 10
1.1.6.2 Pancreas and islet transplantation .................................................................................... 11
1.1.6.3 Other potential therapy ....................................................................................................... 13

1.2 Amyloidogenesis ........................................................................................................................... 15
1.2.1 Amyloid formation and human diseases................................................. 15
1.2.2 IAPP aggregation and islet amyloid deposition...................................... 16
1.2.3 Cytotoxic mechanisms of IAPP aggregates........................................... 19
1.2.4 Islet amyloid and type 2 diabetes........................................................... 21
1.2.5 Amyloid formation and islet transplantation ........................................... 22

1.3 Islet β-cell survival signaling pathways..................................................... 23
1.3.1 Protein Kinase B (PKB) signaling .......................................................... 23
  1.3.1.1 Isoforms and structure of PKB ......................................................... 23
  1.3.1.2 Process of PKB activation ................................................................. 25
    1.3.1.2.1 PI3-kinase-dependent activation of PKB (Figure 1.5)..................... 25
    1.3.1.2.2 PI3-kinase-independent activation of PKB .................................. 28
  1.3.1.3 Regulation of cell survival by PKB .................................................. 29

1.3.2 Other major signaling pathways in islet β-cell survival .......................... 31

1.4 Immunological self-tolerance ..................................................................... 32
  1.4.1 Amino acid catabolism and immune self-tolerance ............................... 33
  1.4.2 Indoleamine 2,3-dioxygenase .............................................................. 34
    1.4.2.1 Structure and expression of IDO ...................................................... 35
    1.4.2.2 IDO and immune regulation .......................................................... 37
    1.4.2.3 Role of IDO in specific physiological and pathological conditions ..... 40
      1.4.2.3.1 IDO and mammalian pregnancy ............................................. 40
      1.4.2.3.2 IDO and autoimmune conditions ......................................... 40
      1.4.2.3.3 IDO and transplantation ....................................................... 41
      1.4.2.3.4 IDO and cancer ................................................................. 42
1.4.2.3.5 IDO and neurological diseases

1.5 Rationale, hypothesis and objectives

Chapter 2: Three-dimensional scaffolds reduce islet amyloid formation and enhance survival and function of cultured human islets

2.1 Introduction

2.2 Materials and methods

2.2.1 Preparation of human fibroblasts

2.2.2 Culture of human islets

2.2.3 Preparation of FPCM and CM islet composites

2.2.4 Islet viability assay

2.2.5 Glucose-stimulated insulin secretion

2.2.6 Immunohistochemistry and detection of islet amyloid

2.2.7 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

2.2.8 Statistical analysis

2.3 Results

2.3.1 Three-dimensional scaffolds improve viability of human islets during in vitro culture.

2.3.2 Human islets embedded within three-dimensional scaffolds have lower β-cell apoptosis and higher islet β/α cell ratio compared with two-dimensional cultured islets.

2.3.3 Three-dimensional scaffolds preserve insulin, PDX1, and GLUT2 mRNA expression in cultured human islets.

2.3.4 Three-dimensional scaffolds improve β-cell function in cultured human islets.
2.3.5 Amyloid formation is significantly lower in human islets embedded within three-dimensional scaffolds than two-dimensional cultured islets .......................................................... 62

2.3.6 Embedding human islets within three-dimensional scaffolds reduces islet Fas expression in cultured human islets ........................................................................................................ 65

2.4 Discussion .................................................................................................................................................. 67

Chapter 3: Amyloid formation reduces phosphorylated PKB levels in cultured islet β-cells via IL-1β signaling pathway ................................................................................................................. 71

3.1 Introduction .................................................................................................................................................. 71

3.2 Materials and methods .......................................................................................................................... 72

3.2.1 Culture of human islets .......................................................................................................................... 72

3.2.2 Animal models .......................................................................................................................................... 73

3.2.3 Mouse islet isolation and culture ............................................................................................................. 73

3.2.4 Culture and treatment of transformed β-cells ......................................................................................... 74

3.2.5 Immunohistochemistry, TUNEL assay and thioflavin S staining ......................................................... 75

3.2.6 Immunoblotting for phosphorylated PKB ............................................................................................ 76

3.2.7 Statistical analysis .................................................................................................................................. 77

3.3 Results ....................................................................................................................................................... 77

3.3.1 Exogenously applied hIAPP reduces β-cell phospho-PKB levels, which is associated with decreased β-cell proliferation and increased β-cell apoptosis ......................................... 77

3.3.2 Endogenous hIAPP aggregates inhibit PKB phosphorylation in human and hIAPP+/− transgenic mouse islet β-cells ............................................................................................................. 79

3.3.3 Prevention of hIAPP aggregation during islet culture by collagen matrix (CM) restores β-cell phospho-PKB levels and markedly reduces β-cell apoptosis ............................................ 81
3.3.4 Inhibition of islet amyloid formation by the amyloid-binding dye Congo red (CR) or collagen matrix (CM) decreases IL-1β expression in islet β-cells during in vitro culture. .. 85
3.3.5 Blockade of IL-1β signaling by anakinra or exenatide restores phospho-PKB levels in human islet β-cells during culture................................................................. 87

3.4 Discussion.............................................................................................................. 89

Chapter 4: Intraperitoneal injection of IDO-expressing fibroblasts protects islet β-cells from immunological attack and reverses hyperglycemia in non-obese diabetic mice............92

4.1 Introduction............................................................................................................ 92

4.2 Materials and methods .......................................................................................... 93

4.2.1 Mice .................................................................................................................. 93

4.2.2 Construction of lenti-viral vectors .................................................................... 94

4.2.3 Isolation, culture and lenti-viral infection of fibroblasts ...................................... 94

4.2.4 Assessment of diabetes and treatment protocol .................................................. 95

4.2.5 Analyses of indoleamine 2,3-dioxygenase (IDO) activity .................................... 96

4.2.6 Evaluation of islet function ............................................................................... 96

4.2.7 Histological analyses and immunostainings ....................................................... 97

4.2.8 Flow cytometry .................................................................................................. 98

4.2.9 In vitro islet studies ............................................................................................ 98

4.2.10 Statistical analysis ............................................................................................. 99

4.3 Results..................................................................................................................... 99

4.3.1 Intraperitoneal injection of 15M IDO-expressing fibroblasts inhibits the progression of diabetes in NOD mice......................................................................................... 99

4.3.2 Enzymatic activity of IDO is a key factor in its therapeutic efficacy ................. 102
4.3.3 Intraperitoneal injection of 15M IDO-expressing fibroblasts improves the functionality of the remaining islet β-cells in newly diabetic NOD mice .................. 104

4.3.4 Intraperitoneal injection of 15M IDO-expressing fibroblasts reduces the infiltration of lymphocytes in islets and maintains insulin-producing islet β-cells in NOD mice........ 105

4.3.5 Intraperitoneal injection of 15M IDO-expressing fibroblasts in NOD mice prevents the accumulation of β-cell specific cytotoxic lymphocytes (CTLs) and IL-17-producing CD4⁺ T cells (Th17 cells) ........................................................................................................ 107

4.3.6 Intraperitoneal injection of 15M IDO-expressing fibroblasts increases the frequency of regulatory T cells (Tregs) ........................................................................................................................................ 110

4.3.7 Islets co-cultured with IDO-expressing fibroblasts have reduced IL-1β expression and β-cell apoptosis .......................................................................................................................... 112

4.4 Discussion ........................................................................................................................................................................ 115

Chapter 5: Conclusions and discussions ......................................................................................................................... 121

5.1 Summary and discussion.................................................................................................................................................. 121

5.2 Significance..................................................................................................................................................................... 128

5.3 Future studies ................................................................................................................................................................. 130

References ........................................................................................................................................................................ 133
List of Tables

Table 2.1 Primary antibodies for immunolabelling human islet sections ........................................... 52
Table 2.2 Secondary antibodies for immunolabelling human islet sections ......................................... 53
Table 2.3 Primers used for RT-PCR ........................................................................................................ 54
Table 3.1 Primary antibodies for immunolabelling islet sections and cells ........................................... 75
Table 3.2 Secondary antibodies for immunolabelling islet sections and cells ..................................... 76
Table 4.1 Primary antibodies for immunolabelling pancreas or islet sections ...................................... 97
Table 4.2 Secondary antibodies for immunolabelling pancreas or islet sections ................................. 98
List of Figures

Figure 1.1 Processing of preproIAPP to mature IAPP ................................................................. 17
Figure 1.2 The amino acid sequence of IAPP in mouse, rat and human. ........................................... 18
Figure 1.3 The schematic process of islet amyloid formation. ............................................................ 19
Figure 1.4 The schematic structure of PKB isoforms ........................................................................ 25
Figure 1.5 PI3-kinase-dependent process of PKB activation ............................................................... 28
Figure 1.6 Three major mechanisms of PKB-mediated cell survival .................................................. 31
Figure 1.7 Tryptophan metabolism along the kynurenine pathway .................................................... 35
Figure 1.8 IDO-mediated immunomodulation via metabolic and signaling pathways ....................... 39
Figure 2.1 Three-dimensional scaffolds improve viability of cultured human islets ....................... 56
Figure 2.2 Caspase-3 activation was markedly reduced in islet β-cells of human islets cultured in
three-dimensional scaffolds, compared with two-dimensional cultured islets .............................. 58
Figure 2.3 Human islets cultured in three-dimensional scaffolds have higher β/α cell ratios,
compared with two-dimensional cultured islets .................................................................................. 59
Figure 2.4 Three-dimensional scaffolds preserve expression of insulin, PDX1, and GLUT2 in
cultured human islets .................................................................................................................. 60
Figure 2.5 Three-dimensional scaffolds enhance β-cell function in human islets during in vitro
culture. .................................................................................................................................... 61
Figure 2.6 Human islets cultured in three-dimensional scaffolds have markedly reduced islet
amyloid formation, compared with two-dimensional cultured islets ........................................... 63
Figure 2.7 In cultured human islets, amyloid-positive islets closely correlate with active caspase-3-positive islet β-cells .......................................................................................... 64
**Figure 2.8** Reduced islet amyloid formation in human islets cultured in three-dimensional scaffolds is associated with lower Fas expression. ................................................................. 66

**Figure 3.1** Aggregates of exogenously applied hIAPP inhibit PKB phosphorylation (activation) in INS-1 β-cells, reduce β-cell proliferation and increase β-cell apoptosis. ........................................... 78

**Figure 3.2** Endogenous hIAPP aggregates reduce phospho-PKB levels in primary islet β-cells during *in vitro* culture. ........................................................................................................... 80

**Figure 3.3** Three-dimensional collagen matrix (CM)-embedded hIAPP<sup>+/−</sup> transgenic mouse islets have markedly decreased hIAPP aggregates and β-cell apoptosis, and increased phosphorylated PKB levels as compared with free-floating (FF) cultured islets......................................................... 82

**Figure 3.4** Collagen matrix (CM) significantly reduces formation of hIAPP aggregates in cultured human islets, which is associated with restored phosphorylated PKB levels and decreased β-cell apoptosis. ......................................................................................................... 84

**Figure 3.5** Prevention of amyloid formation by Congo red (CR) or collagen matrix (CM) reduces IL-1β expression in human islets during culture. ......................................................... 86

**Figure 3.6** Human islets treated with anakinra (An) or exenatide (Ex) have decreased IL-1β expression during culture, which is associated with increased phosphorylated PKB levels in islet β-cells............................................................................................................................................ 88

**Figure 4.1** Intraperitoneal injection of 15×10<sup>6</sup> (15M) IDO-expressing dermal fibroblasts inhibits T1D development in NOD mice. ............................................................................................................. 101

**Figure 4.2** Kynurenine level, an index of IDO enzymatic activity, plays an important role in the efficacy of IDO cell therapy. .............................................................................................................. 103

**Figure 4.3** 15M IDO-expressing fibroblasts restore the functionality of the remaining islet β-cells in diabetic NOD mice. .................................................................................................................. 105
Figure 4.4 IP injection with 15M IDO-expressing fibroblasts decreases the aggressiveness of insulitis in islets of NOD mice. ................................................................. 106

Figure 4.5 Inhibition of β-cell specific autoreactive CD8⁺ T cells by treatment with 15M IDO-expressing fibroblasts in NOD mice. ................................................................. 109

Figure 4.6 15M IDO-expressing fibroblasts decrease CD4⁺IL-17⁺ (Th17) cells in NOD mice. 110

Figure 4.7 15M IDO cell therapy upregulates FOXP3⁺ T cells in NOD mice. ...................... 112

Figure 4.8 Islets cultured with IDO-expressing fibroblasts have reduced IL-1β expression and β-cell apoptosis rate ......................................................................................... 114

Figure 5.1 The schematic summary of research project. ....................................................... 128
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1MT</td>
<td>1-methyl-tryptophan</td>
</tr>
<tr>
<td>2-D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3-D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid β</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>AGC</td>
<td>Protein kinase A/protein kinase G/protein kinase C</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>APCs</td>
<td>Antigen-presenting cells</td>
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<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
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<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
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<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
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<td>Arg</td>
<td>Arginine</td>
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<td>ARG</td>
<td>Arginase</td>
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<tr>
<td>ATG</td>
<td>Anti-thymocyte globulin</td>
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<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutant</td>
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<tr>
<td>BAD</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
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<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>B-cell lymphoma-extra large</td>
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<td>BGL</td>
<td>Blood glucose level</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>Bim</td>
<td>bcl-2 interacting mediator of cell death</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CLNs</td>
<td>Cervical lymph nodes</td>
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<tr>
<td>CM</td>
<td>Collagen matrix</td>
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<td>CPE</td>
<td>Carboxypeptidase E</td>
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<td>CR</td>
<td>Congo red</td>
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<td>CREB</td>
<td>cyclic AMP (c-AMP)-response element binding protein</td>
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<td>CTLs</td>
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<td>Carboxyl-terminal modulator protein</td>
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<td>CVB4</td>
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<td>DCs</td>
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<td>DCD</td>
<td>Donors after cardiac death</td>
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<tr>
<td>DKA</td>
<td>Diabetic ketoacidosis</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
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<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>eIF2α</td>
<td>Eukaryotic initiation factor 2</td>
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<td>Endoplasmic-reticulum-associated protein degradation</td>
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<td>Embryonic stem</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<td>FF</td>
<td>Free-floating</td>
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<td>FGFs</td>
<td>Fibroblast growth factors</td>
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<td>FLICE-like inhibitory protein</td>
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<td>Forkhead box P3</td>
</tr>
<tr>
<td>FPCM</td>
<td>Fibroblasts-populated collagen matrix</td>
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<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
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<tr>
<td>GADA</td>
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<td>HFIP</td>
<td>1,1,1,3,3,3-hexafluoro-2-propanol</td>
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<tr>
<td>HHS</td>
<td>Hyperosmolar hyperglycemic state</td>
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<td>hIAPP</td>
<td>Human islet amyloid polypeptide</td>
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<td>HLA</td>
<td>Human leukocyte antigen</td>
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<td>HM</td>
<td>Hydrophobic motif</td>
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<td>Full Name</td>
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<td>Hsp</td>
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<td>HSPG</td>
<td>Heparan sulfate proteoglycans</td>
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<td>IAA</td>
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<td>IAPP</td>
<td>Islet amyloid polypeptide</td>
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<td>IBMIR</td>
<td>Instant blood mediated inflammatory reaction</td>
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<tr>
<td>ICA</td>
<td>Islet cell auto-antibodies</td>
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<td>IDO</td>
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<td>IFNs</td>
<td>Interferons</td>
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<td>IGRP</td>
<td>Islet-specific glucose-6-phosphatase catalytic subunit-related protein</td>
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<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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</tr>
<tr>
<td>IPGTT</td>
<td>Intraperitoneal glucose tolerance tests</td>
</tr>
<tr>
<td>IPS</td>
<td>Induced pluripotent stem</td>
</tr>
<tr>
<td>ISREs</td>
<td>Interferon stimulatory response elements</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immuno-receptor tyrosine-based inhibitory motifs</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Kyn</td>
<td>Kynurenine</td>
</tr>
<tr>
<td>LNs</td>
<td>Lymph nodes</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPKAPK2</td>
<td>MAP kinase-activated protein kinase 2</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute 2</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLK-3</td>
<td>Mixed-lineage kinase 3</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NOD like receptor-related protein 3</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>NPH</td>
<td>Neural protamine Hagedorn</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>P2C</td>
<td>Protein 2C</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PC</td>
<td>Prohormone convertase</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositol-dependent kinase 1</td>
</tr>
<tr>
<td>PD-L1/2</td>
<td>Programmed cell death ligand 1/2</td>
</tr>
<tr>
<td>PDX1</td>
<td>Pancreatic and duodenal homeobox 1</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PHLPPα</td>
<td>PH domain leucine-rich repeat protein phosphatase</td>
</tr>
<tr>
<td>PI3</td>
<td>Phosphoinositol 3</td>
</tr>
<tr>
<td>PIKK</td>
<td>PI3-kinase related protein kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol (4,5) biphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3,4,5) triphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLNs</td>
<td>Pancreatic lymph nodes</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>p-PKB</td>
<td>Phosphorylated PKB</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase tensin homolog</td>
</tr>
<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphatase non-receptor type 22</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SAP</td>
<td>Serum amyloid P</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress-activated protein kinase</td>
</tr>
</tbody>
</table>
Serine
SHIP  SH2-domain-containing inositol polyphosphate 5-phosphatase
SHPs  Src homology 2 domain phosphotyrosine phosphatases
SOCS3  Suppressor of cytokine signaling 3
SP  Signal peptide
SPK  Simultaneous pancreas-kidney
SPLs  Spleens
STAT  Signal transducers and activators of transcription
STZ  Streptozotocin
T1D  Type 1 diabetes
T2D  Type 2 diabetes
TCRs  T cell receptors
TDO  Tryptophan 2,3-dioxygenase
Teff  Effector T cells
TGF-β  Transforming growth factor-β
Th  T helper
Thio S  Thioflavin S
Thr  Threonine
TNF  Tumor necrosis factor
TORC2  Transducer of regulated CREB protein 2
t-PKB  Total PKB
TRADD  TNF receptor type 1 associated death domain
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Tregs</td>
<td>Regulatory T cells</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>Trp</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VEGFs</td>
<td>Vascular endothelial growth factors</td>
</tr>
<tr>
<td>YAP</td>
<td>Yes-associated protein</td>
</tr>
<tr>
<td>ZnT 8</td>
<td>Zinc transporter 8</td>
</tr>
<tr>
<td>ZnT8A</td>
<td>Zinc transporter auto-antibodies</td>
</tr>
</tbody>
</table>
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Dedication

To my beloved families and medicine
Chapter 1: Introduction

1.1 Type 1 diabetes

“Diabetes mellitus” is derived from the Greek. Diabetes means siphon (to pass through) and mellitus means honeyed or sweet. The name was chosen because excess sugar is detected in the urine of diabetics [1]. Diabetes includes a group of metabolic disorders characterized by chronic hyperglycemia with abnormalities of carbohydrate, fat and protein metabolism that result from defects in insulin production, or its action, or both [2]. Around 347 million people worldwide have been reported to suffer from diabetes [3]. In Canada, more than nine million people live with diabetes or prediabetes [4]. With more than 20 newly diagnosed diabetic patients every hour, it is estimated that the number of diabetic patients worldwide will rise up to 552 million in 2030. Diabetes is predicted to be the seventh leading cause of death in 2030 [5].

Type 1 diabetes (T1D), a juvenile-onset diabetes, accounts for about 5-10% of all cases of diabetes [6]. It is an autoimmune disease, in which insulin-producing islet β-cells are destroyed by autoreactive T cells, resulting in absolute insulin deficiency. The incidence of T1D continues to increase worldwide and it has serious short-term and long-term complications [6,7]. Type 2 diabetes (T2D) is the other main type of diabetes that comprises around 90% of diabetic patients around the world [8]. It mainly occurs in adults, although it is increasingly diagnosed in youth as well [9,10]. Its symptoms are similar to those seen in T1D but it has a different etiology. T2D is due primarily to unhealthy lifestyle, such as physical inactivity [11]. Gestational diabetes is another type of diabetes. This kind of hyperglycemia occurs or first recognized during pregnancy likely due to pregnancy-related factors such as human placental lactogen that interferes with
susceptible insulin receptors [12].

1.1.1 Epidemiology of T1D

It has been reported that the incidence of T1D is increasing at the rate of 3-5% per year worldwide [7]. An epidemiological survey between 1990-1999 showed that T1D incidence rate rose from infancy and peaked during puberty (ages 10-14 years) [13]. However, updated data showed that the annual increase of T1D cases was 5.4% in children aged 0-4 years, which was higher than 5- to 9-year-age group (4.3%) and 10- to 14-year-age group (2.9%). Furthermore, it is predicted that the new T1D cases in children aged 0-4 years will double by 2020 [14]. Several factors have been considered as contributors to this epidemic, including hygiene and nutrition conditions [15,16]. Although T1D mainly occurs in childhood, it has also been reported in adults [17]. The incidence of T1D has a huge geographical variation [18]. For example, China was reported as the country with the lowest incidence rate of T1D, while the UK had roughly 30 times higher T1D incidence rates than China. Finland has the highest T1D incidence rate in the world [19-21]. Interestingly, studies also found that populations with different ethnic backgrounds that live in the same country have similar incidence rates. For example, in the UK children of South Asian ancestry have similar incidence rates for T1D with children from other ethnic lines [22]. Unlike other autoimmune diseases in which female patients are usually more than male patients, the incidence of T1D has no gender differences [23].

1.1.2 Etiology of T1D

It is now well established that a combination of genetic background and predisposed environmental factors are involved in development of T1D [24]. It has been shown that
monozygotic concordance of developing T1D is more than 50%. Moreover, the prevalence of T1D in general population is approximately 0.4%, while the prevalence of T1D in sibling is approximately 6% [25,26]. This evidence strongly supports that genetic factors are closely associated with the susceptibility of T1D. Studies have confirmed that over 40 genes/regions are related to T1D [27]. For example, genes located in the human leukocyte antigen (HLA) complex class II region on chromosome 6p21 have been shown to account for approximately 50% of genetic risks of T1D [28,29]. The examples of these variants are: HLA-DQ (HLA-DQA1 and HLA-DQB) and HLA-DR (HLA-DRB1) which are the major candidates of genetic predisposition to T1D [30]. The region within insulin (INS) gene on chromosome 11p15 is the second most important genetic susceptibility factor for T1D, providing approximately 10% of the genetic risks of T1D [30]. Some other regions of human genome have also been identified as possible contributors for the development of T1D. For example, polymorphisms in protein tyrosine phosphatase non-receptor type 22 (PTPN22) and cytotoxic T lymphocyte antigen 4 (CTLA4) have been shown to be associated with T1D and also several other autoimmune diseases through regulating T cell activation signaling pathway [30,31]. The interleukin-2 alpha chain receptor (IL-2RA) gene region which plays a significant role in controlling T cell proliferation has also been reported to be associated with T1D [27].

The finding that monozygotic concordance rate in T1D is around 50% rather than 100%, suggests that environmental factors are also important in T1D progression. Some investigations have indicated that certain types of viral infection can trigger T1D [32]. For example, Coxsackie virus B4 (CVB4) contains a protein 2C (P2C) that is similar to the enzyme glutamic acid decarboxylase (GAD) in pancreatic islets. Due to this molecular mimicry, autoreactive T cells
can cross-react with CVB4 antigens presented by antigen-presenting cells (APCs) and become activated resulting in development of T1D. CVB4 can also lead to T1D by directly destroying islet β-cells [33-35]. Paradoxically, there is a hygiene hypothesis suggesting that the absence of infections in modern society fails to educate the genetically predisposed immune system to promote self-tolerance thereby increasing the susceptibility of T1D [36,37]. Increasing evidence has shown that the commensal bacterial flora colonizing in our gastrointestinal tract plays an important role in the formation of mature immune system to decrease the susceptibility to T1D. Non-obese diabetic (NOD) mice, an experimental model of T1D, have been found to be prone to diabetes due to deficiency in gut flora, while exposure to certain gut bacteria can prevent development of diabetes in NOD mice [38,39]. Similar evidence has been found in humans. Recent studies have confirmed that children with T1D have distinct microbiota from that found in non-diabetic children [40]. Other environmental factors have also been found to contribute to T1D development such as vitamin D deficiency [41,42].

1.1.3 Pathogenesis of T1D

Due to the deficiency of immune self-tolerance (detailed in Chapter 1.4), several immunological events occur during the development of T1D, including production of auto-antibodies, activation of islet-specific autoreactive lymphocytes and destruction of insulin-producing islet β-cells [43,44]. These immune responses account for the pathogenesis of T1D. They may persist for many years leading to 60-90% islet cell death and/or dysfunction before the onset of clinically apparent T1D [45].
1.1.3.1 Initiation of immune responses in T1D

Islet β-cell death can be induced physiologically or pathologically by viral infections [46,47]. It has been reported that both human and rodents have a transient β-cell death shortly after birth, which is a physiological process for pancreas development [47-49]. Viruses not only directly lyse and release β-cell antigens but they can also indirectly destroy islet β-cells by increasing the secretion of pro-inflammatory cytokines, such as type I interferons (IFNs) [50]. IFN-α can alter the reaction of β-cells to interleukin 22 (IL-22) produced by T cells thereby inducing islet β-cell damage [51]. In normal conditions, IL-22 binds to the receptor on islet β-cells and activates the signal transducers and activators of transcription 3 (STAT3), which stimulate the transcription of pro-survival genes [52]. However, with IFN-α stimuli, islet β-cells switch to STAT1 as the downstream of IL-22 signaling pathway, which enhances the gene transcription and protein expression of tissue damaging factors such as inducible NO synthase (iNOS) [51]. Studies have also shown that early blockade of IFN-α inhibits development of T1D in NOD mice. Additionally, type I IFNs promotes dendritic cells (DCs)-mediated presentation of β-cell antigens to T cells thereby initiating immune responses [53].

1.1.3.2 The role of different cells in immune responses in T1D

In the past decade, many studies have shown that the pathogenesis of T1D is complicated and many cell subsets participate in the related immune responses [44].

CD4 T cells. Activation of CD4 T cells by DCs can promote macrophages to produce pro-inflammatory cytokines and reactive oxygen species, which are toxic to islet β-cells [54]. The CD4 T cells can also stimulate islet antigen-specific B cells to differentiate into antibody-
producing plasma cells [55]. With the help of CD4⁺ T cells, DCs acquire the ability to cross-present antigen to autoreactive CD8⁺ T cells for CD8⁺ T cell activation [56,57]. Studies have shown that depletion of CD4⁺ T cells in NOD mice decreased the incidence of T1D and even reversed overt diabetes, indicating the important role of CD4⁺ T cells in development of T1D [58].

**CD8⁺ T cells.** CD8⁺ T cell-mediated β-cell destruction has been considered as a major mechanism of β-cell damage in T1D [59]. Pancreatic β-cells carry some autoantigens, including preproinsulin signal peptide (SP), insulinoma-associated antigen-2 (IA-2), islet amyloid polypeptide (IAPP), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), zinc transporter 8 (ZnT 8), and glutamic acid decarboxylase 65 (GAD65) [60-63]. These autoantigens are targets for CD8⁺ T cells to destroy β-cells. This cell-mediated immune response is through releasing cytolytic granules containing perforin and granzymes, as well as Fas-Fas ligand (FasL) interaction [44]. Researchers have successfully isolated cytotoxic CD8⁺ T cell clones from the islets of NOD mice [64]. Furthermore, studies have shown that deletion of MHC class I and/or CD8⁺ T cells can prevent T1D in NOD mice [65,66].

**B cell lymphocytes.** Depletion of B cells in NOD mice inhibits the onset of T1D, suggesting a role for B cells in the pathogenesis of T1D [67,68]. Additionally, auto-antibodies produced by B cells have been used to identify people at risk of developing T1D. There are five main auto-antibodies in T1D including islet cell auto-antibodies (ICA), insulinoma associated-2 auto-antibodies (IA-2A), insulin auto-antibodies (IAA), glutamic acid decarboxylase auto-antibodies (GADA) and zinc transporter auto-antibodies (ZnT8A) [45,69]. The activated B cells can also
act as antigen-presenting cells (APCs) to present antigens to CD4$^+$ and CD8$^+$ T cells, further stimulating the anti-β-cell immune responses [70]. Thus, increased insulin-specific B cells accelerate the development of T1D [71].

**Macrophages and natural killer (NK) cells.** Macrophages can be activated by CD4$^+$ T cells to produce pro-inflammatory cytokines, such as interleukin 1β (IL-1β), IFN-γ and tumor necrosis factor (TNF), along with free radicals, which are the mediators of islet β-cell destruction [54]. However, some studies showed that depletion of macrophages had no effect on the incidence of T1D or the time of T1D onset, possibly because their role in β-cell destruction can be replaced by other cell types [72]. The other role of macrophages is removing apoptotic islet β-cells. Defects in this role have been found in the macrophages of NOD mice [73]. The role of NK cells in T1D is controversial. Some studies reported the presence and a pathogenic role for NK cells in T1D, while others failed to detect NK cells in T1D patients [74-77]. It has been indicated that the role of NK cells is associated with virus infections [74,75]. The NK cells appear to be important in patients who have virus infections as triggers of T1D [74].

**Regulatory T cells (Tregs).** Tregs characterized by expression of the forkhead box P3 (FOXP3), play central roles in the control of immune responsiveness and peripheral immunological tolerance [78]. Clinical data have shown that there are less effective Tregs in patients with T1D than those in healthy controls, indicating that Tregs can be a contributing factor in development of T1D [79]. Furthermore, Tregs regulate immune responses via production of anti-inflammatory cytokines such as interleukin 10 (IL-10) and transforming growth factor-β (TGF-β), inhibition of interleukin-2 (IL-2) and destruction of APCs [44].
Invariant NKT (iNKT) cells. iNKT cells have been identified as another population of T cells with regulatory properties in animal models. Increased expression of iNKT can reduce the incidence of T1D in NOD mice [80,81]. The mechanism of iNKT-mediated immunological regulation is through induction of T helper type 2 (Th2) cell responses to islet autoantigens [82-84]. However, the role of iNKT in human T1D is still not well understood [85,86].

1.1.4 Complications and comorbidities

There are short-term and long-term complications associated with T1D. The major short-term complications include hypoglycemia (due to inappropriate dose of insulin therapy), diabetic ketoacidosis (DKA) and hyperosmolar hyperglycemic state (HHS) [87-89]. The long-term complications of T1D are classified as microvascular and macrovascular disorders, which account for most of the morbidity and mortality in patients with T1D [90].

Microvascular disorders. Diabetic nephropathy is the most common cause of renal failure in the developed world. Previous studies showed that 30-40% of patients with diabetic nephropathy develop the end-stage renal diseases, whereas recent studies reported a decreased proportion, possibly due to the better control of glycaemia and hypertension by intensive insulin therapy [91-93]. Approximately, 20–25% T1D patients suffer from diabetic retinopathy, which is the most common cause of acquired blindness in the western countries [94]. Diabetic neuropathy can be divided into two major categories: focal and generalised. Focal neuropathies include carpal tunnel syndrome, peroneal nerve and third cranial nerve palsies, and diabetic amyotrophy (proximal nerve conditions). Sensorimotor polyneuropathy is the major disorder in generalised neuropathy. It can affect both peripheral nerves and autonomic system leading to cardiac
dysfunction, gastroparesis, and erectile dysfunction [95,96].

**Macrovascular disorders.** Cardiovascular diseases, such as myocardial infarction, become the common macrovascular complications in patients with T1D [97]. The risk for cardiovascular events in individuals with T1D is ten-fold higher than that in age-matched non-diabetic populations [98]. Many factors have been found to contribute to the cardiovascular disease in T1D, such as diabetic nephropathy, autonomic neuropathy, dyslipidemia, hypertension, and perhaps some specific microvascular cardiac diseases [6]. Moreover, studies have shown that patients with T1D have less favorable outcomes after suffering cardiovascular diseases compared with non-diabetic populations [99,100].

**Comorbid conditions.** Clinical data have shown that 5% and 3-10% of children with T1D also suffer clinical autoimmune thyroid disease and coeliac disease, respectively [101-104].

### 1.1.5 Diagnosis

The clinical symptoms of T1D include polydipsia, polyuria, polyphagia, unexplained loss of bodyweight, fatigue, blurred vision and susceptibility to certain infections. The criteria for diagnosis of T1D are based on blood glucose measurements including fasting plasma glucose \( \geq 7.0 \text{ mmol/L} (\geq 126 \text{ mg/dL}) \) or random plasma glucose concentrations \( \geq 11.1 \text{ mmol/L} (\geq 200 \text{ mg/dL}) \) plus symptoms of diabetes, or 2 hour post-oral glucose tolerance test (OGTT) \( \geq 11.1 \text{ mmol/L} (200 \text{ mg/dL}) \) [105]. In 2009, the American Diabetes Association (ADA) modified their guidelines for diagnosis of diabetes to include glycated haemoglobin (HbA1c) \( \geq 6.5\% \) [106]. Additionally, islet auto-antibodies in the patient serum have may potentially predict the
occurrence of the disease. The combined measurements of the auto-antibodies such as GADA and IA-2A are recommended for initial confirmation of the suspected diagnosis of T1D [107].

1.1.6 Treatment of T1D

1.1.6.1 Insulin therapy

Different insulin types that are currently used to control blood glucose can be divided into four categories: 1) rapid-acting analog insulin (aspart, glulisine and lispro) that closely mimics physiological prandial insulin secretion; 2) short-acting prandial insulin (regular insulin) with a significant basal component; 3) intermediate-acting insulin [neural protamine Hagedorn (NPH) insulin] that has a broad peak action to provide basal as well as prandial coverage; and 4) long-acting insulin (detemir and glargine) that has minimal peak action and provides basal insulin supply [108]. The goal of insulin therapy in T1D is to mimic the physiological insulin secretion from a normally functional pancreas: (1) continuous basal insulin secretion, which suppresses lipolysis and balances gluconeogenesis with glucose use; and (2) short bursts of prandial insulin secretion (bolus insulin) which stimulates glucose uptake after food consumption and inhibits gluconeogenesis [109]. Thus, insulin replacement regimen for T1D should consist of both basal and bolus insulin components.

In the late 1970s, insulin pump therapy was introduced as a more reliable and safer insulin treatment for T1D. With a small self-inserted catheter, insulin pump therapy replaces the need for repeated injections. Pump devices mimic physiologic insulin release by administration of a 24-hour variable amounts of basal insulin, along with meal-activated bolus insulin [110]. However, insulin pump therapy has its potential adverse events including insertion-site infection
and interruption in insulin delivery (due to a pump malfunction), which could lead to DKA [111].

After the initiation of insulin treatment, remaining β-cells can functionally recover from glucose toxicity within a limited period [45]. This “honeymoon phase” is seen in up to 60% of T1D patients [112]. It occurs more frequently with increasing age of patients at onset and generally lasts 3-6 months [113]. More importantly, patients may require progressive reduction in their initial insulin dosages during this period [114,115].

1.1.6.2 Pancreas and islet transplantation

An alternative approach to control blood glucose and even cure T1D is to transplant insulin-producing tissue obtained from non-diabetic donors. It can be a whole pancreas transplantation or just islet transplantation.

Pancreas transplantation. It has been several decades since the first whole pancreas transplantation was performed [116]. The pancreas and kidney are commonly transplanted simultaneously from the same deceased donor so it is also called simultaneous pancreas-kidney (SPK) transplantation. SPK transplantation has been considered as a reliable clinical treatment for T1D with graft survival rates of up to 86% at 1 year and 70% at 5 years after transplantation [117,118]. In addition, successful β-cell replacement by SPK transplantation can reverse the secondary complications of T1D, such as autonomic neuropathy and retinopathy [119,120]. However, it is a major surgical procedure with procedure-related mortality rates of up to 4%. The major complications of SPK transplantation include graft thrombosis, graft pancreatitis,
pancreatic fistulae, and pseudocyst formation, which are all related to the transplanted pancreatic exocrine tissues rather than the transplanted endocrine islets [121].

**Islet transplantation.** In contrast to the whole-pancreas transplantation, isolated islet transplantation as a cellular transplant approach is less invasive. Studies have shown that islet transplantation has much lower procedure-related morbidity as compared with SPK transplantation [122]. Moreover, islet transplantation can utilize the islets isolated from deceased donor pancreas that are considered unsuitable for whole-pancreas transplantation [123]. The process of islet transplantation consists of four main steps: pancreas donation and retrieval, islet isolation, islet culture, and islet implantation [121]. In the 1990s, the islet graft function rate was only 19% at 3 years after islet transplantation [124]. However, after 2000, with the use of “Edmonton Protocol” and other novel strategies, some leading centers have achieved success with insulin independence rates of 80%-85% at 1 year and 24% at 5 years after transplantation [125]. In 2009, Collaborative Islet Transplant Registry reported that the overall incidence of sustained islet graft function was 66% at 1 year and 45% at 3 years after transplantation [126]. Recently, 78% insulin-independence rate at 3 years after transplantation was also reported [127].

Although islet transplantation is a feasible approach for treatment of T1D, it is still limited by lack of sufficient donors and progressive islet β-cell death and dysfunction during the different stages of the transplantation [128]. Both immunological and non-immunological factors have been shown to contribute to the β-cell graft death and dysfunction. The non-immunological factors include disruption of islet extracellular matrix (ECM) and innervation during islet isolation [129], hypoxia [130], islet amyloid formation [131,132], glucolipotoxicity [133],
endoplasmic reticulum (ER) stress [134] and toxicity of immunosuppressants [135]. The immunological factors contain instant blood mediated inflammatory reaction (IBMIR) which is an innate immune response starting within minutes after islet infusion [130], alloimmunity and recurrent autoimmunity [136]. Many strategies have been proposed to address the above issues and thereby enhancing the graft survival and function. Strategies addressing the shortage of donors include improvement of islet yield from single-donor [137], using living donors and donors after cardiac death (DCD) instead of heart-beating, brain-dead donors [138,139], xenotransplantation with porcine islets and stimulation of β-cell regeneration [140,141]. The strategies preventing β-cell death and dysfunction include restoration of islet ECM [142,143], promotion of vasculogenesis [144], reduction of hypoxia [145], islet amyloid formation [146], metabolic stress [147], alteration of implantation sites [148] and immunomodulation (immunosuppression, tolerance induction and immunoisolation) [149].

1.1.6.3 Other potential therapy
There are also many other potential therapies either in clinical trial or in experimental settings.

β-cell based therapy. In addition to injection of exogenous insulin and islet transplantation, there are other strategies aimed at improving function of islet β-cells during the progression of T1D. For example, treatment with glucagon-like peptide-1 (GLP-1) has been shown to improve the function of residual islet β-cells in early onset of diabetes in NOD mice [150]. Moreover, islet regeneration was stimulated by treatment with gastrin [151] or islet neogenesis associated protein (INGAP) therapy [152]. Stem cells, such as embryonic stem (ES) cells [153] and induced pluripotent stem (IPS) cells [154] can be differentiated to islet β-cells under specific conditions
to compensate for the loss of islet β-cells in T1D.

**Immune-based therapy.** Induction of β-cells must be accompanied by immuno-based therapy in order to successfully protect the newly formed β-cells from autoimmune attack. Moreover, immunomodulation during the “honeymoon phase” of T1D can enhance the function of residual islet β-cells to produce sufficient insulin for normoglycemia [155]. The immune-based therapy can be divided into two categories: autoantigen-specific intervention and non-autoantigen intervention [156]. GAD65 as an autoantigen has been targeted. Vaccination with GAD65 has successfully preserved the function of residual β-cells in patients with recent onset T1D in phase II clinical trial [157,158]. Also, some modulations on systemic immune system rather than specific autoantigens have been proposed, which is referred to as non-autoantigen intervention. For example, depletion of T cells by anti-CD3 antibody or anti-thymocyte globulin (ATG) has significant effect on increasing c-peptide levels in T1D patients [45,159,160]. Besides T cell depletion, B cell depletion by anti-CD20 monoclonal antibody has also been shown to reverse T1D in diabetic NOD mice and preserve c-peptide levels in patients with new onset T1D for one year [161,162]. One of the important targets in non-autoantigen immunomodulation is co-stimulatory molecule, which plays a key role in T cell activation [163]. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and B7-H4 can compete with the co-stimulatory molecules, such as CD28 and provide co-inhibitory signals that inhibit T cell activation [164]. Studies have shown that both CTLA-4.Ig and B7-H4.Ig can inhibit the progression of T1D in NOD mice [165,166]. Some pro-inflammatory cytokines are also targeted for treatment of T1D due to their critical role in the pathogenesis of this disease. For example, anti-IL-1β therapy by both Canakinumab (anti-IL-1β antibody) and Anakinra (IL-1 receptor antagonist) can preserve the
functional mass of residual islet β-cells in patients with recent onset T1D [167]. Recently, cell-based tolerogenic immunotherapy was considered as an attractive and feasible treatment of T1D. Many different types of cells have been modified with immunotolerogenic capacity, such as dendritic cells [168], fibroblasts [169], stem cells [141], splenocytes [170] and Sertoli cells [171].

1.2 Amyloidogenesis

1.2.1 Amyloid formation and human diseases

Native protein structures are important for cells to exert their physiological function. However, due to misfolding, some proteins converse their native soluble conformations to insoluble fibrillar aggregates named amyloid fibrils or plaques [172]. This kind of conformational transitions is linked to many human diseases. For example, fibrillar aggregates have been found in brains of patients with neurodegenerative diseases, such as Alzheimer’s disease (AD) [173,174] and Parkinson’s disease (PD) [175,176]. In AD, extracellular amyloid plaques are formed by aggregation of amyloid β (Aβ) peptide which is the product of amyloid precursor protein (APP) during the inappropriate proteolytic processing [177]. Such aggregates can also localize in other tissues or organs. For example, in type 2 diabetes, pancreatic islet amyloid is formed by the aggregation of human islet amyloid polypeptide (hIAPP) [178,179]. In medullary carcinoma, there is aggregation of calcitonin in the thyroid [180]. Besides localization in one specific tissue, aggregates can also form in multiple tissues to form systemic amyloidosis such as in AA amyloidosis [181]. Importantly, growing evidence suggests a critical pathologic role for these amyloid deposits in different diseases [182-185].
1.2.2 IAPP aggregation and islet amyloid deposition

Islet amyloid was discovered over 100 years ago and its was first described as hyalinization of islets [186]. Its major component is islet amyloid polypeptide (IAPP, or amylin), although islet amyloid contains other components such as serum amyloid P (SAP) component, apolipoprotein E (ApoE) or heparan sulfate proteoglycans (HSPG) [187-190]. IAPP is a 37-amino acid (aa) peptide secreted by islet β-cells with insulin in response to β-cell secretagogues such as glucose [190-193]. The biosynthesis of IAPP is similar to that of insulin. IAPP is first synthesized in the endoplasmic reticulum (ER) as an 89-aa preproIAPP containing a 22-aa signal peptide [194,195]. After the signal peptide is removed, it forms a 67-aa proIAPP. As with proinsulin, processing proIAPP (8 kDa) to IAPP (4 kDa) occurs via sequential cleavage of C-terminus in the Golgi by the prohormone convertase PC1/3 followed by PC2-mediated cleavage of N-terminus in the secretory granules [196,197]. Carboxypeptidase E (CPE) removes the dibasic amino acids at C-terminal of IAPP [198] (Figure 1.1). Before secretion from islet β-cells, mature IAPP is subject to post-translational modifications such as disulfide bridge formation, amidation of C-terminal tyrosine and O-glycosylation of threonines [199,200].
Figure 1.1 Processing of preproIAPP to mature IAPP.
The N-terminal signal sequence is removed from preproIAPP to form proIAPP. ProIAPP is cleaved by PC1/3 at its C-terminus and by PC2 at its N-terminus. The KR residues (red) that remain at the C-terminus after PC1/3 processing are then removed by CPE and IAPP is amidated. IAPP: islet amyloid polypeptide, PC: prohormone convertase, CPE: carboxypeptidase E.

Since aggregation is essential for cytotoxic properties of IAPP, numerous studies have investigated the mechanisms responsible for this aggregation. Different factors have been proposed to induce IAPP aggregates such as overexpression of IAPP, presence of amyloidogenic region and inappropriate processing of proIAPP [131,201,202]. Previous in vitro and in vivo studies have shown that overexpression of hIAPP leads to its fibrinogenesis and islet amyloid deposition, suggesting that the levels of hIAPP released from β-cells may be an important factor in the hIAPP aggregation process [201,203]. Although IAPP is a highly conserved molecule, there is a considerable variation in the sequence of amino acids in the middle segment (20-29 aa) of this peptide in different species (Figure 1.2). This segment of mouse and rat IAPP that do not form aggregates, there are three proline residues which are the breakers of β-sheet conformation.
required for IAPP aggregation. In contrast, this segment in the human IAPP molecule, named as amyloidogenic region, has no proline residues [204,205]. Growing evidence suggests that this amyloidogenic region (20-29 aa) is necessary for human IAPP aggregation both in vitro and in vivo [202,206,207]. Impaired processing of proIAPP has also been suggested to play a key role in islet amyloid formation. The evidence supporting this idea includes: 1. histological assessments have shown the presence of N-terminal flanking region of proIAPP in islet amyloid [208]; 2. in vitro studies have shown that islet amyloid formation correlates with defective proIAPP processing [131,209]; 3. it has also been demonstrated that impaired N-terminal processing of proIAPP in islets lacking PC2 led to IAPP aggregation and β-cell death during in vitro culture [210,211] and that adenoviral-mediated PC2 expression in islets prevented IAPP aggregation and β-cell death by restoring the normal N-terminal proIAPP processing [211].

![Figure 1.2 The amino acid sequence of IAPP in mouse, rat and human.](image)

There is a considerable variation in the segment (20-29aa) of IAPP in different species. In mouse and rat IAPP, there are three proline residues (red) in this segment, which prevent IAPP aggregation. In human IAPP, this segment is named as amyloidogenic region (blue box), which is responsible for hIAPP aggregation.

Classical light microscopy can only detect mature amyloid fibrils representing the end stage of the aggregation [212]. However, the misfolded soluble monomeric IAPP leads to the formation of soluble β-sheet oligomeric state [212-214], which mediates a further progression to protofibrils and insoluble mature amyloid fibrils [215] (Figure 1.3). Whether IAPP aggregation
starts intra-cellularly or extra-cellularly is still controversial. In favour of extracellular aggregation of IAPP, histological analysis of human IAPP transgenic mouse islets showed IAPP-immunoreactive fibrillar aggregates were adjacent to β-cell membranes and islet capillaries [216]. Although full-blown amyloid fibrils reside extracellularly, the argument has been made that the initial site of fibrils may still be intracellular [217-221]. It has been hypothesized that the formation of small intracellular IAPP aggregates leads to β-cell death and can be secreted or exteriorized into extracellular space. These extracellular amyloid fibrils then act as seeds for further propagation of amyloid deposits in the extracellular spaces [205,220]. Identification of the initial site of IAPP aggregation seems to be very important for the development of therapies. For example, based on the hypothesis of intracellular amyloidogenesis, drugs should be designed to cross the β-cell membrane to target and inhibit the initial IAPP aggregates [222].

![Diagram of amyloid formation](image)

**Figure 1.3** The schematic process of islet amyloid formation.

### 1.2.3 Cytotoxic mechanisms of IAPP aggregates

The previous studies have demonstrated that mature amyloid fibrils are the cytotoxic form of hIAPP aggregates [223,224]. However, recent studies have revealed that the soluble oligomeric hIAPP is the major toxic form for islet β-cells [212,225,226]. There are multiple mechanisms for islet β-cell death induced by hIAPP aggregation including cell membrane permeabilization or
disruption [227], endoplasmic reticulum (ER) stress [228], defection in endoplasmic-reticulum-associated protein degradation (ERAD) [229] and unfolded protein response (UPR) [205], stimulation of islet inflammation [230], defects in autophagy [231], as well as receptor-mediated mechanisms involving oxidative stress [232] and the activation of cell death signaling pathways [233].

Several pieces of evidence show that aggregates of endogenous hIAPP and exogenously applied hIAPP have the ability to disrupt cell membranes to exert their cytotoxicity [218,227,234-236]. hIAPP aggregates can interact with the lipid components of cell membrane and make a channel-like pore on β-cell membrane to destabilize the intracellular ion homeostasis, which leads to cell damage [223,237]. For example, exposure to hIAPP aggregates increases intracellular calcium levels in islet β-cells resulting in β-cell apoptosis [238].

It has been shown that ER stress is an important contributor to β-cell apoptosis induced by endogenous aggregation of hIAPP [228,239]. Transgenic animal models overexpressing hIAPP have increased β-cell death mediated by ER stress [240]. However, in cultured islets with physiological levels of hIAPP, the role of ER stress in amyloid-induced cell toxicity is controversial as no detectable ER stress was reported [241]. Defects in ERAD and UPR have also been identified as stimulators of islet β-cell apoptosis with extracellular and intracellular hIAPP aggregates [228,229]. Autophagy plays a key role in degradating and recycling of damaged or dysfunctional cellular components, and also clears ubiquitinated proteins [242,243]. It has been shown that overexpression of hIAPP induces impairment of autophagy in islets, which promotes β-cell apoptosis [231,244,245]. In contrast, enhancement of autophagy protects
β-cells from the toxicity induced by hIAPP aggregates [231,246,247].

hIAPP-induced pro-inflammatory responses or pro-apoptotic signaling pathways also promote islet β-cell apoptosis. For example, pro-inflammatory IL-1β signaling has been reported to be a critical factor contributing to β-cell damage by hIAPP aggregation [230,248]. Moreover, pro-apoptotic c-Jun N-terminal kinase (JNK) signaling pathway activated by ER stress or oxidative stress is another mediator of hIAPP-induced β-cell death via both intrinsic and extrinsic apoptotic pathways [233]. Recently, our research group identified the essential role of Fas as death receptor in hIAPP toxicity and showed that deletion of Fas significantly reduces hIAPP-induced β-cell apoptosis [249].

1.2.4 Islet amyloid and type 2 diabetes

Clinical studies have shown a reduction of islet mass in patients with T2D compared to age-match controls [250-253]. It is intriguing that the reduction of β-cell area in diabetic subjects only occurred in amyloid-containing islets [254]. This indicates the importance of islet amyloid formation in islet β-cell loss in T2D.

Several factors have been found to contribute to IAPP aggregation and amyloid deposition during T2D. For example, over production of insulin in T2D in response to insulin resistance is associated with increased secretion of IAPP due to the parallel regulation of these two β-cell products [255]. Thus, high level of IAPP itself may be an important factor in IAPP accumulation in T2D [201,203]. Increased demand for insulin during T2D may lead to improper proinsulin processing in exhausted or dysfunctional islets. Because proIAPP and proinsulin share the same
processing enzymes, processing of proIAPP may also be impaired leading to increased levels of proIAPP and/or its partially processed intermediates, especially the N-terminal intermediate form [196,197,256]. Binding of proIAPP and its N-terminal intermediate form to heparan sulfate proteoglycan (HSPG) through heparin binding domain then may initiate islet amyloid formation [257]. Also, insufficient proinsulin processing results in decreased production of mature insulin, which has been identified as an inhibitor of islet amyloid [258-260]. Thus, more islet amyloid is formed due to lack of insulin. Lastly, high fat level in some patients with T2D has been proposed as a contributor to islet amyloid formation based on the evidence that most of human IAPP transgenic mouse models form only islet amyloid on high fat diet [261].

1.2.5 Amyloid formation and islet transplantation

In the past two decades, islet amyloid formation has been investigated in both experimental and clinical islet transplantation. In the first study, researchers transplanted human islets into the renal capsule of nude mice. They found IAPP-positive fibrillar deposits in two-thirds of the islet grafts [262]. Following the site used in clinical islet transplantation, investigators also intraportally transplanted human islets into nude mouse recipients and found islet amyloid deposits in the majority of islets in the grafts [219]. Furthermore, islets from hIAPP-expressing transgenic and control mice were transplanted into streptozotocin (STZ)-induced diabetic mice. Results showed that recurrence of hyperglycemia only occurred in mice that had received islets from hIAPP-expressing transgenic mice. Amyloid deposits were detected before the recurrence of hyperglycemia and correlated with progressive β-cell apoptosis [263]. Two studies of clinical islet transplantation were reported by Dr. Westermark’s group showing that amyloid formation occurred in three of four islet grafts after transplantation [264] and islet amyloid deposits were
detected in almost 50% of grafted islets in a diabetic patient who had received three intraportal islet transplantations within 5 years [265]. Furthermore, porcine islet grafts that do not form amyloid deposits have significantly better survival rate and long-term viability as compared with human islet grafts [132]. Taken together, these findings suggest that islet amyloid is a pathological factor contributing to progressive islet β-cell loss during clinical islet transplantation that may eventually lead to islet graft failure.

1.3 Islet β-cell survival signaling pathways

1.3.1 Protein Kinase B (PKB) signaling

Protein kinase B (PKB, also known as Akt) is a serine/threonine kinase that belongs to the cAMP-dependent protein kinase A/protein kinase G/protein kinase C (AGC) kinase family [266]. It was initially discovered as an oncogene within transforming murine leukemia virus AKT8 [267] and first cloned in 1991 by three independent groups [268-270]. PKB signaling pathway is an important regulator of cell survival [271,272]. Furthermore, many studies have elucidated a key role for PKB in the protection of islet β-cells against apoptosis [273-275].

1.3.1.1 Isoforms and structure of PKB

In mammals, PKB subfamily comprises three isoforms, PKBα, PKBβ and PKBγ (Akt1, Akt2 and Akt3, respectively). Although encoded by different genes on different chromosomes, the three isoforms have high homology at protein levels with the same molecular weight (60 kDa) and share a conserved structure which includes an amino-terminal (N-terminal) pleckstrin homology (PH) domain, a central kinase catalytic domain and a carboxyl-terminal (C-terminal) regulatory domain containing the hydrophobic motif (HM) phosphorylation site [FxxF(S/T)Y]
[276,277] (Figure 1.4). The N-terminal PH domain that was first found in pleckstrin contains 100 amino acids. It plays a critical role in membrane translocation of PKB by interacting with phosphatidylinositol (3,4,5) triphosphate (PIP3) [278]. The central kinase catalytic domain of PKB is highly conserved among AGC kinases such as protein kinase A (PKA), protein kinase C (PKC), p70S6 kinase and ribosomal p90R6 kinase [279]. Phosphorylation of the conserved threonine (Thr) residue in this region is necessary for the activation of PKB [280]. The regulatory domain, located in the C-terminal of the molecule consists of 40 amino acids. The FxxF(S/T)Y hydrophobic motif (where x is any amino acid) in this region is the key feature of the AGC kinase family [279]. Additionally, the serine (Ser) residue in this hydrophobic motif is essential for phosphatidylinositol 3 (PI3)-kinase-dependent full activation of PKB. Thus, this motif is thought to be particularly important for the enzymatic activity of PKB [281]. Although the three isoforms have the structural similarity, their major expressions are in different tissues. PKBα is expressed in most tissues of the body, whereas PKBβ and PKBγ are highly expressed in specific tissues. PKBβ is prominently expressed in insulin-responsive tissues, while PKBγ is mainly expressed in brain and testes [282]. All isoforms of PKB are expressed in islet β-cells [283,284] and contribute to regulation of β-cell mass and function [282,285-287].
Figure 1.4 The schematic structure of PKB isoforms.
Three highly conserved isoforms of PKB (PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3) share similar structure including an N-terminal pleckstrin homology (PH) domain, a central kinase domain and a hydrophobic C-terminal regulatory domain. The phosphorylation sites in the kinase and regulatory domains are indicated.

1.3.1.2 Process of PKB activation

1.3.1.2.1 PI3-kinase-dependent activation of PKB (Figure 1.5)

Membrane translocation of PKB. PKB is a downstream component of PI3-kinase, which can be activated by autophosphorylation of receptor tyrosine kinases, stimulation of G-protein-coupled receptors or activation of integrin signaling [288,289]. Activation of PI3-kinase generates the second messenger phosphatidylinositol (3,4,5) triphosphate (PIP3) from phosphatidylinositol (4,5) biphosphate (PIP2). The PH domain of PKB has high affinity for binding with PIP3 [281]. This binding does not directly activate PKB and instead stimulates translocation of PKB from the cytoplasm to the plasma membrane and alters the conformation of PKB for subsequent phosphorylation [290,291]. Inhibition of PI3-kinase by inhibitors such as Wortmannin and LY294002 [292] or inhibition of the conversion of PIP3 to PIP2 by phosphatase tensin homolog (PTEN) [293,294] and SH2-domain-containing inositol polyphosphate 5-phosphatase (SHIP) [295] prevent recruitment of PKB to plasma membrane.
**Phosphorylation of PKB.** Activation of PKB requires multiple phosphorylation processes including phosphorylation of threonine, serine and tyrosine residues. Once recruited to the plasma membrane, PKB is activated by site-specific phosphorylation. A main site of phosphorylation is within the central kinase domain at threonine residue. For example, a key phosphorylation residue in PKBα isoform is Thr308. Phosphoinositide-dependent kinase 1 (PDK1) is required for phosphorylation of PKB on its threonine residue [296]. PDK1 is a 63-kDa serine/threonine kinase containing a C-terminal PH domain that binds to PIP3 with high affinity. After recruitment to the plasma membrane by PIP3, PDK1 mediates threonine phosphorylation on PKB, which leads to partial activation of PKB [280,290,297]. Full activation of PKB also requires phosphorylation on serine residue (such as Ser473 in PKBα isoform) located in the regulatory domain. Serine phosphorylation has been identified as the essential step in the activation process because it stabilizes the active conformation state of PKB [298]. However, the kinases that are responsible for phosphorylation of serine residue on PKB have not been thoroughly understood. PDK1 has been found as a stimulator of serine residue phosphorylation since transfected cells overexpressing PDK1 had increased levels of phosphorylated serine on PKB [299]. The integrin-linked kinase (ILK) is another kinase considered to mediate the phosphorylation of serine residue on PKB based on the finding that an ILK-specific inhibitor blocked serine phosphorylation of PKB [300]. While these studies suggest that PDK1 and ILK play important roles in the phosphorylation of serine, it is still unclear whether they directly phosphorylate PKB. The significance of other kinases in serine phosphorylation of PKB also remains to be determined. These kinases include PKB itself [301], MAP kinase-activated protein kinase 2 (MAPKAPK2) [280], protein kinase C βII (PKCβII) [302] and the members of the atypical PI3-kinase related protein kinase (PIKK) family: DNA-dependent protein kinase (DNA-
PK) [303], ataxia telangiectasia mutant (ATM) [304] and the transducer of regulated CREB protein 2 (TORC2) [305].

Recent reports have unveiled the possible role of tyrosine (Tyr) phosphorylation in the PKB activation. One study showed that phosphorylation of Tyr315 and Tyr326 located within the kinase domain of PKB is required for PKB activation [306]. Also, Tyr474 which is a conserved tyrosine residue in AGC family has been identified as a necessary phosphorylated site for activation of PKB [307]. However, further studies are required to evaluate the significance of tyrosine phosphorylation in PKB activation.

Several proteins have been shown to inhibit activation of PKB by reducing its phosphorylation, including carboxyl-terminal modulator protein (CTMP) [308], TRB3 [309], Keratin K10 [310], protein phosphatase 2A (PP2A) [311] and PH domain leucine-rich repeat protein phosphatase (PHLPPα) [312]. Positive regulators of PKB activation by stimulating its phosphorylation include heat shock protein 90 (Hsp90) [313], heat shock protein 27 (Hsp27) [314], growth factor receptor-bound protein 10 (Grb 10) [315] and Ft 1 [316]. Following activation at the plasma membrane, phosphorylated PKB translocates back to the cytoplasm or the nucleus [317].
Figure 1.5 PI3-kinase-dependent process of PKB activation.
The process includes three major steps: 1) PI3K-induced membrane translocation of PKB via PH domain; 2) phosphorylation of PKB on threonine and serine residues; and 3) cytoplasmic or nuclear translocation of active PKB. GPCR: G-protein-coupled receptors, RTK: receptor tyrosine kinase, PI3K: phosphatidylinositol 3-kinase, PIP3: phosphatidylinositol (3,4,5) triphosphate, PIP2: phosphatidylinositol (4,5) biphosphate, PDK1: phosphoinositide-dependent kinase 1, ILK: integrin-linked kinase, PH: pleckstrin homology, R: regulator, P: phosphorylation.

1.3.1.2.2 PI3-kinase-independent activation of PKB

Evidence suggests that PKB can also be activated by a PI3-kinase-independent manner. One possible mechanism is through protein kinase A (PKA, cyclic adenosine monophosphate [cAMP]-dependent protein kinase). Several reports have shown that cAMP-stimulating agents such as forskolin, prostaglandin-E1, chlorophenylthio-cAMP and 8-bromo-cAMP activate PKB through PKA [318,319]. Furthermore, unlike PI3-kinase-dependent activation of PKB, only phosphorylation of threonine residue is required for activation of PKB by this mechanism [319].
1.3.1.3 Regulation of cell survival by PKB

The PKB signaling pathway has been widely recognized as one of the most important pathways in the regulation of cell survival [273-275,320]. There are three major ways by which PKB mediates cell survival: direct inhibition of pro-apoptotic proteins, regulations of transcriptional factors and modulation of cellular metabolism (Figure 1.6).

Direct inhibition of pro-apoptotic proteins. One of the major functions of PKB is to phosphorylate and block the effect of pro-apoptotic proteins involved in the cell apoptosis. For example, as a pro-apoptotic protein, Bcl-2-associated death promoter (BAD) binds to B-cell lymphoma 2 (Bcl-2) or B-cell lymphoma-extra large (Bcl-XL) and inhibits their pro-survival activities. However, PKB directly phosphorylates BAD on Ser136 and inhibits the activity of BAD, thereby promoting cell survival by releasing Bcl-2 or Bcl-XL [321,322]. PKB has also been shown to directly phosphorylate Ser196 residue on human pro-caspase-9 and block its cleavage to form caspase-9 [323]. Caspase-9 acts as an initiator of caspase-family proteases in mitochondrial pathway of apoptosis [324]. Thus, PKB inhibits cell apoptosis by decreasing caspase-9 formation. However, the PKB phosphorylation site in pro-caspase-9 has not been found in rodent species, such as rat or mouse, and further studies are required to understand the relevance of caspase-9 in PKB-mediated inhibition of cell apoptosis [325]. PKB has been reported to phosphorylate three upstream kinases of the stress-activated protein kinase (SAPK) that increases cell apoptosis in response to stress or cytokines. These phosphorylations inactivate SAPK and promote cell survival [326-328].

Regulation of transcriptional factors. In mammals, the forkhead box O (FoxO) family of
transcription factors consists of four isoforms (FoxO1, FoxO3, FoxO4 and FoxO6), all of which could directly be phosphorylated by PKB [329]. Phosphorylation of FoxO proteins decreases their transcriptional function for their target pro-apoptotic genes including Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL), TNF receptor type 1 associated death domain (TRADD) and bcl-2 interacting mediator of cell death (Bim), thereby inhibiting cell apoptosis [330-332]. Another factor involved in this mechanism is murine double minute 2 (MDM2, or HDM2 in humans), an E3 ubiquitin ligase that induces p53 degradation. p53 is a major promoter of cell death in response to stress by transcriptional regulation of BH3-only proteins, Puma and Noxa [333]. PKB phosphorylates MDM2 on two residues, Ser166 and Ser186, and this phosphorylation facilitates the translocation of MDM2 to the nucleus, where it promotes dysfunction and degradation of p53 thereby inhibiting p53-mediated pro-apoptotic responses [334-336]. PKB has also been shown to stimulate the nuclear translocation and activation of nuclear factor-κB (NF-κB) by phosphorylation and degradation of its inhibitor, IκB kinase (IKK) [337]. Thus, PKB promotes cell survival by increasing the transcription of NF-κB-dependent pro-survival genes including Bcl-XL, Bcl-2 [338,339] and genes of caspase inhibitors such as FLICE-like inhibitory protein (FLIP) [340]. Moreover, two transcriptional factors, c-AMP-response element binding protein (CREB) and yes-associated protein (YAP) can be phosphorylated by PKB to protect cells from apoptosis [341,342].

**Modulation of cellular metabolism.** PKB can also exert its pro-survival effects through regulation of nutrient uptake and cellular metabolism. High insulin levels stimulate PKB phosphorylation and inhibit glycogen synthase kinase 3 (GSK3) in order to promote conversion of glucose to glycogen [343]. GSK3 is involved in many pro-apoptotic signaling pathways such
as mixed-lineage kinase 3 (MLK-3)/c-Jun N-terminal kinase (JNK) pathway [344]. Thus, inhibition of GSK3 by PKB plays a protective role in cell viability [345]. Moreover, it has been found that PKB-mediated regulation of cellular glucose mechanism such as glycolysis, prevents the activation of pro-apoptotic Bcl-2-associated X protein (Bax), thereby improving cell survival [346].

![Figure 1.6 Three major mechanisms of PKB-mediated cell survival.](image)

**Figure 1.6 Three major mechanisms of PKB-mediated cell survival.**

### 1.3.2 Other major signaling pathways in islet β-cell survival

The extracellular signal-regulated kinase (ERK) signaling is the first discovered signaling pathway involved in growth factors-induced pro-survival cellular responses through receptor tyrosine kinases [347]. It has been widely evaluated as a pro-survival signaling in different cell
types including pancreatic islet β-cells [348-350]. It has been shown that ERK activity was dramatically declined after islet isolation which correlated with a decrease in islet β-cell survival during pre-transplant culture [351]. Moreover, 804G matrix has been identified to protect cultured islet β-cells against apoptosis via activation of ERK [274]. Furthermore, it has been shown that the positive effects of GLP-1 on islet β-cell survival are partially dependent on ERK activity [350]. Specific isoforms of signal transducer and activator of transcription (STAT) can also mediate survival signaling pathways in islet β-cells. For example, activation of STAT6 by anti-inflammatory interleukin-13 (IL-13) has cytoprotective effects on islet β-cells and enhances islet β-cell viability during in vitro culture [352].

1.4 Immunological self-tolerance

Under physiological condition, the mammalian immune system shows a balance between responsiveness to pathogenic microorganisms and unresponsiveness to harmless self-antigens (self-tolerance) [353]. Breaking this balance by aberrant immune reactions would lead to the development of serious autoimmune diseases, such as type 1 diabetes [44,354].

There are recessive and dominant mechanisms for achieving immunological tolerance. The recessive tolerance includes induction of autoreactive lymphocyte apoptosis, receptor editing by replacing autoreactive T or B cell receptors with nonreactive receptors, and functional inactivation (anergy) of autoreactive lymphocytes [355-357]. When the recessive tolerance is not efficient to get rid of all autoreactive lymphocytes, the dominant tolerance has been proposed to counter this threat via a specialized population of T cells, called regulatory T cells (Tregs). Tregs have dominant suppressive effects on the autoreactive lymphocytes migrating to the secondary
1.4.1 Amino acid catabolism and immune self-tolerance

Like other cells, immune cells must have sustained access to amino acids to maintain their survival, differentiation, proliferation and function [359-362]. Deprivation of amino acids by catabolism causes immune cell apoptosis and compromises cell activation [363,364]. Thus, targeting amino acid catabolism in autoreactive lymphocytes may play an important role in modulation of immune tolerance and manipulation of autoimmunity.

Tryptophan (Trp) is the least abundant essential amino acid in human body. However, the importance of its catabolism in immune tolerance has been revealed by many recent studies [365,366]. There are three hemoprotein enzymes responsible for catabolizing Trp, including two isoforms of indoleamine 2,3-dioxygenase (IDO1 and IDO2) (detailed in Chapter 1.4.2) and tryptophan 2,3-dioxygenase (TDO) [367]. In the body, TDO is mainly expressed in the liver, but it is also found in other sites, such as skin and brain [364,368]. Stress-related glucocorticoids can induce TDO expression to catabolize L-Trp by oxidative cleavage of the indole ring [369]. It has been shown that Trp deprivation via catabolism induces cell cycle arrest of T lymphocytes and also affects lymphocyte viability and function [360-362].

Similar with Trp catabolism, arginine (Arg) catabolism is also involved in the regulation of immune tolerance [370-372]. Arg can be catabolized by three enzymes including inducible nitric oxide synthase (iNOS) and two arginase isoforms (ARG1, ARG2), all of which can be induced by inflammatory cytokines [373,374]. It has been identified that Arg catabolism plays a critical
role in suppression of antigen-specific T-cell responses [372]. For example, ARG1 can inhibit maternal immune responses to the fetus during mammalian pregnancy by catabolizing and depleting Arg [375].

1.4.2 Indoleamine 2,3-dioxygenase

It has been shown that only about 1% of Trp from food is utilized to synthesize the neurotransmitter, serotonin, with most (>95%) dietary Trp metabolized along the kynurenine (Kyn) pathway (Figure 1.7). As discussed previously, TDO that is mainly expressed in the liver is an enzyme responsible for catabolizing Trp as the first step of the Kyn pathway [376,377]. However, Kyn as the metabolite of Trp has also been detected in many other tissues, such as intestine, suggesting that another enzyme responsible for catabolizing Trp can be expressed in the extrahepatic tissues [378,379]. The enzyme was named indoleamine 2,3-dioxygenase (IDO) that is expressed in various organs, such as lung, small and large intestine, colon, spleen, kidney, stomach, and brain [378-380].
1.4.2.1 Structure and expression of IDO

IDO is a cytosolic enzyme containing a heme prosthetic group. Human IDO consists of 403 amino acids with molecular weight of 45kDa [381,382]. Using X-ray crystallography, it has been shown that the main structure of IDO contains two distinct alpha-helical domains. The large catalytic alpha-helical domain is at C-terminus of the protein and provides a cavity for the heme prosthetic group, while the small non-catalytic alpha-helical domain is at N-terminus. There is also a long loop for connecting these two domains [383].

IDO has a constitutive expression only in lower gastrointestinal tract [384]. However, it can be expressed in many different cells and tissues in an inducible manner. For example, interferon-
gamma (IFN-γ) stimulates IDO expression in various cell types, including trophoblasts, macrophages, dendritic cells, cultured fibroblasts and pancreatic islet β-cells [364,385-388]. Studies have found that IDO also exists in eosinophils and endothelial cells which are important cell types in allergic inflammation [389,390].

IDO expression can be regulated by distinct mechanisms [391-393]. For example, IFN-γ regulates the expression of IDO gene via Janus kinase/signal transducer and activator of transcription 1 (JAK/STAT1) pathway [391]. In murine and human, IDO is encoded by Indo gene which is located on the short arm of chromosome 8 (8p12–8p11) [394]. The promoter of this gene consists of a gamma-activated sequences (GAS) and two interferon stimulatory response elements (ISREs) [364]. After binding with the receptor, IFN-γ activates JAK protein, which can further phosphorylate STAT1. Phosphorylated STAT1 then translocates to nuclei and binds to the promoter of Indo gene thereby stimulating IDO expression [395]. However, in some specific cell types, IFN-γ-mediated IDO expression is inhibited by some cytokines, such as interleukin 4 (IL-4) and interleukin 13 (IL-13) [396,397]. Studies from our and other groups also found that JNK signaling pathway plays a key role in lipopolysaccharide (LPS)-induced IDO expression [393,398]. Moreover, transforming growth factor β (TGFβ), another inducer of IDO expression, has been identified to promote IDO gene transcription via PI3-kinase/PKB signaling pathway [392]. There are also many other stimulators of IDO gene transcription, such as CTLA-4, CpG-rich oligodeoxynucleotides and endogenous thymosin α [399-401]. At the post-translational level, suppressor of cytokine signaling 3 (SOCS3) have been identified to inhibit IDO expression by binding IDO for ubiquitinylation and degradation [402]. Further, certain types of antigen-presenting cells stimulate or inhibit functional IDO expression in response to
different exogenous signals [403,404]. This modulation has been proposed to be important for maintaining the balance of immune system [405].

1.4.2.2 IDO and immune regulation

IDO has significant suppressive effects on effector T cells including inhibition of their viability, function, proliferation and differentiation [365]. It can also promote the differentiation of Tregs [406]. Therefore, it plays a central role in maintenance of immunological tolerance. There are two main mechanisms of IDO-mediated immunological regulations: metabolic immune regulation and signaling immune regulation (Figure 1.8) [407].

**Metabolic immune regulation.** IDO catabolizes the essential amino acid, Trp, to produce Kyn, which provides IDO with a role in metabolic regulation of immune responses. This kind of regulation includes two main ways: generation of a ligand for the aryl hydrocarbon receptor (AhR) via producing Kyn; and induction of amino-acid sensing pathways via depleting Trp. In the first way, Kyn acts as the activating ligand for the transcription factor AhR [408]. It has been shown that activation of AhR has direct effects on suppression of cytotoxic T cells, reduction of immunogenicity of DCs and differentiation of forkhead box (FOX)P3+ regulatory T cells [408,409]. Thus, administration of natural or synthetic Kyn has been identified to promote immune tolerance [410,411]. In the second way, Trp depletion increases the level of uncharged transfer RNA (tRNA) in T cells, which further activates the amino acid-sensitive general control nondepressible-2 (GCN2) kinase [412,413]. Then some downstream transcription factors, such as eukaryotic initiation factor 2 (eIF2α) and activating transcription factor 4 (ATF4) are regulated by the active GCN2 kinase [414,415]. Studies have shown that activation of GCN2
pathway leads to the cell-cycle arrest and functional anergy of CD8+ T cells [362]. It also blocks T helper 17 (Th17) cell differentiation, whereas it enhances the differentiation and function of Tregs [416-419]. Besides modulation of T cells, activation of GCN2 pathway by Trp deprivation also regulates IDO-expressing APCs [420]. Trp starvation can also suppress the nutrient-sensing mammalian target of rapamycin (mTOR) pathway, which is necessary for initiation of ribosomal translation, especially for the translation of T cells and thereby suppressing T cells [412,421-423].

**Signaling immune regulation.** IDO can also regulate the immune responses by an enzymatic activity-independent way. In this way, due to containing immuno-receptor tyrosine-based inhibitory motifs (ITIM), IDO acts as a direct intracellular signaling molecule in APCs that express it [424]. In the presence of TGF-β, IDO can be activated by phosphorylation of ITIM. Active IDO then triggers a variety of downstream signaling effectors including Src homology 2 domain phosphotyrosine phosphatases (SHPs) and noncanonical (anti-inflammatory) NF-κB, which can induce further production of TGF-β and type I IFNs, and also favor a bias of the APCs toward a tolerogenic phenotype [400,425,426]. Thus, with this positive feedback loop, IDO can keep its own activation to induce immune tolerance.
Figure 1.8 IDO-mediated immunomodulation via metabolic and signaling pathways.
IDO-mediated Trp depletion and Kyn production can regulate immune cells via amino-acid sensing pathway (GCN2 and mTOR) and AhR pathway, respectively. IDO can also regulate immune responses via an enzymatic activity-independent signaling pathway with TGF-β. Thus, immuno-tolerance is induced by IDO through suppression of effector T cell proliferation, survival and function, as well as stimulation of Treg differentiation and function. IDO: indoleamine 2,3-dioxygenase, Trp: tryptophan, Kyn: kynurenine, GCN2: control nondepressible-2, mTOR: mammalian target of rapamycin, AhR: aryl hydrocarbon receptor, TGF-β: transforming growth factor beta, SHPs: Src homology 2 domain phosphotyrosine phosphatases, NF-κB: nuclear factor-κB, IFN-α/β: interferon-alpha/beta.

Although most of related studies focus on IDO-mediated regulation of T cells, there are some findings showing that IDO can also modulate the viability and function of B cells [427-429], as well as the proliferation of NK cells [430,431].
1.4.2.3 Role of IDO in specific physiological and pathological conditions

1.4.2.3.1 IDO and mammalian pregnancy

During the mammalian pregnancy, the fetus successfully survives by defending itself from maternal immunological attack. IDO has been proposed to be responsible for this phenomenon due to its immunosuppressive effects and high expressions during the pregnancy. This hypothesis has been initially proven by using pregnant mouse models [432]. During human pregnancy, IDO can be produced by blastocysts as early as day 6. Then it is continuously expressed in the villous stoma and on the fetal membranes by different types of cells including syncytiotrophoblasts, extravillous cytotrophoblasts, and macrophages [388,433]. Studies have identified that the hormones, such as chorionic gonadotrophin that are highly produced during pregnancy can enhance the expression of IDO [434]. Furthermore, it has been reported that defective activation of IDO during pregnancy can lead to several complications including abortion. For example, changes of IDO activity or level may be involved in the pathogenesis of pre-eclampsia, which is a severe pregnancy complication [435,436].

1.4.2.3.2 IDO and autoimmune conditions

Animal studies have identified that IDO is also responsible for regulating the progression of autoimmune diseases, such as T1D [401,437]. For example, in prediabetic NOD female mice, IDO-expressing splenic dendritic cells have a transient dysfunction of suppressing cytotoxic T lymphocytes (CTLs) [438]. Additionally, deprivation of IDO expression and activity by depletion of DCs or treatment with IDO inhibitors, such as 1-methyl-tryptophan (1MT) increases insulitis severity and accelerates the development of T1D in NOD mice [72]. Accordingly, immunotherapy based on inducing IDO can significantly prevent T1D or slow the disease
progression [437,439]. In murine models, pharmacologic inhibition of IDO also exacerbates many other autoimmune diseases including experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease and rheumatoid arthritis [440-443]. Moreover, evidence has shown that pregnancy regresses the autoimmune diseases due to the high expression of IDO, which further confirms that IDO might play an important role in regulating autoimmunity [406].

1.4.2.3.3 IDO and transplantation

Based on its protective effects during pregnancy, IDO has been proposed to play an essential role in prevention of allograft rejections after transplantation [444]. It has been reported that IDO which is expressed by epithelial cells has immunoprotective effects on cardiac allografts with CD40-Ig treatment [445]. Recently, IDO-expressing cells have been found to induce donor specific tolerance even without the help of immunosuppressive drugs. For example, IDO has been shown to protect murine renal, heart or islet allografts against rejection without additional immunosuppressants [169,445-447]. Additionally, our group induced IDO expression in bystander fibroblasts and found that these IDO-expressing fibroblasts resulted in a five-fold decrease of cell proliferation rate in allogeneic lymphocytes during in vitro culture [169]. However, there is no significant difference in cell proliferation between fibroblasts with or without IDO expression. [448]. These findings indicate that IDO expression successfully suppresses the proliferation of allogeneic lymphocytes rather than the fibroblasts. As such, this approach can be used to specifically inhibit the allo-immunity without compromising non-immune cells.
1.4.2.3.4  IDO and cancer

IDO expression has been detected in 25 different types of human cancers, indicating its important role in the development of cancer therapy [449]. Both tumor cells and DCs in tumor-draining lymph nodes (LNs) express IDO [450,451]. IDO expression by tumor cells contributes to generating the local immune suppressive microenvironment [449,452]. In animal studies, developing tumors increase IDO expression by DCs in tumor-draining LNs [451]. This process is regulated by transcription factor forkhead box O3 (FoxO3) which mediates the binding of CTLA-4-expressing Tregs to B7 molecules on DCs thereby inducing IDO expression by DCs [453-455]. Reciprocally, IDO expression can stimulate the differentiation of Tregs via the GCN2 and AhR pathways which was discussed above [408,419]. Thus, there is a positive reinforcement between IDO and Tregs, which exerts immunosuppressive effects in the tumor microenvironment. Accordingly, blocking IDO by IDO-inhibitors is considered as a cancer immunotherapy and several drugs are now in Phase I clinical trials [449,456,457]. However, some studies found an opposite role of IDO in cancer by indicating that IDO-mediated tryptophan depletion can act as a negative modulator of tumor growth [458-460]. It has been identified that IFN-γ-induced IDO expression has a suppressive effect on tumor cell proliferation via tryptophan deprivation [459].

1.4.2.3.5  IDO and neurological diseases

Both clinical studies and basic researches have shown that IDO plays a critical role in the development of neurologic depression [461-464]. For example, increased plasma Kyn levels have been detected in depressive patients and high IDO expression induced by chronic stress causes the depressive behavior in animal models [463,465]. The depression possibly results from
Trp catabolism by IDO. Trp is the substrate for synthesis of serotonin (5-HT) which is responsible for balancing mood [466,467]. Thus, reduced level of serotonin by depleting Trp via IDO leads to the depression [466,468]. Additionally, neurotoxic IDO activity in brain has also been proposed to contribute to the pathogenesis of dementia in patients with specific chronic infection or neurodegenerative disorders such as Alzheimer’s disease, suggesting the importance of IDO-inhibitors in treatments with these neurological diseases [469-471].

1.5 Rationale, hypothesis and objectives

In T1D, there is a progressive loss of insulin-producing islet β-cells due to immunological attack by autoreactive T cells. Thus, β-cell replacement by islet transplantation provides a feasible approach for treatment of T1D. However, the efficacy of this treatment is currently limited by insufficiency of available islet donors and poor long-term survival of islet grafts [472,473]. A considerable number of islets are lost during the pre-transplantation culture period [131,474,475]. Formation of toxic protein aggregates named islet amyloid and loss of islet extracellular matrix (ECM) are two important non-immune factors that contribute to β-cell dysfunction and death both during pre-transplant islet culture [131] and in islet grafts [132]. In addition to non-immunological detrimental factors, it has been shown that transplanted islets are susceptible to the recurrent autoimmunity, which finally results in islet graft failure [130,476]. This suggests that regulation of autoimmunity is also important for improving the outcome of islet transplantation. More importantly, inhibition of autoimmunity itself is able to prevent or reverse T1D [156,166,477]. Thus, the goal of this study was to identify the new strategies to protect islet β-cells from the toxicity induced by non-immune or autoimmune factors thereby
enhancing islet β-cell viability and function both in vitro and in vivo. This goal has been achieved by pursuing the following three specific objectives:

**Objective 1: To investigate the effects of three-dimensional (3-D) scaffolds on survival and function of human islets during in vitro culture (Chapter 2).**

We developed two 3-D scaffolds, collagen matrix (CM) alone or human fibroblast-populated collagen matrix (FPCM). The collagen provides surrogate ECM materials for islets and the fibroblasts maintain the scaffold structure and release different growth factors [478]. We used isolated human islets embedded in collagen matrix without (CM) or with fibroblasts (FPCM) to:

*Objective 1.1:* Examine whether CM and/or FPCM can improve the viability and function of human islets during in vitro culture.

*Objective 1.2:* Test whether CM and/or FPCM can reduce islet amyloid formation in human islets during in vitro culture.

**Objective 2: To investigate the effects of islet amyloid formation on PKB activation (phosphorylation) in islet β-cells during in vitro culture (Chapter 3).**

Islet amyloid formation, a pathological hallmark of pancreas in T2D, also forms in pre-transplant cultured human islets and transplanted islets [205,219,249]. It acts as a non-immune factor contributing to islet β-cell death and dysfunction, which may finally lead to islet graft failure [230,249]. Multiple signaling pathways have been proposed to be involved in the cytotoxicity induced by islet amyloid formation [230,233,248,249]. PKB is a key effector of the PI3-kinase signaling pathway and plays a significant role in the regulation of islet β-cell viability, proliferation and function both in vitro and in vivo [285-287]. Thus, we investigated the effects of
islet amyloid formation on PKB activation in cultured islet β-cells. We used isolated human islets and transgenic mouse islets to:

Objective 2.1: Examine whether islet amyloid formation reduces PKB phosphorylation in β-cells.

Objective 2.2: Test whether reduction of islet amyloid formation by CM restores PKB phosphorylation.

Objective 2.3: Examine whether the effects of islet amyloid formation on PKB phosphorylation are mediated by IL-1β.

Objective 3: To investigate the factors influencing the efficacy of IDO cell therapy for T1D reversal in newly-onset diabetic NOD mice and the potential mechanisms (Chapter 4).

Induction of immunological self-tolerance can inhibit the progression of T1D through protecting islet β-cells from autoimmune attack and preserving the mass of functional β-cells to sustain glucose homeostasis [171]. Indoleamine 2,3-dioxygenase (IDO) is an enzyme responsible for catabolizing the essential amino acid, tryptophan, to kynurenine. It has been elucidated that decreased tryptophan level and increased production of tryptophan metabolite-kynurenine have effects on both suppressing toxic effector T cells and boosting regulatory T cells [387,479].

Under this objective, dermal fibroblasts were transduced with lenti-viral vector to stably express IDO. We used these IDO-expressing fibroblasts as a cell therapy to:

Objective 3.1: Investigate the factors determining the efficiency of IDO-cell therapy in treatment of T1D in NOD mice.

Objective 3.2: Scrutinize the potential immunological mechanisms of the optimized IDO-cell therapy.
In summary, we hypothesized that: 1). 3-D scaffolds can prevent toxic islet amyloid formation and restore amyloid-induced reduction of phosphorylated PKB levels thereby enhancing islet β-cell viability and function during *in vitro* culture; 2). treatment with optimized IDO-expressing fibroblasts would successfully reverse recent onset T1D in NOD mice by protecting the survival and function of residual islet β-cells.
Chapter 2: Three-dimensional scaffolds reduce islet amyloid formation and enhance survival and function of cultured human islets

2.1 Introduction

In the past decade, replacement of pancreatic β-cells with human islet transplantation has provided a feasible approach for treatment of patients with type 1 diabetes [125,480,481]. However, human islet transplantation is currently limited by low numbers of available pancreatic donors, a significant loss of functional islet mass during islet isolation and after transplantation, and poor long-term survival of islet grafts [472,473,482]. A study from the Edmonton Group showed that, although 80% of human islet graft recipients achieve insulin independence during the first year, only 10% of patients remain insulin independent by 5 years after transplantation [481]. Both immune and non-immune factors contribute to reduced β-cell mass and function in transplanted islets, which eventually leads to islet graft failure [149,472].

Nonimmune-mediated islet β-cell death occurs during the various steps of islet transplantation, including islet isolation, culture, and implantation [127,472,483]. A considerable number of islets are lost during the pre-transplant culture period [131,474,475], as well as in the first days after transplantation [484-486]. Formation of islet amyloid toxic protein aggregates and loss of islet extracellular matrix are two important non-immune factors that contribute to β-cell dysfunction and death both during pre-transplant islet culture [131] and in islet grafts [132].

Islet amyloid, formed by aggregation of islet amyloid polypeptide, is a pathological characteristic
of the pancreas in type 2 diabetes that contributes to progressive β-cell death in this disease [205]. Several studies have shown that toxic islet amyloid polypeptide aggregates also form in cultured and transplanted human islets [131,132,264,265]. Similar to that seen in type 2 diabetes, formation of islet amyloid in both cultured and transplanted human islets is associated with β-cell dysfunction and death and contributes to islet graft failure [132,205,264,265].

In natural pancreatic tissue, islet cells are surrounded by a network of proteins and polysaccharides named extracellular matrix (ECM) [129]. In addition to its structural support, ECM also provides a variety of signaling that plays important roles in maintaining islet cell viability and function [487-491]. However, enzymatic and mechanical stresses induced by islet isolation destroy the structure of islet ECM and disturb the islet-ECM interactions, which may contribute to islet graft failure [492]. In order to restore islet ECM, our research group previously developed a composite scaffold that consists of fibroblasts embedded within collagen matrix [493]. In this model, the collagen provides surrogate ECM materials for islets and the fibroblasts maintain the scaffold structure and release different growth factors [478].

In the present study, we used human islets isolated from cadaveric pancreatic donors to test whether collagen matrix (CM) alone or human fibroblast-populated collagen matrix (FPCM) can reduce formation of β-cell toxic amyloid aggregates in human islets during culture and enhance islet viability and function.
2.2 Materials and methods

2.2.1 Preparation of human fibroblasts

Human fibroblasts were explanted from foreskin samples of infants in accordance with the ethical guidelines set forth by the University of British Columbia. Briefly, foreskin samples from infants undergoing elective circumcision were obtained under local anesthesia. Skin samples were washed six times in sterile 1 X PBS supplemented with a 1% antibiotic-antimycotic mixture (100 µg/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B) (Life Technologies-Invitrogen, Auckland, New Zealand). Epidermal and dermal layers were separated by treatment with dispase (40 mg/mL; Life Technologies-Invitrogen) for 2 hours at 37°C. After removal of the epidermal layer, dermal tissues were minced into small pieces (1 to 2 mm in diameter) and transferred into 60 X 15-mm Petri dishes, and fibroblasts were cultured following the established protocol [494]. Cultured fibroblasts (~80% confluent) were trypsinized and cultured in 75-cm² culture flasks. Passages 3 to 7 of cultured fibroblasts were used for the experiments.

2.2.2 Culture of human islets

Human islets used in all studies of this thesis were isolated from cadaveric pancreatic donors by Ike Barber Human Islet Transplant Laboratory (Vancouver, BC, Canada) in accordance with approved procedures and guidelines by the Clinical Research Ethics Board of the University of British Columbia. Islets were hand-picked, and islet purity was assessed by dithizone staining. Human islets (purity ~90%) were then cultured in noncoated 48-well plates under three different conditions: free-floating in Ham’s/F10 medium (Sigma-Aldrich, St. Louis, MO, USA), embedded within collagen matrix/Ham’s/F10 (CM), or embedded within fibroblast-populated
collagen matrix/Ham’s/F10 (FPCM). For all three conditions, islets were cultured for 7 days in a humid atmosphere (95% air, 5% CO2) at 37°C. Ham’s/F10 medium was supplemented with 6 mmol/L glucose, 12 mmol/L HEPES, 2 mmol/L L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The medium was replaced every 48 hours. For amyloid studies, islets were cultured in high-glucose (16 mmol/L) medium from day 4 to day 7, to potentiate islet amyloid formation in human islets.

2.2.3 Preparation of FPCM and CM islet composites

CM and FPCM were established as described previously [493], with some modifications. Briefly, CM for islet culture was prepared by adding 60 µL of 3 X Ham’s/F10 medium (Sigma-Aldrich), 10 µL of 0.1 mol/L NaOH, 21 µL of fetal bovine serum (Life Technologies-Invitrogen), and 150 µL of 5 mg/mL acid-extracted fetal bovine type I collagen (Sigma-Aldrich) per well in 48-well plates (final collagen concentration: 2.4 mg/mL). To prepare FPCM, 10^4 human fibroblasts were added to the collagen matrix just described. Human islets (100 islets/well) were then added to each well containing either CM or FPCM before solidification of the collagen.

2.2.4 Islet viability assay

Islet cell viability was assessed by a viability/cytotoxicity assay kit (Life Technologies-Invitrogen, Eugene, OR, USA) according to the manufacturer’s instructions. Briefly, islet samples (100 islets/well) were incubated with the working solution containing ethidium homodimer-1 (4 mmol/L) and calcein AM (2 mmol/L) in 1 X PBS for 30 minutes at room temperature under dark conditions. Islets were examined under an Axiovert 200M fluorescence
microscope (Carl Zeiss, Oberkochen, Germany). Live cells with intact membrane were detected by the green fluorescence generated by ubiquitous intracellular esterase in those cells; dead cells with damaged membrane were detected by ethidium homodimer-1 staining (red fluorescence). The proportion of live and dead islet cells were analyzed by flow cytometry performed on dispersed islet cells.

2.2.5 Glucose-stimulated insulin secretion

FPCM and CM composites were digested by incubating composites in type I collagenase solution (4 mg/mL; Sigma-Aldrich) at 37°C for 5 minutes. Collagenase activity was then stopped by adding supplemented Ham’s/F10 medium and human islets were hand-picked from digested matrices under a microscope. Islets (50 islets/well) were then pre-incubated (1 hour) in Krebs-Ringer bicarbonate buffer containing 10 mmol/L HEPES (pH 7.4), 0.25% bovine serum albumin, and 1.67 mmol/L glucose in 48-well plates at 37°C, followed by 1 hour incubation in Krebs-Ringer bicarbonate buffer containing either 1.67 mmol/L glucose (basal insulin release) or 16.7 mmol/L glucose (glucose-stimulated insulin release). Human islets in each well were lysed in 100 µL lysis buffer containing 1 mol/L HCl and 0.1% bovine serum albumin. The incubation medium and islet lysates were collected and centrifuged (13,000 X g) for 10 minutes at 4°C, and the supernatants were frozen at -20°C until assayed. Insulin levels in medium and islet lysates were measured using a human specific insulin enzyme-linked immunosorbent assay (ELISA) kit (Alpco Diagnostics, Salem, NH, USA).

2.2.6 Immunohistochemistry and detection of islet amyloid

Paraffin-embedded sections (5 µm) of islets cultured in HAM’s F10 (two-dimensional), acellular
CM, or FPCM were deparaffinized and hydrated by incubation in xylene and ethanol. To retrieve cell surface antigens, a steamer heating pretreatment with citrate buffer was performed before blocking with 2% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS (PH 7.4). For double immunostainings of insulin/glucagon or insulin/ cleaved (active) caspase-3, islet sections were incubated with primary antibodies at 4°C overnight, then rinsed with PBS and incubated with secondary antibodies for 1 h at room temperature. Sections were then mounted with a mounting medium containing nuclear dye DAPI (Vector laboratories, Burlingame, CA). For detection of islet amyloid, islet sections were immunostained for insulin as detailed above, followed by incubation with 0.5% (w/v) thioflavin S solution (Sigma-Aldrich) for 5 minutes at room temperature. For triple immunostainings of insulin/Fas/thioflavin S (Thio S) or insulin/cleaved (active) caspase-3/Thio S, islet sections were immunolabelled for insulin and Fas, or cleaved (active) caspase-3 and then islet sections were incubated for 5 minutes at room temperature with 0.5% thioflavin S solution. The list of primary and secondary antibodies used for immunostainings are shown in Table 2.1 and Table 2.2, respectively. Islet images were captured using a Zeiss Axioplan 2 microscope and AxioVision image analysis software.

<table>
<thead>
<tr>
<th>Staining</th>
<th>Antibody and dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin/Glucagon</td>
<td>Guinea pig anti-insulin (1:750)</td>
<td>Dako, Carpinteria, CA</td>
</tr>
<tr>
<td></td>
<td>Rabbit anti-glucagon (1:750)</td>
<td>Dako, Carpinteria, CA</td>
</tr>
<tr>
<td>Insulin/Cleaved caspase-3/ (Thio S)</td>
<td>Guinea pig anti-insulin (1:500)</td>
<td>Dako, Carpinteria, CA</td>
</tr>
<tr>
<td></td>
<td>Rabbit anti-cleaved caspase-3 (1:100)</td>
<td>R&amp;D Systems, Burlington, ON, CA</td>
</tr>
<tr>
<td>Insulin/Thio S</td>
<td>Guinea pig anti-insulin (1:750)</td>
<td>Dako, Carpinteria, CA</td>
</tr>
<tr>
<td>Insulin/Fas/Thio S</td>
<td>Guinea pig anti-insulin (1:750)</td>
<td>Dako, Carpinteria, CA</td>
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<tr>
<td></td>
<td>Rabbit anti-Fas (1:100)</td>
<td>Cell Signaling, Pickering, ON, CA</td>
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### Table 2.2 Secondary antibodies for immunolabelling human islet sections

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<th>Antibody and dilution</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin/Glucagon</td>
<td>Texas Red-conjugated anti-guinea pig IgG (1:750)</td>
<td>Jackson Labortories, West Grove, PA, USA</td>
</tr>
<tr>
<td></td>
<td>Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:750)</td>
<td></td>
</tr>
<tr>
<td>Insulin/Cleaved caspase-3</td>
<td>FITC-conjugated goat anti-guinea pig IgG (1:1000)</td>
<td>Abcam, Cambridge, MA, USA</td>
</tr>
<tr>
<td></td>
<td>Rhodamine-conjugated anti-rabbit IgG (1:1000)</td>
<td>Chemicon International, Temecula, CA</td>
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<tr>
<td>Insulin/Cleaved caspase-3</td>
<td>Texas Red-conjugated anti-guinea pig IgG (1:750)</td>
<td>Jackson Labortories, West Grove, PA, USA</td>
</tr>
<tr>
<td>Thio S</td>
<td>Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000)</td>
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<td>Insulin/Fas/Thio S</td>
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<tr>
<td></td>
<td>Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200)</td>
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</table>

#### 2.2.7 Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

CM and FPCM composites were digested using type-I collagenase (4mg/ml; Sigma-Aldrich) as described above and islets were hand-picked from cell suspensions. Total RNA was isolated from human islets using an RNeasy kit (Qiagen, Valencia, CA). cDNA was synthesized using SuperScript first-strand synthesis system for RT-PCR (Life Technologies-Invitrogen). Polymerase chain reaction (PCR) was then performed using primers shown in Table 2.3. Amplified PCR products were then separated by 1% agarose gel electrophoresis and visualized with SYBR Safe DNA gel staining (Life Technologies-Invitrogen) under UV light.
Table 2.3 Primers used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (F); Reverse primer (R)</th>
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<tbody>
<tr>
<td>Insulin</td>
<td>F: 5’-AGGCCATCAAGCAGATCACTGTCCCT-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-ACGCTTCTGCAGGGACCCCT-3’</td>
</tr>
<tr>
<td>PDX1</td>
<td>F: 5’-GCGGTCCTGGAGGAGGCCCAA-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GGCGCGGCGCCGTAGATGTAC-3’</td>
</tr>
<tr>
<td>GLUT2</td>
<td>F: 5’-TGGGTGGGTGCTTGGGGGAC-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CCCCTGAGAGCGGTGGGAGC-3’</td>
</tr>
<tr>
<td>Fas</td>
<td>F: 5’-GCTGGGCATCTGGACCTCCTCCT-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-TTGGCAGGGCACGCAGTCTCG-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5’-GCGATTGTCTCTCAATGACCA-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-TGGAGGGAGATGCTCAGTG-3’</td>
</tr>
</tbody>
</table>

2.2.8 Statistical analysis

Data are expressed as means ± SEM. Statistical analyses were performed using one-way analysis of variance (one-way ANOVA), followed by post hoc multiple comparison tests. P < 0.05 was taken as significant. All experiments were performed in triplicate and were repeated three times.

2.3 Results

2.3.1 Three-dimensional scaffolds improve viability of human islets during in vitro culture

To examine the effects of the 3-D scaffold and the potential role of fibroblasts in enhancing islet survival in human islets, isolated human islets were embedded within collagen matrix with human fibroblasts (FPCM) or without (CM). Free-floating human islets cultured in standard two-dimensional (2-D) culture plates were used as control. Islet morphology was monitored during culture, and viability testing was performed on islets before and after 7 days culture under each condition. Embedded human islets within both CM and FPCM preserved normal islet morphology, whereas 2-D cultured islets gradually lost their normal morphology during culture.
(Figure 2.1A). Furthermore, detection of live and dead islet cells by calcein AM and ethidium homodimer-1 staining showed that both CM- and FPCM-embedded human islets had significantly lower numbers of dead cells, compared with those in 2-D cultured islets (Figure 2.1A). These findings were further confirmed by quantification of dead islet cells by flow cytometry performed on dispersed human islet cells from each culture condition (Figure 2.1B and C). Islet cell viability was ~90% before culture and was reduced to 63% in 2-D cultured islets after 7 days of culture, whereas cell viability in CM- and FPCM-embedded islets was ~83% and ~92%, respectively. Maintenance of cell viability in 3-D scaffold cultured islets closely correlated with a reduced number of dead cells in CM (~16%) and FPCM (~8%) cultured islets, compared with 2-D cultured islets (~38%). There was no significant difference in cell viability between CM- and FPCM-embedded human islets.
Figure 2.1 Three-dimensional scaffolds improve viability of cultured human islets.
Human islets were cultured in 3-D fibroblast-populated collagen matrix (FPCM), collagen matrix (CM), or 2-D Ham’s/F10 medium. Islet cell viability was assessed pre-culture (day 0) and after 7 days of culture using a live/dead viability/cytotoxicity assay kit. (A) Islet morphology and viability in different culture conditions. Note loss of normal islet morphology during 2-D culture (bright-field images, taken under inverted microscope). Live islet cells are shown in green, dead islet cells in red. Original magnification, X400. (B) Representative histograms of flow cytometry assays performed on dispersed human islet cells. (C) Quantification of live and dead islet cells in 2-D and 3-D cultured human islets by flow cytometry. Data are expressed as means ± SEM of...
three independent experiments performed in triplicate. *P < 0.01 vs 2-D cultured islets, one-way ANOVA.

2.3.2 Human islets embedded within three-dimensional scaffolds have lower β-cell apoptosis and higher islet β/α cell ratio compared with two-dimensional cultured islets

To identify whether the dead islet cells detected with ethidium homodimer-1 staining were β-cells, paraffin-embedded islet sections were double-immunostained for insulin and cleaved (active) caspase-3 before and after 7 days of culture under two-dimensional, CM, or FPCM conditions. The percentage of cleaved caspase-3-positive and insulin-positive islet cells was quantified under the three culture conditions. As expected, the number of cleaved caspase-3-positive (apoptotic) islet cells was low in pre-culture human islets, but increased markedly during 7 days of culture under the 2-D condition (Figure 2.2). The majority of apoptotic cells in cultured islets were β-cells. Interestingly, islets embedded within both CM and FPCM had markedly lower numbers of apoptotic β-cells (Figure 2.2). This decrease in the number of apoptotic islet β-cells in CM and FPCM cultured islets was associated with higher numbers of insulin-positive islet cells (Figure 2.3) and higher β/α cell ratios in CM- and FPCM-embedded islets compared with 2-D cultured islets (Figure 2.3), suggesting that 3-D scaffolds reduce β-cell death and preserve β-cell viability in human islets during culture. Co-culture of human islets with human fibroblasts further reduced β-cell apoptosis during culture (Figure 2.3).
Figure 2.2 Caspase-3 activation was markedly reduced in islet β-cells of human islets cultured in three-dimensional scaffolds, compared with two-dimensional cultured islets. (A) Double immunofluorescence staining for cleaved (active) caspase-3 and insulin of paraffin-embedded human islet sections before (day 0) and after 7 days of culture under three different conditions. DAPI was used as a nuclear counterstain. The boxed region corresponds to the inset at higher magnification. Original magnification: X400; X1000 (inset). (B) The number of active caspase-3-positive and insulin-positive islet cells and the total number of β-cells were counted in islets for each culture condition in a minimum of 20 islets per condition. Data are expressed as means ± SEM of three independent experiments. *P < 0.05 vs 2-D cultured islets, one-way ANOVA.
Figure 2.3 Human islets cultured in three-dimensional scaffolds have higher $\beta/\alpha$ cell ratios, compared with two-dimensional cultured islets.

(A) Double immunofluorescence staining for insulin and glucagon of paraffin-embedded human islet sections (5 $\mu$m) before and after 7 days of culture under three different conditions (FPCM, CM, and 2-D culture). DAPI was used as a nuclear counterstain. Original magnification: X400. (B) Proportion of insulin and glucagon positive islet cells before and after 7 days of culture. The $\beta/\alpha$ cell ratio was calculated by counting the number of $\beta$-cells and $\alpha$-cells in each islet in a minimum of 40 islets per condition. Data are expressed as means $\pm$ SEM of three independent experiments. *P $<$ 0.01 vs 2-D cultured islets, one-way ANOVA.
2.3.3 Three-dimensional scaffolds preserve insulin, PDX1, and GLUT2 mRNA expression in cultured human islets

We tested the effects of 3-D scaffolds on mRNA expression of three key β-cell genes: insulin (INS), pancreatic and duodenal homeobox 1 (PDX1), and glucose transporter 2 (GLUT2). Islet culture in standard 2-D plates resulted in reduced mRNA expression of the genes for insulin, GLUT2, and PDX1, but their expression was preserved in CM- and FPCM-embedded islets during 7 days of culture. There was no significant difference in mRNA expression of any of these genes between CM- and FPCM-embedded islets (Figure 2.4).

![Figure 2.4](image)

**Figure 2.4** Three-dimensional scaffolds preserve expression of insulin, PDX1, and GLUT2 in cultured human islets.

Before and after 7 days of culture under three different conditions (CM, FPCM, and 2-D culture), expression of insulin, PDX1, GLUT2 mRNA in human islets was assessed by performing RT-PCR. Data were normalized to the level of GAPDH mRNA, which was used as internal control. 3-D scaffolds preserved expression of insulin, PDX1, and GLUT2 in islet β-cells during culture. Results are representative of three independent experiments.

2.3.4 Three-dimensional scaffolds improve β-cell function in cultured human islets

We examined the effects of 3-D scaffolds on human islet function by assessment of islet insulin response to elevated glucose (16.7 mmol/L) and insulin content. After 7 days in 2-D culture,
human islets lost insulin response to elevated glucose and had significantly lower islet insulin content, compared with pre-culture islets (Figure 2.5). Embedding human islets within CM partially restored islet insulin response to elevated glucose, and culture in FPCM restored it almost completely (Figure 2.5A). Furthermore, both CM- and FPCM embedded islets had islet insulin content similar to that of pre-culture islets (Figure 2.5B). These findings suggest that 3-D scaffolds, particularly FPCM, improve β-cell function, and preserve insulin content in cultured human islets.

**Figure 2.5 Three-dimensional scaffolds enhance β-cell function in human islets during in vitro culture.**

Islet function was assessed by measuring (A) glucose-stimulated (16.7 mmol/L) insulin secretion and (B) islet insulin content in isolated human islets before and after 7 days of culture under three different conditions using a human specific ELISA. Glucose-stimulated insulin secretion was depicted as fold increase over basal release (1.67 mmol/L glucose). Islet insulin content was reported as a percentage, with insulin content of pre-culture (day 0) islets taken as 100%. Islet cultured in 3-D CM and FPCM scaffolds had a markedly higher insulin response to elevated glucose and insulin content, compared with 2-D cultured islets. Maximal islet function was observed in FPCM cultured islets. Results are expressed as means ± SEM of three independent experiments. *P < 0.05 vs 2-D cultured islets; †P < 0.05 vs CM cultured islets, one-way ANOVA.
2.3.5 Amyloid formation is significantly lower in human islets embedded within three-dimensional scaffolds than two-dimensional cultured islets

To examine the effects of 3-D scaffolds on islet amyloid formation, human islets were embedded within either CM or FPCM or were cultured in standard 2-D culture plates. All culture conditions included high glucose (16 mmol/L), to potentiate amyloid formation. Paraffin-embedded islet sections from human islets before and after 7 days culture in three conditions were immunostained for insulin and amyloid (thioflavin S) and the proportion of thioflavin S (amyloid)-positive islets and islet amyloid area were assessed. Islet amyloid was not detectable in any of the fresh human islet preparations, but was easily detectable in islets after 7 days of culture in 2-D condition (Figure 2.6A). Interestingly, islets embedded within CM and FPCM had markedly lower number of thioflavin S-positive islets (Figure 2.6B), as well as significantly lower islet amyloid area compared with 2-D cultured islets (Figure 2.6C). Importantly, FPCM-embedded islets had less amyloid formation than CM-embedded islets, suggesting that collagen matrix and human fibroblasts have additive effects in prevention of amyloid formation in cultured human islets (Figure 2.6).
Figure 2.6 Human islets cultured in three-dimensional scaffolds have markedly reduced islet amyloid formation, compared with two-dimensional cultured islets.

(A) Double immunostaining of paraffin-embedded islet sections for insulin and thioflavin S before and after 7 days of culture under three different conditions (CM, FPCM, and 2-D culture) with elevated glucose to potentiate amyloid formation (arrows). Original magnification, X400. Quantification of the number of thioflavin S (amyloid)-positive islets (B) and amyloid-positive islet area (C) in human islets cultured under three different conditions. A minimum of 50 islets per condition were analyzed. *P < 0.01 vs 2-D cultured islets; †P < 0.05 vs CM cultured islets, one-way ANOVA.
Sections from human islets before culture and after culture for 7 days under the three conditions (all with 16 mmol/L glucose concentration) were triple-stained for insulin, cleaved (active) caspase-3, and amyloid (thioflavin S). There was a close correlation between amyloid-positive and active caspase-3-positive islet β-cells (Figure 2.7).

![Image of staining results](image)

**Figure 2.7 In cultured human islets, amyloid-positive islets closely correlate with active caspase-3-positive islet β-cells.**

Triple immunofluorescence staining of paraffin-embedded human islet sections (5 µm) for insulin, cleaved (active) caspase-3, and thioflavin S before (day 0) and after 7 days of culture in three different conditions (FPCM, CM, and 2-D culture) with elevated glucose to potentiate amyloid formation. The boxed region corresponds to the inset. Original magnification: X400; X1000 (inset).
2.3.6 Embedding human islets within three-dimensional scaffolds reduces islet Fas expression in cultured human islets

Sections of human islets embedded within either CM or FPCM or cultured under the 2-D condition (all with 16 mmol/L glucose concentration) were immunostained for insulin, Fas and amyloid (thioflavin S). Consistent with recent findings from our group [249], amyloid deposition in cultured human islets was associated with up-regulation of the cell death receptor Fas. Interestingly, the lower amyloid formation in human islets cultured in CM or FPCM than in 2-D cultured islets was associated with reduced expression of Fas (Figure 2.8A). We also tested mRNA levels of Fas in human islets before and after culture under different conditions. Similarly, Fas mRNA level was significantly lower in human islets cultured in 3-D scaffolds, compared with islets cultured under the 2-D condition (Figure 2.8B).
Figure 2.8 Reduced islet amyloid formation in human islets cultured in three-dimensional scaffolds is associated with lower Fas expression.

(A) Triple immunofluorescence staining for insulin, Fas and thioflavin S (amyloid) of paraffin-embedded human islet sections (5 µm thick) before and after 7 days of culture under three different conditions (FPCM, CM, and 2-D culture) with elevated glucose to potentiate amyloid formation. The boxed regions correspond to the inset. Original magnification: X400; X1000 (inset).

(B) Total RNA was isolated from human islets before and after 7 days of culture under three different conditions (FPCM, CM, and 2-D culture). Expression of Fas mRNA was assessed by performing RT-PCR. Data were normalized to the level of GAPDH mRNA, which was used as internal control.
2.4 Discussion

In the present study, we demonstrated that the 3-D scaffolds markedly reduced formation of toxic amyloid aggregates in human islets. We further demonstrated that culture of human islets within the 3-D scaffolds markedly improved islet morphology, viability, and function during *in vitro* pre-transplant culture. We believe that these findings are best explained in terms of the 3-D scaffolds acting as a surrogate of islet ECM and thus enhancing islet viability and function.

Adult human islets are surrounded by ECM, which is closely associated with a capsule consisting of a single layer of fibroblasts and collagen fibers produced by these cells [129]. Several studies have shown that islet morphology, function, and viability are heavily influenced by the islet ECM [487-490,495]. The previous studies have shown that loss of ECM and disturbances of islet-ECM interaction caused by islet isolation and culture process are important factors contributing to islet β-cell dysfunction and death, both during *in vitro* culture and in islet grafts [492,493,496]. Accordingly, different approaches have been tested, by our research group and by others, to mimic islet ECM and to re-establish islet-ECM interactions. These approaches include using various types of collagen [497,498], synthetic polymers [499], and small intestinal submucosa [500,501] as scaffold matrices to improve viability and function of isolated islets and thereby increasing the success rate of islet transplantation.

We previously introduced a novel 3-D scaffold, consisting of fibroblasts embedded in collagen matrix, to mimic the native ECM of islets. Our studies suggested that type I collagen is a favorable matrix for our proposed model, compared with other types of collagen matrices, because of its slower biodegradation rate. Type I collagen also provides the natural environment
of the fibroblasts that we used in this FPCM scaffold. Additionally, we have previously shown the important role of type I collagen in improving islet viability and function in animal experiments [493,502].

In the present study, we demonstrated that our proposed 3-D scaffolds also successfully enhance the viability and function of human islets during in vitro culture and we further showed that both of the 3-D scaffolds (CM and FPCM) preserve the expression of several key β-cell genes, including the genes encoding PDX1 and GLUT2. PDX1 is a transcription factor that plays a central role in modulation of the pancreatic β-cell function and viability by regulating some other important genes, including insulin, somatostatin, and islet amyloid polypeptide genes [503,504]. Down-regulation of PDX1 expression in β-cells may lead to β-cell failure in animal models [505]. In humans, missense mutations of the PDX1 gene result in defective insulin secretion and the development of diabetes [506-509]. GLUT2 is an important gene for glucose sensing of the pancreatic β-cell, which participates in the pathway for glucose-stimulated insulin secretion related to islet β-cell function [510]. A disordered glucose-stimulated insulin release in intrahepatic islets is associated with decreases in the expression of GLUT2 and of PDX1 [511,512]. Consistent with these findings, in the present study we demonstrated that both CM and FPCM preserve expression of PDX1 and GLUT2, with enhanced islet viability and function.

Recent studies have shown that islet amyloid, known to be a pathological characteristic of type 2 diabetes, also forms in cultured and transplanted human islets [131,132,265]. Early amyloid formation during pre-transplant culture of human islets may act as a seed for propagation of further amyloid deposition in islet grafts after transplantation into recipients with type 1 diabetes.
Amyloid formation in islets is associated with β-cell dysfunction and death, both in vitro and in vivo [131,132,265]. A growing body of evidence suggests that formation of toxic amyloid in human islets contributes to islet graft failure [132,263,265]. Reducing amyloid formation, especially at the early stage of amyloid formation during pre-transplant islet culture, may thus protect islets from amyloid toxicity and further improve survival of human islets in islet grafts.

One of the key findings of the present study is the demonstration that culture in CM and FPCM markedly reduced islet amyloid formation, compared with 2-D culture. Importantly, the effects of FPCM on preventing amyloid formation were more profound than those of CM, suggesting that collagen matrix and fibroblasts have additive effects in prevention of islet amyloid formation. The additive effects of fibroblasts in reducing amyloid formation in human islets during 3-D culture are likely mediated indirectly, via enhanced viability and function of islet β-cells. Fibroblasts produce fibronectin, which balances synthesis and degradation of matrix and maintains the structural integrity of collagen matrix in FPCM [493]. Also, previous studies from our research group and those of others have shown that fibroblasts produce and secrete angiogenic factors [513] and growth factors [478,493] that can promote islet survival and vascularization.

The recent study from our group has shown that islet amyloid formation is associated with upregulation of the cell death receptor Fas in cultured human islets [249]. Thus, we evaluated the levels of Fas in embedded and non-embedded cultured human islets. Reduced amyloid formation in human islets embedded within 3-D scaffolds was associated with lower Fas expression, compared with 2-D cultured islets. Taken together, these findings suggest that improving β-cell
viability and function by embedding within 3-D scaffolds results in reduced amyloid formation, leading to decreased Fas expression and decreased amyloid-induced β-cell death in cultured human islets.

In summary, our findings show that embedding human islets within the 3-D scaffolds described here decreases amyloid formation and increases viability and function of human islets, and that collagen matrix and fibroblasts have additive effects in enhancing islet function and reducing amyloid formation.
Chapter 3: Amyloid formation reduces phosphorylated PKB levels in cultured islet β-cells via IL-1β signaling pathway

3.1 Introduction

Islet amyloid polypeptide (IAPP or amylin) composed of 37-amino acid residues is secreted with insulin from islet β-cells [187,189,514]. This soluble polypeptide hormone has a physiological role in the regulation of postprandial glycemia through suppression of glucagon release and control of gastric emptying. IAPP also decreases food intake and participates in the regulation of body weight [515-517]. However, during islet transplantation, hIAPP aggregates to form islet amyloid deposition, which contributes to β-cell apoptosis and dysfunction, and eventually leads to islet graft failure [219,230,249,264]. It has been unveiled that the mechanisms of cytotoxicity induced by islet amyloid involve different signaling pathways [233,518]. For example, interleukin 1β (IL-1β) signaling has been identified as one of the mediators of amyloid-induced islet β-cell damage in both type 2 diabetes and islet transplantation [230,248,249].

Protein kinase B (PKB or Akt) signaling pathway plays a significant role in the regulation of cell survival and function [272,519]. In pancreatic islets, PKB is predominantly expressed in β-cells [283,520]. Additionally, activation (phosphorylation) of PKB enhances islet β-cell viability, proliferation and function both in vitro and in vivo [285-287]. Thus, regulation of PKB activity may provide a potential approach for modulation of islet β-cell mass and function [274,521].

Islet extracellular matrix (ECM) is known as another important factor in maintaining islet β-cell
survival and function [489,522]. Accordingly, restoration of islet ECM which is lost during islet isolation process and re-establishment of islet-ECM interactions is an attractive approach for improving islet graft survival [493]. In the previous study (Chapter 2), we used three-dimensional type 1 collagen matrix (CM) to mimic the native islet ECM and showed that CM-embedded human islets had markedly reduced formation of toxic amyloid fibrils during in vitro culture [146].

In the present study, we used transformed β-cells and primary islets from human and transgenic mice with β-cell-specific hIAPP expression to examine the effects of exogenously applied and endogenously formed hIAPP aggregates on PKB phosphorylation in β-cells. We also tested whether blocking islet amyloid formation by CM and/or prevention of amyloid-induced IL-1β production can restore phosphorylated PKB levels in cultured islet β-cells, thereby providing a potential strategy to enhance the viability of islet β-cells during pre-transplant culture and improve the outcome of clinical islet transplantation.

### 3.2 Materials and methods

#### 3.2.1 Culture of human islets

Isolated human islets (purity ~90% as assessed by dithizone staining) were cultured at different conditions: free-floating (FF) in Ham’s/F10 medium (Invitrogen, Burlington, ON, CA) or CMRL culture medium (Mediatech, Herndon, VA, USA), with the amyloid-binding dye Congo red (CR; Sigma-Aldrich, Oakville, ON, CA) at final concentration of 25 μmol/L, exenatide (Byetta; Amylin Pharmaceuticals, San Diego, CA, USA) at final concentration of 10nmol/L, anakinra (Kineret; Sobia Pharmaceuticals, Denton, MD, USA) at final concentration of 10μg/mL, or
embedded within collagen matrix/Ham’s/F10 (CM) as a 3-D scaffold. CM was established as described in Chapter 2.2.3. For all the conditions, islets were cultured for 7 days in a humid atmosphere (95% air, 5% CO₂) at 37°C. Culture medium was supplemented with 11.1 mmol/L glucose, 50U/mL penicillin, 50 µg/mL streptomycin and 50µg/mL gentamycin, 0.5% (w/v) BSA (in Ham’s/F10) and 10% (vol./vol.) FBS (in CMRL). The culture medium was replaced every 48 hours.

3.2.2 Animal models

Hemizygous C57BL/6 hIAPP transgenic mice (hIAPP⁺⁻) expressing β-cell-specific hIAPP were kindly provided by Dr. S. Kahn (Department of Medicine, University of Washington, Seattle, WA, USA) and maintained by breeding with DBA/2J mice (Jackson Laboratory, Bar Harbor, ME, USA). Male hIAPP⁺⁻ mice fed high fat diet form islet amyloid in vivo and develop diabetes in about one year [216]. However, during in vitro culture, with elevated glucose levels, isolated hIAPP⁺⁻ mouse islets form islet amyloid within days [211, 249]. All mice were fed mouse chow containing 9% (w/w) fat (Purina 5021; LabDiet, Richmond, IN, USA). In all studies presented in this thesis, mice were cared and maintained according to the guidelines and principles of Laboratory Animal Care and the standard procedures established by the Canadian Council on Animal Care and the University of British Columbia’s Animal Policy and Welfare Committee.

3.2.3 Mouse islet isolation and culture

Wild-type (hIAPP⁺⁻) and transgenic (hIAPP⁺⁺) mice (8-12 weeks old) were anaesthetised with tribromoethanol (0.02ml/g body weight, i.p.) and terminated by cervical dislocation. The pancreatic islets were isolated as described before [249]. Briefly, pancreases were distended
through the pancreatic duct with 2 mL calcium-free Hanks’ buffer containing 1000U/mL of ice-cold collagenase (Type XI; Sigma-Aldrich, Oakville, ON, CA). The distended pancreases were then removed and incubated at 37°C for 14 min with addition of 2 mL collagenase (1,000U/mL in Hanks’ buffer), followed by gentle shaking for 2 min. Digestion was stopped by adding ice-cold Hanks’ buffer containing 1 mmol/L CaCl₂. Islets were purified by passing the digested pancreatic tissue through 70 µm mesh cell strainers (BD Biosciences, Oakville, ON, CA). Hand-picked isolated islets (purity >95%) were cultured overnight for recovery and then cultured in non-coated 48-well plates (50 islets/well) under three different conditions: free-floating (FF) in Ham’s/F10 medium, free-floating in Ham’s/F10 medium with Congo red (25 µmol/L) or embedded within collagen matrix/Ham’s/F10 (CM). For all conditions, islets were cultured for 7 days in a humid atmosphere (95% air, 5% CO₂) at 37°C. Ham’s/F10 medium was supplemented with 16.7 mmol/L glucose, 0.5% (w/v) BSA and antibiotics as described for human islets.

3.2.4 Culture and treatment of transformed β-cells

INS-1 (832/13) cells, a transformed rat β-cell line, were kindly provided by Dr. C. Newgard (Duke University Medical Center, NC, USA). Cells were grown in RPMI-1640 (Invitrogen) containing 11.1 mmol/L glucose supplemented with 10% (v/v) FBS, 50 U/mL penicillin, 50 µg/mL streptomycin and 50 µmol/L 2-mercaptoethanol. Cells were passaged twice per week. Lyophilised hIAPP or rIAPP (Bachem, Torrance, CA, USA) were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma-Aldrich) and incubated at room temperature (1 h). Aliquots were frozen (-80°C) and lyophilised. INS-1 cells seeded in 8-well chamber slides (150,000 cells/well) were treated with hIAPP (10 µmol/L) or rIAPP (10 µmol/L) that had been prepared freshly by dissolving lyophilised peptide in culture medium and then cultured for 24 hours. The
amyloid-binding dye Congo red was added to the culture medium 30 min before addition of hIAPP at a final concentration of 25 µmol/L.

3.2.5 Immunohistochemistry, TUNEL assay and thioflavin S staining

Fixed islet sections or cells were immunostained for insulin/phosphorylated PKB (p-PKB), insulin/A11, insulin/TUNEL, insulin/thioflavin S (Thio S), insulin/PCNA, insulin/p-PKB/Thio S or insulin/IL-1β/Thio S. The immunohistochemistry protocol and image capture system used for these studies were the same as described in Chapter 2.2.6. For cells stained with PCNA were counterstained with the nuclear dye Hoechst for quantification studies. For double insulin/TUNEL immunostaining, islet sections or cells were immunolabelled with insulin then incubated with TUNEL reaction mixture (Roche Diagnostics, Laval, QC, CA) for 30 min at room temperature. The list of primary and secondary antibodies used for immunostainings are shown in Table 3.1 and Table 3.2, respectively.

Table 3.1 Primary antibodies for immunolabelling islet sections and cells

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<th>Antibody and dilution</th>
<th>Manufacturer</th>
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Table 3.2 Secondary antibodies for immunolabelling islet sections and cells

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3.2.6 Immunoblotting for phosphorylated PKB

About 100 human islets were lysed in 30 μL lysis buffer containing 50 mmol/L Tris–HCl (pH8.0), 150 mmol/L NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulphate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mmol/L PMSF and 10 μg/ml aprotinin for 25 min on ice and vortexed every 5 min. Samples were centrifuged (15,000g, 10 min, 4°C) and the supernatant fractions were frozen at −70°C until analysis. Aliquots of protein (15μg) from islet lysates were electrophoresed on polyacrylamide gel, then incubated with rabbit anti-phosphorylated PKB (1:750, Cell Signaling, catalogue no. 4060) that detects PKB levels only when phosphorylated at Ser473 (or its equivalent sites on PKBβ-Ser474 and PKBγ-Ser472) or rabbit anti-total PKB (1:750, Cell Signaling, catalogue no. 4691) that detects all three forms of total PKB levels for 1 h at room temperature. Membranes were then washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG for GAPDH (Amersham, Baie D’Urfé, QC, CA) at 1:5000 for 1 h. Immunodetection was performed using an enhanced chemiluminescence detection kit (Amersham). Protein bands on the films were analyzed by densitometry using Image Lab
software (Bio-Rad, Mississauga, ON, CA).

3.2.7 Statistical analysis

Data are reported as means ± SEM. Statistical analyses were performed using one-way analysis of variance (one-way ANOVA), followed by post hoc multiple comparison tests. P < 0.05 was considered statistically significant. All experiments were repeated at least three times.

3.3 Results

3.3.1 Exogenously applied hIAPP reduces β-cell phospho-PKB levels, which is associated with decreased β-cell proliferation and increased β-cell apoptosis

INS-1 β-cells were treated with synthetic hIAPP with or without the amyloid-binding dye Congo red (CR; 25 µmol/L) or treated with non-fibrillogenic rIAPP for 24 h. The previous studies from our group have shown that Congo red at this concentration does not have any toxic effects on β-cells [523]. After 24 h culture, hIAPP-treated INS-1 β-cells had markedly lower phospho-PKB (p-PKB) levels (Figure 3.1A), higher apoptotic rate (Figure 3.1B) and lower proliferation (Figure 3.1C) than non-fibrillogenic rIAPP-treated or untreated cells, all of which were restored by Congo red (Figure 3.1A-C). These findings suggest that hIAPP fibrils reduce PKB phosphorylation in β-cells.
Figure 3.1 Aggregates of exogenously applied hIAPP inhibit PKB phosphorylation (activation) in INS-1 β-cells, reduce β-cell proliferation and increase β-cell apoptosis. INS-1 β-cells were treated with synthetic hIAPP (10 µmol/L) with or without CR (25 µmol/L), or treated with non-fibrillogenic rIAPP (10 µmol/L) for 24 h. (A) Phosphorylated PKB (p-PKB) was assessed by double immunolabelling for insulin/p-PKB. The squares (dashed white lines) correspond to the insets (at top right of the relevant images) at higher magnification. Original magnification: X400; X1000 (inset). The proportion of (B) TUNEL-positive (apoptotic) and (C) PCNA-positive (proliferative) β-cells were quantified in each condition. Quantifications represent a minimum of ten microscopic fields each containing 200 to 300 INS-1 cells. Results are expressed as means ± SEM of three independent studies performed in triplicated. *P < 0.01 vs non-treated and rIAPP-treated groups; †P < 0.01 vs hIAPP-treated group, one-way ANOVA.
3.3.2 Endogenous hIAPP aggregates inhibit PKB phosphorylation in human and hIAPP+/− transgenic mouse islet β-cells

We further examined whether biosynthetic hIAPP aggregates can reduce PKB phosphorylation in primary islet β-cells during culture. Freshly isolated human and mouse islets were cultured for 7 days in the presence or absence of Congo red with 11.1 and 16.7 mmol/l glucose, respectively, to potentiate amyloid formation. As expected, thioflavin S (amyloid)-positive islets were not detectable in pre-culture (Day 0) hIAPP+/− wild-type and hIAPP+/− transgenic mouse islets. During 7 days of culture, amyloid formed in hIAPP+/− transgenic mouse islets which was associated with lower β-cell phospho-PKB levels as compared with wild-type islets without amyloid formation. Treatment with Congo red significantly decreased amyloid formation and increased β-cell phospho-PKB levels in hIAPP+/− transgenic mouse islet β-cells (Figure 3.2A). Similarly, formation of hIAPP aggregates in human islets during culture was associated with reduced PKB phosphorylation in β-cells, which was restored by treatment with Congo red (Figure 3.2B).
Figure 3.2 Endogenous hIAPP aggregates reduce phospho-PKB levels in primary islet β-cells during *in vitro* culture.

(A) Freshly isolated hIAPP<sup>−/−</sup> (wild-type) and hIAPP<sup>+/−</sup> mouse islets (8 to 12 weeks old) were cultured for 7 days in Ham’s/F10 (16.7 mmol/l glucose, to potentiate amyloid formation in
hIAPP\textsuperscript{+/-} transgenic mouse islets) with or without Congo red (CR; 25µmol/l). On day 0 and day 7, phosphorylated PKB and islet amyloid were detected by staining for insulin/p-PKB/Thio S. (B) Isolated human islets were cultured in the medium with 11.1 mmol/l glucose (to allow amyloid formation) in the absence or presence of Congo red (CR; 25µmol/l) for 7 days. Paraffin-embedded human islet sections were stained for insulin/p-PKB/Thio S. The squares (dashed white lines) correspond to the enlarged areas in each image. Original magnification: X400; X1000 (inset).

3.3.3 Prevention of hIAPP aggregation during islet culture by collagen matrix (CM) restores β-cell phospho-PKB levels and markedly reduces β-cell apoptosis

Human islets and hIAPP\textsuperscript{+/-} transgenic mouse islets were embedded within the three-dimensional collagen matrix (CM) or cultured free-floating (FF) in standard two-dimensional culture plates in high glucose (11.1 mmol/l or 16.7 mmol/l) to potentiate amyloid formation. Before and after 7 days of culture, hIAPP aggregates were detected by insulin/thioflavin S (amyloid) and insulin/A11 (oligomer) staining (Figure 3.3A & 3.4A). The proportion of A11 (oligomer) or thioflavin S (amyloid)-positive islets was also assessed.

The number of A11-positive islets was ~5% in pre-culture (Day 0) hIAPP\textsuperscript{+/-} transgenic mouse islets and was increased to ~20% in FF-cultured islets after 7 days of culture, whereas CM-embedded islets had only ~8% A11-positive islets (Figure 3.3B). Furthermore, islet amyloid fibrils were not detectable in any of the fresh hIAPP\textsuperscript{+/-} transgenic mouse islets before culture (Day 0), but were easily detectable in islets after 7 days of culture in FF condition (~18%). In contrast, islets embedded within CM had much lower number of thioflavin S-positive islets (~5%) as compared to FF-cultured islets (Figure 3.3C). Immunolabelling of paraffin-embedded hIAPP\textsuperscript{+/-} transgenic mouse islet sections for insulin and phospho-PKB revealed that amyloid formation in FF-cultured islets was associated with decreased number of phospho-PKB-positive
β-cells. However, reduction of islet amyloid formation by CM significantly increased phosphorylated PKB levels (Figure 3.3A) and decreased apoptotic rate in islet β-cells (Figure 3.3A & D).

Figure 3.3 Three-dimensional collagen matrix (CM)-embedded hIAPP+/− transgenic mouse islets have markedly decreased hIAPP aggregates and β-cell apoptosis, and increased phosphorylated PKB levels as compared with free-floating (FF) cultured islets. (A) Paraffin-embedded hIAPP+/− transgenic mouse islet sections were immunolabelled for insulin/A11, insulin/Thio S, insulin/p-PKB/Thio S, or insulin/TUNEL before (Day 0) and after 7 days of culture in two different conditions (FF or CM). The arrows point to the regions corresponding to the insets at higher magnification. Original magnification: X400; X1000 (inset). The proportions of (B) A11 (oligomer)-positive and (C) Thioflavin S (amyloid)-positive islets to total number of islets in each condition were calculated. (D) The proportion of apoptotic β-cells in each condition was quantified. A minimum of 30 islets per condition were analyzed. Results are expressed as means ± SEM of three independent studies. *P < 0.01 vs Day 0 (pre-culture islets); #P < 0.05 vs FF (free-floating islets), one-way ANOVA.
Similarly, in 7-day cultured human islets, CM markedly reduced the number of A11-positive and thioflavin S-positive islets as compared with two-dimensional free-floating (FF) culture (Figure 3.4A-C), which were associated with higher β-cell phospho-PKB levels (Figure 3.4A) and less apoptotic human islet β-cells (Figure 3.4A & D). However, there was no detectable difference between phospho-PKB protein levels in CM-embedded and FF-cultured human islets by Western blot (Figure 3.4E), likely because the human islet lysates contain some islets have no hIAPP aggregates, which mask the changes in islet β-cell phospho-PKB levels induced by hIAPP aggregates.
Figure 3.4 Collagen matrix (CM) significantly reduces formation of hIAPP aggregates in cultured human islets, which is associated with restored phosphorylated PKB levels and decreased β-cell apoptosis.

(A) Paraffin-embedded sections of human islets before (Day 0) and after 7 days of culture in two different conditions (FF and CM) were immunolabelled for insulin/A11, insulin/Thio S, insulin/p-PKB/Thio S, or insulin/TUNEL. The arrows point to the regions corresponding to the insets at higher magnification. Original magnification: X400; X1000 (inset). The proportions of (B) A11 (oligomer)-positive and (C) Thioflavin S (amyloid)-positive islets to total number of islets in each condition were calculated. (D) The proportion of apoptotic β-cells in each condition was quantified. A minimum of 30 islets per condition were analyzed. Results are expressed as
means ± SEM of three independent studies. *P < 0.01 vs Day 0 (pre-culture islets); **P < 0.05 vs FF (free-floating islets), one-way ANOVA. (E) Western blot analysis was performed to assess phosphorylated PKB (p-PKB) and total PKB (t-PKB) levels in the lysates from freshly isolated or 7-day cultured human islets (FF or CM, with 11.1mmol/L glucose). The immunoblots are representative of three independent experiments.

3.3.4 Inhibition of islet amyloid formation by the amyloid-binding dye Congo red (CR) or collagen matrix (CM) decreases IL-1β expression in islet β-cells during in vitro culture

Paraffin-embedded sections of human islets embedded within collagen matrix (CM) or cultured under 2-D condition with or without the amyloid-binding dye CR were immunostained for insulin, IL-1β and amyloid (thioflavin S). After 7 days of culture, CR-treated (Figure 3.5A) and CM-embedded (Figure 3.5B) human islets had less amyloid deposition and markedly lower IL-1β expression as compared with FF-cultured islets with no treatment, suggesting that amyloid formation is associated with increased IL-1β production. This finding is consistent with the recent findings from other studies showing that hIAPP aggregates induce islet IL-1β production [230,248,524].
Figure 3.5 Prevention of amyloid formation by Congo red (CR) or collagen matrix (CM) reduces IL-1β expression in human islets during culture.

(A) Human islets were cultured for 7 days with or without the amyloid-binding dye Congo red (CR; 25μmol/l). On day 0 and day 7, IL-1β and islet amyloid were detected by immunolabelling for insulin/IL-1β/Thio S. (B) Paraffin-embedded sections of human islets before (Day 0) and after 7 days of culture in two different conditions (FF and CM) were immunolabelled for insulin/IL-1β/Thio S. Images are the representative results of three independent experiments. Original magnification: X400; X1000 (inset).
3.3.5 Blockade of IL-1β signaling by anakinra or exenatide restores phospho-PKB levels in human islet β-cells during culture

We further tested whether IL-1β signaling is involved in hIAPP-induced reduction of phospho-PKB in islet β-cells. Freshly isolated human islets were cultured for 7 days with high glucose concentration (11.1 mmol/L) in the presence or absence of anakinra, a clinically approved IL-1 receptor antagonist (IL-1Ra). Islet amyloid formation and expressions of IL-1β and phospho-PKB were detected by immunolabelling of paraffin-embedded human islet sections with insulin/IL-1β/Thio S or insulin/p-PKB. Pre-culture (Day 0) human islets with little or no detectable islet amyloid formation had low IL-1β expression (Figure 3.6A) and high phospho-PKB levels (Figure 3.6B). After 7 days of culture, anakinra-treated human islets had markedly lower IL-1β immunoreactivity (Figure 3.6A), which was associated with increased phospho-PKB levels in β-cells as compared with non-treated human islets (Figure 3.6B). Similar results were obtained from human islets treated with exenatide, a GLP-1 receptor agonist. Exenatide-treated human islets showed a remarkable decrease in IL-1β production (Figure 3.6A) and higher β-cell phospho-PKB levels as compared with non-treated human islets (Figure 3.6B).
Figure 3.6 Human islets treated with anakinra (An) or exenatide (Ex) have decreased IL-1β expression during culture, which is associated with increased phosphorylated PKB levels in islet β-cells. Human islets were cultured for 7 days in the absence (FF) or presence of anakinra (An; 10
µg/mL) or exenatide (Ex; 10nmol/L). Paraffin-embedded sections from pre-culture (Day 0) and 7-day cultured islets were immunolabelled for (A) insulin/IL-1β/Thio S or (B) insulin/p-PKB. Images are the representative results of three independent studies. The arrows point to the regions corresponding to the insets at higher magnification. Original magnification: X400; X1000 (inset).

3.4 Discussion

Islet amyloid formation contributes to islet β-cell apoptosis during pre-transplant culture and in islet graft [132,249,263,265], although the underlying mechanisms are still not fully understood. Several pro-apoptotic signaling pathways, including JNK and Fas have been shown to mediate amyloid-induced β-cell death [233,249]. In contrast, PI3-Kinase/PKB signaling pathway exerts a significant anti-apoptotic effect on islet β-cells [274,521]. In the present study, using transformed β-cells (INS-1 cells) and primary human and mouse islets, we demonstrate that the formation of hIAPP aggregates can inhibit PKB phosphorylation in β-cells, likely via promoting IL-1β production. We also show that blockade of islet amyloid formation and/or IL-1β activity restores reduced phospho-PKB levels in islet β-cells and enhances islet β-cell viability during in vitro culture.

The hIAPP-treated INS-1 cells had markedly lower phospho-PKB levels, reduced proliferation and higher apoptosis than non-fibrillogenic rat IAPP-treated or non-treated cells, all of which were restored by treatment with the amyloid inhibitor Congo red. These findings suggest that aggregation of exogenously applied hIAPP can inhibit PKB phosphorylation in β-cells and this reduction of phospho-PKB levels is associated with hIAPP-induced β-cell damage, consistent with the findings from a previous study in transformed β-cells [521]. Furthermore, our study demonstrates that endogenously formed hIAPP aggregates in both cultured human and hIAPP+/−.
transgenic mouse islets decrease PKB phosphorylation in β-cells, leading to a significantly higher β-cell apoptosis as compared to pre-culture islet β-cells. Taken together, these findings suggest that hIAPP-induced reduction in PKB phosphorylation may contribute to its β-cell toxicity.

It has been widely shown that loss of extracellular matrix (ECM) during islet isolation contributes to islet β-cell death and dysfunction, resulting in islet graft failure [489,492,496]. Accordingly, our research group introduced a 3-D scaffold, consisting of type 1 collagen matrix (CM) as surrogate ECM materials to re-establish islet-ECM contact. Moreover, our previous study in Chapter 2 showed that CM-embedded human islets had markedly reduced islet amyloid formation during in vitro culture as compared with 2-D free-floating (FF) cultured human islets [146]. In the present study, using human and hIAPP+/− transgenic mouse islets, we further identified that CM not only prevented deposition of mature islet amyloid fibrils but also inhibited the formation of small hIAPP aggregates (oligomers) which has been generally accepted as the major toxic form contributing to amyloid-induced islet β-cell death [225,525,526]. In addition, by prevention of hIAPP aggregation, CM significantly increased phospho-PKB levels in cultured primary islet β-cells, which was associated with reduced β-cell apoptosis, suggesting a possible mechanism by which CM can improve islet β-cell survival via inhibition of hIAPP-induced phospho-PKB reduction.

Consistent with previous findings from our group and those of others [248,249,524,527], in the present study, we found that formation of islet amyloid induces IL-1β production during culture. Furthermore, reducing amyloid formation in human islets by treatment with Congo red or
collagen matrix significantly lowered islet IL-1β levels as compared with non-treated islets. One possible mechanism for amyloid-induced IL-1β production is through activation of NOD like receptor-related protein 3 (NLRP3) inflammasome which promotes caspase-1-mediated cleavage of proIL-1β to form mature IL-1β [248,528]. Moreover, both islet β-cells and non-islet cells (e.g. macrophages) have been reported as the sources of IL-1β in islets positive for amyloid formation [524,527].

To determine if the effects of islet amyloid on PKB phosphorylation are mediated by IL-1β in islet β-cells, we treated cultured human islets with or without IL-1β blockers. Anakinra, an IL-1 receptor antagonist (IL-1Ra), competes with IL-1β for binding to IL-1 receptor 1 (IL-1R1) on β-cells, thereby blocking IL-1β signaling pathway [529]. We found anakinra-treated human islets with lower IL-1β expression had significantly elevated phospho-PKB levels in islet β-cells as compared with non-treated cultured human islets. Similarly, cultured human islets treated with exenatide, a GLP-1 receptor agonist, had decreased IL-1β immunoreactivity associated with higher phospho-PKB levels in β-cells as compared with non-treated human islets. These findings suggest that IL-1β may play an important role in mediating hIAPP-induced phospho-PKB reduction in islet β-cells during in vitro culture.

In summary, our findings show that hIAPP aggregation reduces PKB phosphorylation in islet β-cells, likely via IL-1β signaling. Furthermore, results of these studies suggest that inhibition of hIAPP aggregation and/or blockade of IL-1β action can enhance β-cell survival by restoring phosphorylation of PKB.
Chapter 4: Intraperitoneal injection of IDO-expressing fibroblasts protects islet β-cells from immunological attack and reverses hyperglycemia in non-obese diabetic mice

4.1 Introduction

Type 1 diabetes (T1D) is a devastating autoimmune condition in which there is decreased insulin production due to immuno-destruction of pancreatic insulin-producing β-cells [530,531]. Overwhelming evidence has shown that β-cell death and dysfunction in T1D are mediated by cytotoxic T-cells via Fas/FasL interaction or perforin/granzyme system [44,532]. However, recent studies have indicated that T-cells alone are not fully responsible for β-cell destruction in T1D. Pro-inflammatory cytokines such as IL-1β and IL-17 are likely to make a great contribution to β-cell toxicity during the progression of T1D [533-535]. Thus, it has been suggested that a combination of controlling the cytotoxic T cells and blocking the pro-inflammatory cytokines would be a feasible approach of inhibiting β-cell destruction in T1D [536,537]. As such, in this study, we have used intraperitoneal (IP) injection of primary dermal fibroblasts expressing indoleamine 2,3-dioxygenase (IDO) as a new approach of protecting islet β-cells from toxicity induced by both autoreactive T cells and the pro-inflammatory cytokines at the onset of diabetes in non-obese diabetic (NOD) mice.

IDO is an enzyme responsible for catabolizing the essential amino acid, tryptophan (Trp), to kynurenine (Kyn) [385,538,539]. It has been shown that both decreased Trp level and increased Kyn production suppress the cytotoxic T lymphocytes (CTLs) [362,418,540,541]; while induce
and propagate regulatory T cells (Tregs) [542-544]. These findings indicate the potential role of IDO in regulation of immunotolerance [424,438]. We used dermal fibroblasts as a carrier for IDO protein in our cell therapy model. Primary dermal fibroblasts are easy to obtain and maintain in culture therefore, they are suitable for being used in cell therapies with high quantities of cells. In addition, fibroblasts are able to produce interleukin-1 receptor antagonist (IL-1Ra) [545-547], which functions as a competitor for releasable IL-1β in binding to IL-1 receptor type 1 (IL-1R1) and thereby inhibiting IL-1β activity [529,548].

We have previously demonstrated that intraperitoneally injecting a high number (20x10^6 cells/mouse) of IDO-expressing dermal fibroblasts into early diabetic NOD mice significantly inhibits the development of T1D [547]. This finding sets the stage for the current study through which an optimized number of IDO-expressing dermal fibroblasts required to prevent the progression of T1D in NOD mice was determined. Further, the possible role of this IDO cell therapy in modulation of autoimmune responses was elucidated. The findings of this study collectively demonstrated that IP injection of 15x10^6 (15M) IDO-expressing dermal fibroblasts is sufficient to reverse new onset of T1D in NOD mice through inducing Tregs as well as inhibiting β-cell specific autoreactive CD8^+ T cells and IL-17-producing CD4^+ T cells (Th17 cells). Additionally, the significant impact of IDO-expressing fibroblasts on blockade of IL-1β signaling pathway in cultured islet β-cells was also identified.

4.2 Materials and methods

4.2.1 Mice

NOD (female) and C57BL/6 (male and female) were purchased from the Jackson Laboratories.
(Bar Harbor, ME) and maintained in pathogen-free facilities of Jack Bell Research Centre or Blusson Spinal Cord Centre (iCORD). NOD mouse is the most commonly used mouse model of T1D. It develops insulitis at around 3-4 weeks of age, leading to β-cell destruction. The overt diabetes usually occurs at around 12-14 weeks of age [549]. Diabetes is more prevalent in the female NOD mice with an incidence ranging from 60% to 80% [550].

4.2.2 Construction of lenti-viral vectors

The human IDO gene (NM_002164) was kindly provided by Dr. JM Carlin at Miami University. It was amplified by PCR using the forward (F) and reverse (R) primers sequences as follows: F: 5’GGGGACAAAGTTTGTACAAAAAAGCAGGCTTCACCATGGCACACGCTATGGAAAAC-TCCTGG-3’; R: 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTCTAACCCTCCTTC-AAAAGGGATTTC-3’. The amplified product was subcloned into a lenti-viral pLC-E expression vector modified from the lenti-viral backbone FUGWBW [551]. The cloned plasmid DNA was then amplified in competent DH10-B bacteria and purified by using the Qiagen Plasmid DNA Maxi-prep kit (Qiagen, Valencia, CA). Sequence of the IDO/pLC-E construct was evaluated by doing DNA sequencing analysis. A sequence encoding the Blasticidin-resistance gene was also incorporated into the lenti-viral vector for selection of IDO-expressing cells. Lenti-viral vector carrying human IDO gene plus Blasticidin-resistance gene were then used for transduction of mouse dermal fibroblasts as further described in the following section.

4.2.3 Isolation, culture and lenti-viral infection of fibroblasts

Skin pieces obtained from male C57BL/6 mice at 6-8 weeks of age were shaved and washed three times in sterile Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies-
Invitrogen, Burlington, ON, CA) supplemented with antibiotic-antimycotic mixture (100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B) (Gibco, Invitrogen Incorporation, NY, USA). Fibroblasts were cultured following the established protocol [169]. Upon reaching confluence, the cells were trypsinized, split for subculture at a ratio of 1:4, and reseeded onto 75-cm² cell culture flasks (BD Biosciences, MA) with DMEM containing 10% FBS (Hyclone Laboratories, Inc, Logan, UT, USA) and antibiotic-antimycotic mixture. Passage 3 to 4 of cultured fibroblasts were used for transduction with IDO lenti-viral vectors.

C57BL/6 mouse dermal fibroblasts were transduced with a medium containing 50% IDO lenti-viral vectors and 50% supplemented DMEM. After 24 hours, the conditioned media was replaced. On days 5 and 9 post transduction, fibroblasts were treated with Blasticidin (8µg/ml; Sigma-Aldrich, Oakville, ON, CA) for obtaining pure IDO-expressing cells by day 12. Successful IDO gene transduction was confirmed by measurement of L-kynurenine levels in conditioned media of the cells and PCR analysis for detection of IDO mRNA expression. Then IDO-expressing cells were subcultured and used for this study.

### 4.2.4 Assessment of diabetes and treatment protocol

NOD mouse blood glucose levels (BGLs) were monitored twice per week using an Accu-Chek Compact Plus blood glucose monitoring system (Roche diagnostics, Laval, QC, CA). Mice with a random blood glucose measurement of greater than 13mM on two consecutive assessments were considered diabetic. The threshold of 13mM was rationalized based on the previous observation that our batches of NOD mice continued to have high BGLs after reaching this level of hyperglycemia. Newly diabetic NOD mice were randomly assigned to be intraperitoneally
injected with either $10 \times 10^6$ (10M) or $15 \times 10^6$ (15M) IDO-expressing mouse dermal fibroblasts. The numbers of IDO-expressing fibroblasts selected in this study were based on our previous findings [547]. BGLs of treated mice were checked twice per week and remission of diabetes was defined as BGLs consistently remained below 10mM.

4.2.5 **Analyses of indoleamine 2,3-dioxygenase (IDO) activity**

The biological activity of IDO was reflected by the kynurenine levels due to its ability to catabolize tryptophan to kynurenine. Accordingly, before injection, the activity of IDO in transduced dermal fibroblasts was evaluated by measuring kynurenine levels in the conditioned media following the previously established protocol [552]. Briefly, proteins in conditioned media were precipitated by using 30% trichloroacetic acid. Samples were centrifuged then 0.5 mL of supernatant from each sample was incubated with equal volume of Ehrlich’s reagent (Sigma-Aldrich) for 10 min at room temperature. Absorbance of the solution was measured at 490nm by spectrophotometry. The levels of kynurenine were calculated using an equation obtained from the standard curve. After injection of IDO-expressing fibroblasts, blood samples were obtained from mouse tail vein once per week. Concentrations of plasma kynurenine were measured by high-performance liquid chromatography (HPLC) [553].

4.2.6 **Evaluation of islet function**

Intraperitoneal glucose tolerance tests (IPGTT) were performed in the mice (receiving 15M IDO-expressing fibroblasts) at 15 weeks post-cell injection. Age-matched non-diabetic mice were used as controls. After a 16 h overnight fast, glucose (2 mg/g body weight) was intraperitoneally injected into nonanesthetized mice. Blood samples were obtained from tail vein and BGLs were
measured at 0, 15, 30, 60, and 120 minutes. Then area under the curve was evaluated by using SigmaPlot software (Systat Software Inc., San Jose, CA). At 8 weeks post-cell injection, blood samples were taken from the mice treated with 10M or 15M IDO-expressing fibroblasts then plasma c-peptide levels were measured using specific ELISA kit (ALPCO Diagnostics, Salem, NH, USA).

### 4.2.7 Histological analyses and immunostainings

For detection of insulitis, paraffin-embedded sections (5 μm) of pancreases were stained with hematoxylin-eosin (H-E) (Sigma-Aldrich, St, Louis, MO, USA) and analyzed by light microscopy. Paraffin-embedded sections of pancreases or islets were double immunostained with insulin/glucagon, insulin/IL-1β, insulin/CD3 or insulin/cleaved (active) caspase-3. The immunohistochemistry protocols and image capture system used for these studies were the same as described in Chapter 2.2.6. The list of primary and secondary antibodies used for immunostainings are shown in Table 4.1 and Table 4.2, respectively.

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<th>Antibody and dilution</th>
<th>Manufacturer</th>
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<td>Rabbit anti-glucagon (1:750)</td>
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<td>Rabbit anti-cleaved caspase-3 (1:100)</td>
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Table 4.2 Secondary antibodies for immunolabelling pancreas or islet sections

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<td></td>
<td>Rhodamine-conjugated anti-rabbit IgG (1:1000)</td>
<td>Chemicon International, Temecula, CA</td>
</tr>
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</table>

4.2.8 Flow cytometry

Antibodies detecting CD4 (RM 4-5 or GK 1.5), B220 (RA3-6B2), CD8 (53-6.7), CD25 (pc 61.5) were purchased from eBioscience (San Diego, CA, USA). PE-conjugated NRP-V7 tetramer was a generous gift from Dr. Rusung Tan (University of British Columbia). Peripheral blood mononuclear cells (PBMCs) and cells from spleens (SPLs), pancreatic lymph nodes (PLNs) and cervical lymph nodes (CLNs) were fluorescently labelled by indicated antibodies in flow cytometry buffer (PBS containing 1% FBS). For analysis of intracellular cytokines (IL-17 and FOXP3), the cells were permeabilized with Permeabilization Buffer (eBioscience) then stained intracellularly with anti-mouse IL-17a (eBio17B7; eBioscience) or FOXP3 (FJK-16s; eBioscience). Flow cytometry was performed on an Acccuri C6 machine and data analysis was conducted with FSC Express V3.

4.2.9 In vitro islet studies

Isolated human islets were received from Ike Barber Human Islet Transplant Laboratory (Vancouver, BC, Canada) as described in Chapter 2.2.2. C57BL/6 mouse (6-10 weeks of age)
islets were isolated following the process described in Chapter 3.2.3. Human or mouse islets were then cultured under three different conditions for 7 days: in Ham’s/F10 medium (Sigma-Aldrich) alone, in Ham’s/F10 medium with recombinant human IL-1Ra (500 ng/mL; R&D Systems) or with human or mouse fibroblasts expressing IDO (5 x 10^4 fibroblasts/well) in a medium containing 50% Ham’s/F10 medium and 50% DMEM. In all three conditions, islets were cultured in a humid atmosphere (95% air, 5% CO2) at 37°C. Ham’s/F10 medium was supplemented with glucose (final concentrations: 11.1mmol/L for human islets; 16.7mmol/L for mouse islets), 12 mmol/L HEPES, 2 mmol/L L-glutamine, 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The medium was replaced every 48 hours. Paraffin-embedded sections (5 µm) of these islets were immunostained with insulin/IL-1β or insulin/cleaved caspase-3 as described in Chapter 4.2.7.

4.2.10 Statistical analysis

Data are expressed as means ± SEM of three or more independent sets of experiments. Paired data were evaluated by Student’s t test, and a one-way analysis of variance (ANOVA) was used for multiple comparisons. P values < 0.05 were considered statistically significant.

4.3 Results

4.3.1 Intraperitoneal injection of 15M IDO-expressing fibroblasts inhibits the progression of diabetes in NOD mice

We have previously showed that IP injection of 20x10^6 IDO-expressing fibroblasts into NOD mice stops the progression of diabetes [547]. This finding sets the stage to ask the question of what would be the optimized number of IDO-expressing fibroblasts that is needed to reverse
T1D in NOD mice? To answer this question, either 10x10^6 (10M) or 15x10^6 (15M) IDO-expressing fibroblasts were injected into the peritoneal cavity of NOD mice which were at the onset of diabetic development. Blood glucose levels (BGLs) of these mice were monitored twice per week. The results showed that the BGLs in 5 out of 6 (83%) mice treated with 15M IDO-expressing cells recovered to normal up to 120 days (the endpoint of this study). However, this was not the case for mice receiving 10M IDO-expressing cells. The finding showed that only 1 out of 6 (17%) diabetic mice treated with 10M IDO-expressing cells was reversed to normoglycemic status (Figure 4.1A-B). Further, mice receiving 15M IDO-expressing fibroblasts had a higher survival rate as compared to that of mice receiving 10M IDO-expressing cells (Figure 4.1C). Body weight as an index for diabetic status was measured once weekly and results showed that NOD mice receiving 15M IDO-expressing cells gradually stopped losing body weight, whereas the mice receiving 10M IDO-expressing cells kept losing body weight (Figure 4.1D).
Figure 4.1 Intraperitoneal injection of $15 \times 10^6$ (15M) IDO-expressing dermal fibroblasts inhibits T1D development in NOD mice.

(A) Blood glucose levels (BGLs) in each mouse treated with either $15 \times 10^6$ (15M, blue line) or $10 \times 10^6$ (10M, red line) IDO-expressing fibroblasts (n=6). (B) The quantification of diabetic status. (C) Kaplan-Meier plot for survival. (D) Averaged body weights of mice receiving either 15M or 10M IDO-expressing cells (n=5).
4.3.2 Enzymatic activity of IDO is a key factor in its therapeutic efficacy

To evaluate the importance of enzymatic activity of IDO expressed by fibroblasts in reversing the progression of T1D in NOD mice, kynurenine (Kyn), a tryptophan metabolite, as an index for IDO activity was measured in the conditioned media of IDO-expressing fibroblasts as well as in plasma of mice receiving IP injections of IDO-expressing dermal fibroblasts. The results showed that one batch of IDO-expressing fibroblasts had a remarkably lower Kyn level (44.67 µM) after transduction with IDO gene as compared with other sets of transduced fibroblasts (Figure 4.2A). Further, the mouse injected with 15M cells from this batch of IDO-expressing fibroblasts had lower plasma Kyn levels over time as compared with mice treated with 15M cells from other sets of IDO-expressing fibroblasts (Figure 4.2B). Interestingly, the low enzymatic activity seen in this batch of IDO-expressing fibroblasts before and after IP injection into the NOD mouse was associated with the unsuccessful reversion of hyperglycemia in this particular mouse (Figure 4.1A: 15M-6). In contrast, normoglycemia occurred in the mouse (Figure 4.1A: 10M-6) receiving 10M IDO-expressing fibroblasts that had markedly higher level of kynurenine (80.69 µM) in the culture medium than other sets of fibroblasts (Figure 4.2A). This mouse also had higher plasma kynurenine levels over time than other mice treated with 10M IDO-expressing cells (Figure 4.2B). These data show that there is positive correlation between the level of kynurenine production and efficacy of injected cells in controlling the progression of diabetes in NOD mice.
Figure 4.2 Kynurenine level, an index of IDO enzymatic activity, plays an important role in the efficacy of IDO cell therapy.

(A) Kynurenine levels in the conditioned media of fibroblasts transduced with IDO gene (red arrow points to Kyn level: 44.67 µM; green arrow points to Kyn level: 80.69 µM, n=6). (B) Plasma kynurenine levels in the mice treated with 15M or 10M IDO-expressing fibroblasts (blue line with triangle: mouse receiving 15M IDO-expressing fibroblasts with Kyn level: 44.67 µM; red line with triangle: mouse receiving 10M IDO-expressing fibroblasts with Kyn level: 80.69 µM, n=4).
4.3.3 Intraperitoneal injection of 15M IDO-expressing fibroblasts improves the functionality of the remaining islet β-cells in newly diabetic NOD mice

To evaluate the functionality of the remaining β-cells in NOD mice treated with 15M IDO-expressing fibroblasts, intraperitoneal glucose tolerance test (IPGTT) was performed at 15 weeks after cell injection. Results showed that mice receiving 15M IDO-expressing fibroblasts had a comparable glucose tolerance as that shown in age-matched non-diabetic mice (Figure 4.3A-B). To further investigate the islet functionality, plasma c-peptide levels were measured in mice receiving either 10M or 15M IDO-expressing fibroblasts at 8 weeks post injections. We chose this time point based on the fact that most of mice treated with 10M IDO-expressing cells were kept up around 9 weeks. As the control group, we used non-diabetic NOD mice that were age-matched with mice treated with 15M IDO-expressing cells. Mice receiving 15M IDO-expressing cells had a significantly higher level of c-peptide in their plasma than that in the mice intraperitoneally injected with 10M IDO-expressing cells (Figure 4.3C). However, there was no significant difference of c-peptide levels between mice treated with 15M IDO-expressing cells and non-diabetic mice. These results suggest that the remaining islet β-cells in NOD mice receiving 15M IDO-expressing fibroblasts have comparable functionality as β-cells in non-diabetic mice.
105

Figure 4.3 15M IDO-expressing fibroblasts restore the functionality of the remaining islet β-cells in diabetic NOD mice.
(A) Blood glucose concentrations during IPGTT (n=3). (B) The area under the IPGTT curves. (C) Plasma c-peptide levels of mice were measured by using a mouse ELISA kit. Results are expressed as means ± SEM of three independent experiments. *P < 0.05 vs 10M group, one-way ANOVA.

4.3.4 Intraperitoneal injection of 15M IDO-expressing fibroblasts reduces the infiltration of lymphocytes in islets and maintains insulin-producing islet β-cells in NOD mice

To examine the effect of IDO-expressing cell treatment on infiltrated immune cells in islets, mouse pancreases were harvested at the endpoints of the study. Mice with normoglycemia were kept until 120 days, while mice with hyperglycemia were euthanized before 120 days due to general morbidity. Mouse pancreases were sectioned and stained with H-E, insulin/CD3 or insulin/glucagon. Islets in the mice receiving 10M IDO-expressing fibroblasts showed a widespread infiltration of lymphocytes including CD3+ T cells with almost no remaining insulin
producing β-cells. In contrast, mice receiving 15M IDO-expressing fibroblasts showed a significantly less infiltration of lymphocytes in islets and there was also restoration of insulin-producing islet β-cells (Figure 4.4A). These findings were further confirmed by calculating insulitis scores. Results showed 50% of the islets in NOD mice treated with 15M IDO-expressing cells were free of insulitis. However, only 10% of islets in mice receiving 10M IDO-expressing cells showed free of infiltrated immune cells. Severe insulitis was found in 40% of islets in mice receiving 10M IDO-expressing cells (Figure 4.4B).

![Figure 4.4 IP injection with 15M IDO-expressing fibroblasts decreases the aggressiveness of insulitis in islets of NOD mice.](image)

(A) Representative images of pancreas sections stained with hematoxylin-eosin (H-E), insulin/CD3 or insulin/glucagon. The arrow points to the infiltration of lymphocytes. Magnification of images: X400. (B) Insulitis scores were calculated according to H-E staining. Scores were given as follows: free of infiltrates = score 1; <25% infiltrates per islet = score 2;
25-50% infiltrates per islet = score 3; >50% infiltrates per islet = score 4. Five animals from each group are included.

4.3.5 Intraperitoneal injection of 15M IDO-expressing fibroblasts in NOD mice prevents the accumulation of β-cell specific cytotoxic lymphocytes (CTLs) and IL-17-producing CD4+ T cells (Th17 cells)

To explore the potential mechanisms involved in effectiveness of 15M IDO-expressing cells in reversion of diabetes progression, we first investigated the effects of this treatment on a population of autoreactive β-cell specific CD8+ CTLs and also Th17 cells. We used NRP-V7 tetramer for detection of autoreactive CTLs that specifically destroy islet β-cells by recognition of a peptide NRP-V7 on the MHC class I molecules. To achieve this, blood was collected from mice injected with either 10M or 15M IDO-expressing fibroblasts after 0, 1, 1.5, 2 months post onset of hyperglycemia. Blood taken from diabetic mice receiving daily injection of insulin without cell treatment (untreated) was used as control. As mice receiving 15M IDO-expressing fibroblasts showed a promising result, another set of blood samples were collected from these mice at 3 months post cell injection. NRP-V7-reactive CTLs and Th17 cells in peripheral blood mononuclear cells (PBMCs) were detected using flow cytometry. The result showed that mice injected with 15M IDO-expressing cells had a gradually decreased frequency of NRP-V7-reactive CTLs. The ratio of these cells from 2.07%±0.14% at the onset of diabetes was reduced to 0.27%±0.11% at 3 months post cell injection (Figure 4.5A-B). Further, the frequency of Th17 cells was also decreased over time in this group of mice. The frequency of Th17-positive cells from 7.77%±0.22% at the onset of diabetes was reduced to 3.78%±0.3% at 3 months post treatment (Figure 4.6A-B). Additionally, mice receiving 15M IDO-expressing cells had significantly lower ratios of NRP-V7-reactive CTLs (Figure 4.5A-B) and Th17 cells (Figure
4.6A-B) in PBMCs than those in mice receiving either nothing (untreated mice) or 10M IDO-expressing cells at different time points. At the endpoints of the study, cells harvested from spleens (SPLs), pancreatic lymph nodes (PLNs) or cervical lymph nodes (CLNs) were analyzed for the presence of NRP-V7-reactive CTLs and Th17 cells. Mice injected with 15M IDO-expressing dermal fibroblasts had markedly less autoreactive CD8\(^+\) T cells in SPLs, PLNs and CLNs as compared with those taken from mice receiving either nothing (untreated mice) or 10M IDO-expressing cells (Figure 4.5C-D). The frequencies of Th17 cells in SPLs and PLNs isolated from mice with 15M IDO cell therapy were also significantly decreased as compared to those taken from untreated controls (Figure 4.6C-D).
Figure 4.5 Inhibition of β-cell specific autoreactive CD8⁺ T cells by treatment with 15M IDO-expressing fibroblasts in NOD mice.

NRP-V7 MHC I tetramer levels in mouse peripheral blood monocytes (PBMCs) were detected by doing flow cytometry. (A) Representative plots of CD8 vs. NRP-V7 MHC I tetramer. (B) Quantitative data. At the endpoints, NRP-V7 MHC I tetramer levels in cells from mouse spleens (SPLs), pancreatic lymph nodes (PLNs) or cervical lymph nodes (CLNs) were analyzed. (C) Representative plots of CD8 vs. NRP-V7 MHC I tetramer. (D) Quantitative data. Three animals from each group are included. Results are expressed as means ± SEM of three independent studies. *P < 0.05 vs 10M and untreated groups. #P < 0.05 vs 15M group, one-way ANOVA.
Figure 4.6 15M IDO-expressing fibroblasts decrease CD4⁺IL-17⁺ (Th17) cells in NOD mice. The frequency of Th17 cells in PBMCs of mice from different groups was detected using flow cytometry. (A) Representative plots of CD4 vs. IL-17A. (B) Quantitative data. At the endpoints, Th17 cells in mouse SPLs, PLNs or CLNs were detected. (C) Representative plots of CD4 vs. IL-17A. (D) Quantitative data. Three animals from each group are included. Results are expressed as means ± SEM of three independent studies. *P < 0.05 vs 10M and untreated groups. #P < 0.05 vs 15M group, one-way ANOVA.

4.3.6 Intraperitoneal injection of 15M IDO-expressing fibroblasts increases the frequency of regulatory T cells (Tregs)

Development of diabetes in NOD mice is due to an imbalance of regulatory and cytotoxic T cells. Thus, we further investigated the effects of IDO cell therapy on modulation of Tregs. We measured the frequency of CD4⁺CD25⁺FOXP3⁺ Tregs in PBMCs at different time points and in
SPLs, PLNs or CLNs at the endpoints. The results showed that IP injection of 15M IDO-expressing fibroblasts increased the ratio of CD4⁺CD25⁺FOXP3⁺ Tregs from 5.65%±1.03% at 1 month post treatment to 10.67%±1.07% at 3 months post treatment. Moreover, at 2 months following treatment, mice injected with 15M IDO-expressing cells had a significantly higher frequency of CD4⁺CD25⁺FOXP3⁺ Tregs than that in mice receiving either nothing (untreated mice) or 10M IDO-expressing cells (Figure 4.7A-B). At the endpoints, mice intraperitoneally injected with 15M IDO-expressing fibroblasts were sacrificed and the frequencies of Tregs in SPLs, PLNs or CLNs were evaluated. The result showed marked increases in the ratios of CD4⁺CD25⁺FOXP3⁺ Tregs in all three different organs as compared to those taken from mice receiving either nothing (untreated mice) or 10M IDO-expressing cells (Figure 4.7C-D). These findings collectively suggest that the reversal of hyperglycemia in mice treated with 15M IDO-expressing fibroblasts is, at least in part, due to induction of CD4⁺CD25⁺FOXP3⁺ Tregs in NOD mice.
Figure 4.7 15M IDO cell therapy upregulates FOXP3⁺ T cells in NOD mice. CD25⁺FOXP3⁺ T cells in PBMCs of mice at different time points were detected. (A) Representative plots of CD25 vs. FOXP3. (B) Quantitative data. At the endpoints, CD25⁺FOXP3⁺ T cells in different mouse organs (SPLs, PLNs or CLNs) were detected. (C) Representative plots of CD25 vs. FOXP3. (D) Quantitative data. Three animals from each group are included. Results are expressed as means ± SEM of three independent studies. *P < 0.05 vs 10M and untreated groups, one-way ANOVA.

4.3.7 Islets co-cultured with IDO-expressing fibroblasts have reduced IL-1β expression and β-cell apoptosis

It is well established that IL-1β contributes to islet β-cell death and dysfunction during in vitro culture [554-556]. Furthermore, IL-1β has been shown to be involved in the pathogenesis of T1D [534,557]. As such, we have conducted a set of experiments through which the effects of IDO-
expressing fibroblasts on IL-1β expression in islet β-cells during *in vitro* culture were examined. Mouse or human islets were cultured alone (IA), treated with recombinant human IL-1Ra (+IL-1Ra) or co-cultured with mouse or human IDO-expressing fibroblasts (+IDO-fib) for 7 days. Paraffin-embedded sections of these islets were then double stained with insulin (red)/IL-1β (green) or insulin (green)/active caspase-3 (apoptotic marker, red). The findings showed that both mouse and human islets co-cultured with IDO-expressing fibroblasts for 7 days had lower IL-1β expression in β-cells and significantly less β-cell apoptosis as compared with islets cultured alone. Interestingly, there were no detectable differences of IL-1β expression as well as β-cell apoptosis rate between islets co-cultured with IDO-expressing fibroblasts and islets treated with IL-1Ra (Figure 4.8). This finding reveals that IDO-expressing fibroblasts is likely to be responsible for reducing β-cell toxicity induced by IL-1β during the development of T1D.
Figure 4.8 Islets cultured with IDO-expressing fibroblasts have reduced IL-1β expression and β-cell apoptosis rate.

Mouse or human islets were cultured alone (IA), treated with IL-1 receptor antagonist (+IL-1Ra) or co-cultured with IDO-expressing fibroblasts (+IDO-fib) for 7 days. Paraffin-embedded sections from pre-culture (D0) and 7-day cultured islets were double immunolabelled for (A) insulin and IL-1β or (B) insulin and active caspase-3 (aCasp3) (upper panel: mouse islets, lower panel: human islets). Images are the representative results of three independent studies. The arrows point to the regions corresponding to the insets at higher magnification. Original magnification: X400; X1000 (inset). (C) and (D): The number of active caspase-3-positive and insulin-positive islet cells and total number of β-cells were counted in mouse (C) and human (D) islets from each culture condition in a minimum of 20 islets per condition. Data are expressed as means ± SEM of three independent experiments. *P < 0.05 vs IA group, one-way ANOVA.
4.4 Discussion

Our previous success in demonstrating that IP injection of a high number (20x10^6 cells/mouse) of IDO-expressing primary dermal fibroblasts prevents the development of new onset diabetes in NOD mice sets the stage for the current study through which: 1) The optimized number of IDO-expressing fibroblasts required to inhibit the immuno-destruction of islet β-cells and reverse hyperglycemia in NOD mice was determined, 2) The importance of kynurenine level, as index of IDO enzymatic activity for efficacy of IDO cell therapy in preventing the progression of diabetes has been identified, 3) The effectiveness of IDO-expressing cell therapy on modulation of β-cell specific autoreactive CD8^+ T cells, IL-17-producing Th17 cells and regulatory T cells (Tregs) was elucidated, finally, 4) The effect of IDO-expressing fibroblasts on IL-1β expression in human and mouse primary islet β-cells and its relationship with regulation of islet β-cell survival during in vitro culture have been shown.

Similar to “the honeymoon phase” in patients with T1D [113], in NOD mice with recent onset diabetes, there is a pool of functionally “exhausted” or degranulated islet β-cells that have not been destroyed. These β-cells may immediately recover to produce a significant amount of insulin for glucose homeostasis after they are exempted from autoimmune attack [558]. In the current study, we used fibroblasts expressing indoleamine 2,3-dioxygenase (IDO) as an immune modulator and found that optimized IDO-expressing fibroblasts functionally recovered the remaining islet β-cells and successfully reversed hyperglycemia in a prominently high percentage of NOD mouse recipients due to modulation of several possible immunological contributing factors of T1D.
Firstly, we identified that $15 \times 10^6$ (15M) was the optimized number of IDO-expressing dermal fibroblasts to sufficiently reverse the course of T1D development in newly diabetic NOD mice. IP injection of 15M IDO-expressing fibroblasts significantly reduced infiltration of lymphocytes into islets and improved the functionality of the remaining islet β-cells, which produced enough insulin to normalize the blood glucose levels and body weight in 5 out of 6 recipients. Our previous study showed that injection of $20 \times 10^6$ (20M) IDO-expressing fibroblasts successfully reversed hyperglycemia in 82% diabetic NOD mice [547], which suggests the comparable efficacies between 15M and 20M IDO-expressing cell therapies.

Additionally, we found that IDO enzymatic activity is another important contributing factor to the efficacy of this cell therapy. The mouse that received 15M transduced fibroblasts from a batch with the lowest IDO activity did not go back to normoglycemia. On the other hand, the mouse treated with 10M fibroblasts with highest IDO activity became normoglycemia around 14 days after cell injection. These findings suggest that not only the number of the cells, but also enzymatic activity of IDO is an important factor in the efficacy of IDO cell therapy on inhibition of T1D development in NOD mice.

It is well established that IDO has the ability of catabolizing the essential amino acid, tryptophan, to kynurenine metabolites and thereby generating an immune tolerance in different physiological and pathological conditions such as that seen in mammalian pregnancy [432,436], autoimmunity [559,560] and transplantation [561,562]. Based on this body of evidence, we further investigated the underlying immunological mechanisms by which our 15M IDO-expressing fibroblasts altered the progression of T1D in NOD mice. The important role of β-cell specific autoreactive
CD8\(^+\) T cells in the pathogenesis of diabetes is underscored by the findings that no occurrences of diabetes in NOD mice is observed in the absence of MHC class I and autoreactive CD8\(^+\) T cells [65]. Additionally, transgenic NOD mice over-expressing autoreactive CD8\(^+\) T cell receptors (TCRs) develop T1D at a greatly accelerated rate [563]. NRP-V7 tetramer has been proven to be a great detector for this specific population of cytotoxic T lymphocytes (CTLs) [564]. Thus, in this study, we used NPR-V7 tetramer to track the \(\beta\)-cell specific autoreactive CTLs in PBMCs, SPLs, PLNs or CLNs. We observed that IP injection of 15M IDO-expressing fibroblasts suppressed the autoreactive CD8\(^+\) T cells in mouse PBMCs over time. Moreover, mice treated with 15M IDO-expressing fibroblasts had significantly lower ratios of autoreactive CTLs in PBMCs, SPLs, PLNs and CLNs as compared with those in mice receiving either no cell treatment or 10M IDO-expressing cells. These findings reveal that 15M IDO-expressing fibroblasts are able to inhibit the accumulation of autoreactive CTLs involved in destruction of islet \(\beta\)-cells and thereby reverse the progression of T1D at an early stage when the remaining \(\beta\)-cells are able to produce enough insulin for glucose homeostasis.

Interleukin (IL)-17-producing Th17 cells have been characterized as a new subset of CD4\(^+\) T helper cells. Pathogenic Th17 cells can disturb the balance between effector T (Teff) and Treg cells [565]. Stimulation of CTL responses by Th17 cells has also been identified [566,567]. The role of Th17 cells in mediating pathogenesis of T1D is increasingly realized. Studies show that cells polarized to Th17 cells can induce T1D in lymphopenic NOD SCID mice [568]. Th17 cells have also been found to be increased in the peripheral blood and PLNs of patients with T1D [569,570]. In our \textit{in vivo} studies, data showed that the frequency of Th17 cells in PBMCs of mice intraperitoneally injected with 15M IDO-expressing fibroblasts was significantly reduced.
as compared with that found in mice receiving either no cell treatment or 10M IDO-expressing fibroblasts. In addition, mice treated with 15M cells had markedly lower frequencies of Th17 cells in SPLs and PLNs than those in untreated diabetic mice. The decreased frequency of Th17 cells in mice treated with 15M cells is likely to be associated with the reduced autoreactive CTLs and the increased Tregs found in this group of mice. However, we found no significant differences in the ratios of Th17 cells in CLNs among three groups. Nevertheless, the frequency of Th17 cells in general was lower in CLNs as compared to PBMCs and SPLs in all groups. This suggests that Th17 cells are less abundant in lymphoid tissues remote to inflammation site (i.e. pancreas) in T1D.

CD4⁺CD25⁺FOXP3⁺ regulatory T cells (Tregs) are major contributors in maintaining immune tolerance [571]. There are reports showing that Tregs may be defective in NOD mice, which is correlated with T1D onset [86,572]. Additionally, loss of regulatory factors such as FOXP3 accelerates the onset of T1D and enhances disease severity [573]. In this study, we found that IP injection of 15M IDO-expressing fibroblasts in diabetic mice gradually and significantly increased the frequencies of CD4⁺CD25⁺FOXP3⁺ Tregs in blood and lymphoid tissues of these mice as compared with those found in either untreated mice or mice treated with 10M IDO-expressing fibroblasts. The increase in Tregs is considered to be important for enhancing the inhibition of CTLs in order to further protect islet β-cells.

In previous studies, different types of cells have been used for expressing IDO, such as dendritic cells [386] or Sertoli cells [171]. However, in our study, we transduced IDO gene into dermal fibroblasts due to several reasons. Firstly, dermal fibroblasts are easy for acquisition and
maintenance as compared with other cell types [574]. Secondly, fibroblasts can act as antigen presenting cells [575]. Importantly, dermal fibroblasts have been identified to express interleukin-1 receptor antagonist (IL-1Ra) which binds to the same receptor of interleukin-1β (IL-1β) [529,547,576]. IL-1β as one of the key innate immune mediators has been found to be elevated in the early course of T1D and contribute to β-cell apoptosis and dysfunction [534]. Thus, we co-cultured islets with IDO-expressing fibroblasts to see whether IDO-expressing fibroblasts can inhibit IL-1β activation through blocking IL-1 receptors in order to prevent islet cells from IL-1β-induced apoptosis. Our results showed that both human and mouse islets co-cultured with IDO-expressing fibroblasts had markedly lower IL-1β expression and apoptotic rate in β-cells. These findings indicate that injected IDO-expressing fibroblasts may serve as an anti-inflammatory mediator. In a clinical setting, investigators have found that either suppression of inflammatory responses alone, such as treatment with anakinra (IL-1 receptor blocker) or inhibition of adaptive immunity alone is not effective for reversing hyperglycemia in recent onset T1D, suggesting the necessity of using a combination treatment [167,577]. In fact, IP injection of IDO-expressing fibroblasts in NOD mice at the early stage of diabetes has a potential to overcome both problems of inflammatory responses by controlling the level of pro-inflammatory cytokines such as IL-1β and IL-17, and the adaptive autoimmunity by modulating the autoreactive T cells and Tregs.

In conclusion, our current study shows that $15 \times 10^6$ IDO-expressing dermal fibroblasts with effective kynurenine level can reverse the progression of T1D in NOD mice. This seems to happen through induction of Tregs as well as inhibition of β-cell specific CTLs and IL-17-
producing Th17 cells, which successfully attenuates insulitis and restores the functionality of the remaining islet β-cells in newly diabetic NOD mice.
Chapter 5: Conclusions and discussions

5.1 Summary and discussion

In type 1 diabetes, insulin-producing islet β-cells are progressively destroyed by autoimmune attack. Thus, islet β-cell replacement by islet allo-transplantation provides an effective treatment for T1D but it is currently restricted by poor long-term survival of islet grafts [149,472,473,482]. Studies have shown that the major loss of islet β-cell mass and function occurs during islet isolation as well as following pre-transplant culture due to the detrimental effects induced by different non-immune factors, such as loss of islet ECM or formation of toxic islet amyloid [131,475,482,492]. As such, in this project, we developed new strategies to protect islet β-cells from the toxicity induced by non-immune or autoimmune factors and thereby improving β-cell viability and function (Figure 5.1).

Islet ECM constitutes the native islet microenvironment and provides a variety of signaling that regulates islet cell morphology, differentiation, proliferation, survival and function [487-489,578]. However, its destruction by the mechanical and enzymatic stresses generated during islet isolation leads to islet β-cell death and dysfunction contributing to the final islet graft failure [492,579]. Therefore, restoration of islet ECM and re-establishment of islet-ECM interactions during pre-transplant culture are essential for improving the outcome of islet transplantation. Accordingly, we developed two novel three-dimensional (3-D) scaffolds, collagen matrix (CM) alone or human fibroblasts-populated collagen matrix (FPCM) to provide the surrogate ECM for islets during in vitro culture. Our scaffolds have several advantages over other ECM-based approaches. Firstly, 3-D structure of our scaffolds mimics islet environment in native tissue more
closely as compared to 2-D culture systems. It also plays an important role in preservation of the morphology, survival and function of islet β-cells [488,580-582]. Secondly, type I collagen was applied to our scaffolds. As one of the most common collagen types in islet ECM, type I collagen is essential for implementing the physiological functions of islet ECM such as improving islet β-cell survival and stimulating insulin secretion [495,583]. In addition, type I collagen has slower biodegradation rate than other types of collagen in islet ECM [584]. Thirdly, fibroblasts were populated into our 3-D scaffold. Fibroblasts not only produce type I collagen to maintain the integrity of the scaffold but they also provide angiogenic and growth factors including vascular endothelial growth factors (VEGFs) and fibroblast growth factors (FGFs) to further promote islet viability and vascularization [493,513,585]. We embedded isolated human islets within these scaffolds instead of culturing them in 2-D regular condition as free-floating islets. After 7 days of culture, we found that both CM and FPCM can preserve islet morphology as well as expression of several key β-cell genes including insulin, PDX1 and GLUT2. Moreover, human islets embedded within CM or FPCM have significantly increased viability, glucose-stimulated insulin secretion and insulin content compared to 2-D cultured islets. These findings are consistent with previous studies from our group which reported that CM and FPCM promote mouse islet cell viability and function both in vitro and in vivo [493]. Studies from other research groups also demonstrated that isolated rat or canine islets embedded within type I collagen matrix had a remarkable reduction in islet cell death [586] and a significant enhancement in insulin secretion [490,495].

Growing evidence supports that formation of toxic protein aggregates named islet amyloid is a
major non-immune factor contributing to islet graft failure. Previous studies have shown that amyloid formation occurs in both pre-transplant cultured and transplanted human islets, and contributes to the progressive islet β-cell death [131,132,205,264,265]. Our study is the first to assess islet amyloid formation in islets embedded within 3-D scaffolds. First, using thioflavin S staining which can only detect fibrillar form of islet amyloid, we found that islet amyloid fibrils were not detectable in any of the fresh human islet preparations used in this study prior to in vitro culture but were easily detectable in human islets after 7 days of culture in 2-D regular condition. This finding is consistent with our previous studies that showed islet amyloid formation in human islets following 7 days of culture [320,527]. Nevertheless, human islets embedded within 3-D scaffolds, either CM or FPCM, had markedly reduced fibrillar human islet amyloid polypeptide (hIAPP) aggregates as compared with 2-D cultured islets. As previously discussed in Chapter 1.2.2, there are different forms of hIAPP aggregates during the process of islet amyloid formation. Increasing evidence suggests that toxic properties are mainly due to prefibrillar oligomeric aggregates [205,212,226,239,587]. Similar with fibrillar aggregates, hIAPP oligomers can also disrupt islet β-cell membranes and break the intracellular ion homeostasis, resulting in cell apoptosis [212]. Accordingly, we further investigated hIAPP oligomers in both human and hIAPP-expressing transgenic mouse islets during in vitro culture by staining with A11 antibody. Same as fibrillar aggregates, we found that the percentage of human and transgenic mouse islets with hIAPP oligomers was very low before culture, but was dramatically increased in 2-D free-floating (FF) cultured islets after 7 days. Interestingly, the number of islets containing hIAPP oligomers was markedly decreased in CM-embedded islets as compared with FF-cultured islets. These findings collectively indicate the important impact of 3-D scaffolds on prevention of islet amyloid formation during in vitro culture.
Islet amyloid formation exerts its cytotoxicity by different mechanisms [227,230,233]. Our findings showed that following 7 days of culture, scaffold-embedded human islets with less islet amyloid formation had lower Fas expression as compared to that shown in 2-D cultured islets. This finding is consistent with other studies from our lab which reported that deletion of Fas significantly reduces amyloid-induced β-cell apoptosis [249]. We further looked at the effects of islet amyloid formation on PI3-kinase signaling pathway, which plays an important role in regulation of islet β-cell survival, proliferation and function [285-287]. To this end, protein kinase B (PKB), the key effector of this signaling pathway, was specifically investigated. First, we identified that exogenously applied IAPP aggregates decreased PKB activation (phosphorylation) in pancreatic β-cells during in vitro culture, which was associated with reduced β-cell proliferation and increased β-cell apoptosis. This finding is consistent with the previous studies showing that addition of synthetic hIAPP to cultured INS-1 β-cells decreases phosphorylated PKB levels leading to cell death [521,588]. We further demonstrated that endogenously formed hIAPP aggregates in primary human and hIAPP-expressing transgenic mouse islets inhibited PKB phosphorylation in β-cells. Prevention of hIAPP aggregation by islet amyloid inhibitor, Congo red (CR) or collagen matrix (CM) restored phosphorylated PKB levels in islet β-cells, which was associated with reduced islet β-cell apoptosis. This is the first report to show the relationship between endogenous hIAPP aggregates and PKB phosphorylation in islet β-cells and these findings suggest that reduction of PKB phosphorylation may act as one of the mechanisms by which hIAPP aggregation exerts its toxicity in islet β-cells.

It has been shown that hIAPP aggregates activate NLRP3 inflammasomes to produce pro-inflammatory IL-1β, which then leads to islet β-cell death and dysfunction [230,248,528].
However, the underlying mechanisms of IL-1β-induced islet β-cell toxicity have not been fully understood. Results from our experiments in Chapter 3 confirmed that hIAPP aggregates can induce IL-1β production in human islets during in vitro culture. More importantly, these studies provided evidence to show that hIAPP aggregation induces reduction of PKB phosphorylation in islet β-cells, at least partially, via stimulation of IL-1β production, providing a potential mechanism by which IL-1β may mediate islet β-cell toxicity induced by hIAPP aggregation.

In addition to non-immune factors, immune response to graft tissue is also a major contributing factor to islet graft failure [121,149]. To prevent allograft rejection, syngeneic islets from young non-diabetic NOD mice were transplanted into diabetic NOD mice. However, the grafted islets were still destroyed, attributing to recurrent autoimmune responses [589]. Moreover, it has been revealed that the recurrent autoimmunity can facilitate the alloimmune responses in animal models [590]. This body of evidence suggests that recurrence of autoimmunity is a key causative factor of islet graft failure and the control of autoimmunity is essential for improving the outcome of islet transplantation [590-594]. More importantly, inhibition of autoimmunity itself is able to reverse T1D without exogenous islet transplantation [156,166,477]. In a clinical setting, many patients (~60%) experience a “honeymoon period” (or remission phase) shortly after T1D initial diagnosis. During this period, exogenous insulin therapy is beneficial for functional recovery of remaining islet β-cells (10%-30%) in these patients. The recovered islet β-cells have the ability of secreting enough insulin for normalizing blood glucose levels and reversing T1D. However, in the presence of autoimmunity, these recovered islet β-cells will be progressively destroyed by autoimmune attack and ultimately patients without β-cells will be dependent on injection of exogenous insulin [45,595]. This natural progression of T1D in patients provides
supportive evidence that controlling autoimmunity by immunoregulation is very important for reversing the progression of T1D.

As introduced in Chapter 1.4, IDO is an enzyme responsible for catabolizing the essential amino acid, tryptophan, to kynurenine and other metabolites. It is well established that IDO expression also suppresses the viability, proliferation and function of effector T cells, as well as inducing the differentiation of regulatory T cells (Tregs) [387,479], which indicates its important role in the protection of semi-allogeneic fetus during pregnancy and prevention of allograft rejection during transplantation [432,444]. Accordingly, in this project, we applied a lenti-viral vector to dermal fibroblasts and used these IDO-expressing fibroblasts in a cell therapy approach for treating recent onset diabetes in NOD mice. NOD mice have been broadly used as a suitable animal model for investigating T1D. This mouse model shares similar genetic backgrounds, biochemical alterations and symptoms with T1D in human [596]. For example, similar to “honeymoon period” in diabetic patients, in newly diabetic NOD mice, there is also a pool of live islet β-cells with aberrant functions, which can be recovered to produce a significant amount of insulin for reversing hyperglycemia [558].

In this study, we identified some important factors that are involved in the efficacy of IDO-expressing fibroblast therapy, including the number of the injected cells and enzymatic activity of IDO. We demonstrated that 15x10⁶ (15M) is the optimized number of IDO-expressing fibroblasts required to reverse hyperglycemia and prevent loss of body weight in 83% of new onset diabetic NOD mice. Furthermore, our findings showed that optimized IDO-expressing cell therapy resulted in decreased frequency of β-cell specific autoreactive CD8⁺ T cells and IL-17-
producing Th17 cells, as well as increased frequency of CD4\(^+\)CD25\(^+\)FOXP3\(^+\) Tregs and therefore significantly reduced the aggressiveness of insulitis in islets and preserved the function of remaining islet \(\beta\)-cells manifested as restoring insulin secretion to reverse T1D. In this study, we transduced bystander dermal fibroblasts with the IDO gene rather than directly delivering the IDO gene in islet cells, with the purpose of better protecting islet cells. Another key reason for choosing dermal fibroblasts was their capacity for producing interleukin-1 receptor antagonist (IL-1Ra) which can block the pro-apoptotic IL-1\(\beta\) signaling pathway by competitive binding to IL-1\(\beta\) receptor [529,547,576]. IL-1\(\beta\) has been identified to stimulate the expansion of autoreactive T cells and attenuate the function of Tregs, which contribute to the pathogenesis of T1D [597]. In contrast, the increase in IL-1Ra: IL-1\(\beta\) ratio seems to be correlated to the diabetic remission in new onset T1D patients [598]. Accordingly, we further investigated the effects of IDO-expressing fibroblasts on IL-1\(\beta\) expression in islets during \textit{in vitro} culture. Results showed that both human and mouse islets co-cultured with IDO-expressing fibroblasts had remarkable lower IL-1\(\beta\) expression in \(\beta\)-cells, which was associated with significantly reduced \(\beta\)-cell apoptosis as compared to that of islets cultured alone. In addition, our previous study showed that fibroblasts also produced co-inhibitory molecules, such as programmed cell death ligand 1 and 2 (PD-L1 and PD-L2), which play important roles in tolerance induction [547,599]. Last but not least, fibroblasts can secrete some growth factors, such as FGFs, which further improve the survival and function of islet \(\beta\)-cells [146,493]. In summary, the above findings provide evidence to show that optimized IDO-expressing fibroblasts can successfully control autoimmunity and therefore preserve the viability and function of remaining islet \(\beta\)-cells, which can reverse T1D in newly diabetic NOD mice.
Figure 5.1 The schematic summary of research project.

Three-dimensional scaffolds, collagen matrix (CM) alone and human fibroblasts-populated collagen matrix (FPCM) markedly reduce toxic islet amyloid formation and significantly enhance the viability and function of human islet β-cells during in vitro culture (Chapter 2). Islet amyloid formation inhibits PKB phosphorylation in cultured islet β-cells, at least partially, via IL-1β signaling pathway (Chapter 3). Indoleamine 2,3-dioxygenase (IDO) is an enzyme responsible for catabolizing the essential amino acid, tryptophan, to kynurenine. Dermal fibroblasts transduced with the IDO gene (IDO-expressing fibroblasts) efficiently inhibit β-cell specific autoreactive CD8+ T cells and IL-17-producing CD4+ T cells (Th17 cells); They upregulate regulatory T cells (Tregs) in diabetic NOD mice as well. IDO-expressing fibroblasts also block IL-1β signaling pathway in cultured islet β-cells by expression of IL-1 receptor antagonist (IL-1Ra). Therefore, IDO-expressing fibroblasts inhibit autoimmune responses and preserve the survival and function of remaining islet β-cells in newly diabetic NOD mice, which are able to produce enough amount of insulin to reverse hyperglycemia (Chapter 4). In summary, the cell- and matrix-based approaches developed in this project can significantly improve survival and function of islet β-cells both in vitro and in vivo.

5.2 Significance

Type 1 diabetes (T1D), as the most common type of diabetes in children and adolescents, has high morbidity and mortality due to its devastating complications [6,7,13]. However, treatment
of this severe disease remains inadequate. Although exogenous insulin injection can ameliorate the symptoms and complications of hyperglycemia, it does not reverse the progression of T1D [600]. Additionally, insulin therapy may result in life-threatening risks such as hypoglycemia [601]. Replacement of β-cells by islet transplantation provides a promising approach for treatment of T1D, but it has limited long-term efficacy due to progressive islet β-cell death and dysfunction during islet isolation, pre-transplant culture and post transplantation. Thus, development of feasible new approaches for curing T1D is necessary.

In this project, we developed two novel 3-D scaffolds, collagen matrix (CM) and human fibroblast-populated collagen matrix (FPCM) which remarkably prevent toxic islet amyloid formation and significantly enhance the viability and functionality of human islets during in vitro culture. These findings provide the fundamental evidence to support the potential for our scaffolds acting as new strategies to improve the quality and quantity of human islets during pre-transplant culture and thereby improving the outcome of clinical islet transplantation. In addition, we speculate that in translational applications of this model for transplantation, a patient’s own fibroblasts could be used for preparation of the 3-D scaffold to be transplanted. Moreover, we have previously shown that, unlike their rapid proliferation in regular culture medium, fibroblasts have a very low proliferation rate in collagen matrix [493], and therefore the islet/fibroblast ratio does not significantly change during culture in matrix. This evidence further confirms that our 3-D scaffolds have promising prospects of application in clinical islet transplantation.

Aggregation of human islet amyloid polypeptide (hIAPP), a hallmark of pancreas in T2D, also occurs during pre-transplant culture and post-transplantation, accounting for non-immune β-cell
death during islet transplantation. In the present study, we discovered that hIAPP aggregates inhibit PKB phosphorylation in islet β-cells during \textit{in vitro} culture, which indicates a potential pathway by which hIAPP aggregates lead to islet β-cell death and dysfunction. This finding also implies a potential role for PKB as a therapeutic target for protection of islet β-cells from the toxicity induced by hIAPP aggregation thereby enhancing the survival and function of islet β-cells used for clinical islet transplantation.

In this study, we also developed a cell therapy, containing dermal fibroblasts with stable IDO expression, which has the ability to successfully inhibit autoimmune responses and induce immunological tolerance. Therefore, it shows a long-term effect on reversing T1D in diabetic NOD mice. The fact that high IDO expression occurs in some physiological conditions, such as mammalian pregnancy [433], indicates that our IDO cell therapy could be a relatively safe approach for T1D patients. These findings collectively suggest that the IDO cell therapy could be a suitable therapeutic strategy to treat T1D in clinical setting.

Overall, our new strategies developed in this project protect islet β-cells from toxicity induced by non-immune or autoimmune factors and therefore improve β-cell viability and functionality which supports their potential to be exploited as feasible approaches for treatment of T1D.

5.3 Future studies

In \textit{Chapter 2}, we chose type 1 collagen as the surrogate of islet ECM in the development of 3-D scaffolds based on its advantages [495,583,584]. However, in native tissue, the composition of islet ECM is complex and each component has its own properties and functions [602]. In order to
better mimic the native islet ECM components, the scaffolds can be improved by combining other important matrix proteins, such as collagen IV and laminin, which are two major constituents of islet perivascular basement membrane and are essential for maintaining islet vasculature [603-605]. Moreover, we demonstrated that our 3-D scaffolds significantly reduced islet amyloid formation but the underlying mechanisms remain unknown. It has been shown that impaired prohIAPP processing occurs during islet culture, leading to increased levels of immature (pro)hIAPP which potentiates hIAPP aggregation and islet amyloid deposition [320].

We speculate that enhancement of β-cell function by embedding human islets within our 3-D scaffolds may restore impaired prohIAPP processing, leading to lower production of immature (pro)hIAPP forms from β-cells and thereby reducing islet amyloid formation in cultured human islets. To verify this hypothesis, the cellular protein levels of prohIAPP, its intermediates and mature IAPP form in the scaffold-embedded or 2-D cultured human islets following 7-day culture need to be detected by Western blot. If it proves to be true, a virtuous cycle will be set up showing that improvement of β-cell function at early stages of islet culture by the 3-D scaffolds has preventative effects on islet amyloid formation, which can further reduce amyloid-induced islet β-cell toxicity, leading to enhanced β-cell survival and function.

As previously discussed in detail, phosphorylated PKB mediates cell survival via different signaling pathways involving many downstream factors, such as FoxO or GSK3 [329,345]. In Chapter 3, we have shown that islet amyloid formation inhibits PKB phosphorylation in islet β-cells, which is associated with reduced β-cell survival. However, we have not further explored which downstream factors of PKB signaling pathway participate in this process. As such, further
investigation of PKB downstream factors may provide us more potential targets for prevention of amyloid-induced islet β-cell death.

As an integrating vector, lenti-viral vector provides an efficient gene transduction in both dividing and non-dividing cells. Thus, in Chapter 4, we used lenti-viral vectors to ensure the stable IDO expression in transduced fibroblasts. However, considering clinical translation of our cell therapy model, stable non-viral gene-transduced methods need to be further explored. Additionally, IDO-expressing fibroblasts injected into the mice may also play a role as non-professional antigen-presenting cells (APCs) [575], which implies that the source of fibroblasts could be another important contributing factor to the efficacy of IDO cell therapy and as such, the IDO-expressing syngeneic (NOD) or allogeneic (C57BL/6) dermal fibroblasts may have different therapeutic effects on reversing the progression of T1D in NOD mice. In the present study, the significant effect of IDO-expressing fibroblasts on inhibition of pro-inflammatory IL-1β expression in islet β-cells was only identified using in vitro models. This exciting finding intrigues us to further find out the role of IL-1Rα produced by IDO-expressing fibroblasts in alleviating inflammation and protecting islet β-cells at the early stage of T1D in NOD mice. Finally, as discussed before, the recurrence of autoimmunity after islet transplantation is another important contributing factor to islet graft failure [130,476]. Based on this fact, another area that we would like to investigate in the future is the effect of our IDO cell therapy on prevention of recurrent autoimmunity in islet transplantation.
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