Immunoregulatory Role of Human Islet Amyloid Polypeptide through FoxP3+CD4+CD25+ T Regulatory Cells

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DECLARATION OF ORIGINALITY

The work contained in this thesis is completely original with novelty. It has been carried out by the candidate, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. No portion of this thesis has been submitted which has been accepted for the award of any other degree or diploma in any university or other tertiary institution.

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Abstract in English

Abstract of thesis entitled: Immunoregulatory Role of Human Islet Amyloid Polypeptide through FoxP3+CD4+CD25+ T Regulatory Cells
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Background: Islet amyloid polypeptide (IAPP, also known as amylin) is a 37-amino acid peptide principally co-secreted with insulin from the β-cells of the pancreatic islets. Some of the physiological actions of human amylin (hIAPP) include glucose regulation, suppression of appetite and stimulation of renal sodium and water reabsorption. Amylin deficiency and diminished post-prandial amylin response have been reported in advanced stages of type 1 and type 2 diabetes. In autopsy specimens of type 2 diabetes, amyloid is found in 40-90% of cases. During the characterization of islet morphology of aged hIAPP transgenic mice, I observed pathological features suggestive of immune dysregulation. Review of literature also suggested possible immuno-modulating functions of human amylin in *in vitro* experiments. Since autoimmunity and innate immunity are implicated in aging and diabetes, I explored the immunological role of amylin with particular focus on CD4+CD25+ T regulatory cells and toll-like receptors (TLR) which are known mediators of autoimmunity and innate immunity respectively.

Hypothesis: Human amylin may have immunomodulating effects which may have implications on pathogenesis of autoimmune type 1 diabetes.

Objectives:

1. I systemically characterized the morphological, functional and immune regulatory role of human amylin in aged hIAPP transgenic mice which include metabolic profiles, plasma levels of amylin and insulin as well as morphological changes of pancreatic lymph nodes (PLN).
2. I then examined splenic expression of TLR-4 associated changes in cytokines (TNF-α, TGF-β, and IL-6).
3. I also examined the expression level of receptor activity modifying proteins (RAMPs) in pancreas and spleen.
4. I finished by investigating the role of human amylin on stimulating CD4+CD25+ T regulatory (Treg) cells in hIAPP transgenic mice and peripheral blood monocytes (PBMC) from healthy subjects.

Materials and Methods: Male hemizygous hIAPP transgenic mice (n=32) and their nontransgenic littermates (n=20) were fed with normal chow and studied longitudinally up to 18 months of age with measurement of plasma insulin, glucose and amylin at regular intervals. Detailed oral glucose tolerance test, intra-peritoneal insulin tolerance test, insulin and amylin protein expression were examined at 3, 7, 12 and 18 months of age. Histological changes of pancreas and spleen including changes in CD4+CD25+ T regulatory cells and cytokines were examined at 12 and 18 months.

Results:

1. With aging, the hIAPP transgenic mice demonstrated increased plasma amylin, decreased plasma insulin, reduced insulin to amylin ratio and improved insulin sensitivity ($p<0.05$).

2. The aged hIAPP transgenic mice showed changes in immune function as indicated by:
    a、 Reduced number and size of PLN ($p<0.05$).
    b、 Decreased expression level of TLR-4 in splenocytes ($p<0.05$).
    c、 Increased expression of transforming growth factor-beta (TGF-β) and tumor necrosis factor-alpha (TNF-α) protein but decreased level of IL-6 in splenocytes ($p<0.05$).
3. The changes in the levels of immune cytokines such as IL -1, IL-2, IL-4, IL-10, IL-17, interferon-γ and GM-CSF were similar between hIAPP transgenic and nontransgenic mice ($p>0.05$).
4. The levels of RAMP1, RAMP2, and RAMP3 were higher in the spleen of hIAPP transgenic mice than nontransgenic mice ($p<0.05$).
5. The hIAPP transgenic mice showed higher percentage of CD4+CD25+ Treg cells compared with nontransgenic littermates. Treatment with human amylin, but not rat amylin, increased the percentage of FoxP3+CD4+CD25+ Treg cells in both splenic T lymphocytes of hIAPP transgenic mice and PBMCs of healthy subjects *ex vivo* ($p<0.05$).

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Conclusions: Human amylin may play an important role in modulating immunity mainly through stimulating CD4+CD25+ Treg cells, decreasing PLN and altering expression of TLR-4 and cytokines. If these findings are confirmed in *in vivo* model, human amylin has the potential to become a novel and promising therapy to prevent and reverse autoimmune disease such as autoimmune type 1 diabetes.

Abstract in Chinese

摘要

論文題目：人類淀粉樣蛋白通過**CD4+CD25+**調節性**T**細胞的免疫調節作用

由香港中文大學赫蘭於 **2010** 年 **7** 月呈交，為哲學博士 (內科學科) 論文

背景：

胰島淀粉樣多肽（IAPP，也叫做 amylin）是一種與胰腺β細胞胰島素共分泌的 37 個氨基酸多肽。人類 amylin 的主要生理學作用包括血糖調節，抑制食欲和刺激腎臟鈉/水重吸收等。已有研究報道在 1 型和 2 型晚期糖尿病患者中 amylin 缺乏和餐後反應降低。在 2 型糖尿病患者的屍檢樣本中，發現 40－90%均有淀粉樣沉積。在 hIAPP 轉基因老鼠的胰島形態觀察實驗中，有病理學特徵可能提示有免疫系統改變。有關文獻也提示 amylin 在體外實驗中具有免疫調節功能。在糖尿病和衰老病人中也存在自身免疫和固有免疫的异常，因此我側重研究 amylin 在刺激 CD4+CD25+ T 調節性細胞和 Toll 樣受體方面的免疫調節功能，因爲它們被認爲分別在自身免疫和固有免疫中具有重要介導作用。

假說：

Amylin 具有重要的免疫調節功能并因此影響 1 型自身免疫糖尿病的發病機制。

目的：

此研究的目的在於了解人類 amylin 的免疫調節作用以及相關的機制。具體如下：

1. 描述 hIAPP 轉基因老鼠隨年齡的代謝性指標概述，檢測 hIAPP 轉基因老鼠血漿中 amylin 和胰島素水平，檢測 hIAPP 轉基因老鼠胰腺淋巴結的形態學改變。
2. 檢測 hIAPP 轉基因老鼠脾臟中 TLR-4 的表達變化，檢測 hIAPP

轉基因老鼠脾臟中免疫調節細胞因子，比如 TNF-α, TGF-β 和 IL-6 的變化，檢測 hIAPP 轉基因老鼠脾臟和胰腺中 RAMPs 的表達水平。

3. 觀察人類 amylin 在 hIAPP 轉基因老鼠和體外刺激正常人外週血單個核細胞產生 FoxP3+CD4+CD25+調節性 T 細胞中的作用。

方法：

用普通飼料喂養雄性雜合子 hIAPP 轉基因老鼠并觀察至 18 個月。檢測血漿中 amylin 和 insulin 表達水平的變化。在 3，7，12 和 18 個月時測定葡萄糖耐量血糖，胰島素耐量血糖，胰島素以及 amylin 蛋白表達水平。流式細胞儀檢測 CD4+CD25+調節性 T 細胞比例以及炎症性細胞因子的變化。檢測 12 和 18 個月胰臟和脾臟的病理學變化。

結果：

1. 隨著年齡的增長， hIAPP 轉基因老鼠顯示血漿 amylin 升高，insulin 降低，insulin/amylin 比值降低，以及胰島素敏感性增高。
2. 12 及 18 個月的 hIAPP 轉基因老鼠顯示出免疫學功能的變化。
   A. 胰腺淋巴結數量以及面積均降低。
   B. hIAPP 轉基因老鼠 TLR-4 表達水平降低。
   C. hIAPP 轉基因老鼠 TNF-α, TGF-β 表達增加，IL-6 表達降低。
3. hIAPP 轉基因老鼠免疫細胞因子如 IL -1, IL-2, IL-4, IL-10, IL-17, interferon-γ 和 GM-CSF 的水平無顯著變化。
4. 總體來講，hIAPP 轉基因老鼠脾臟 RAMP1，RAMP2 和 RAMP3 的表達均增加。
5. hIAPP 轉基因老鼠 CD4+CD25+調節性 T 細胞數量增加。體外實驗用人 amylin 而不是老鼠 amylin 來處理 hIAPP 轉基因脾細胞和正常人外週血單個核細胞，可以增加 CD4+CD25+調節性 T 細胞的數量。

結論：人類 amylin 通過 CD4+CD25+調節性 T 細胞，胰腺淋巴結，TLR 和細胞因子調節免疫功能方面具有重要作用。如果這些研究得到證實，人類 amylin 將成為可預防和逆轉自身免疫性疾病如糖尿病的新治療方法。

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Caren L HE
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List of Abbreviations

ADM	adrenomedullin
bp	base pair
CBA	cytometric bead array
CGRP	calcitonin gene related peptide
CNS	central nervous system
Da	Dalton
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
FBG	fasting blood glucose
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCRs	G protein-coupled receptors
HFIP	hexafluoroisopropanol
hIAPP	human islet amyloid polypeptide
HOMA-IR	homeostasis model assessment of insulin resistance
HRP	horseradish peroxidase
IAPP	islet amyloid polypeptide
IFN	interferon
IGT	impaired glucose tolerance
IL	interleukin
IVGTT	intravenous glucose tolerance test
LPS	lipopolysaccharide
mins	minutes
MUP	4-methylumbelliferyl phosphate
MW	molecular weight
NF-κB	transcription factor nuclear factor κB
NOD MICE	non-obese diabetic
OGTT	oral glucose tolerance test
PAS	periodic acid schiff
PBMC	Peripheral blood mononuclear cells
PCR	polymerase chain reaction
PLN	pancreatic lymph node

RAMP	receptor (G protein-coupled) activity modifying protein
s	seconds
SD	Standard deviation
SE	Standard error (SE=SD/$\sqrt{n}$)
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBST	Tris-buffered saline Tween-20
TFA	trifluoroacetic acid
TGF	transforming growth factor
Th	T helper lymphocytes
TLR	toll-like receptor
TNF	tumor necrosis factor

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Introduction

1.1 Islet amyloid polypeptide (IAPP)

Islet amyloid polypeptide (IAPP, amylin) is a 37-amino acid protein which is co-secreted with insulin from pancreatic β cells (in a roughly 1:20 amylin: insulin ratio) [1, 2]. Amylin is expressed as prepropolypeptide consisting of 93 (murine) or 89 (human) amino acids which is processed enzymatically with the removal of amino- and carboxy-terminal propeptide segments. The human form of amylin has the amino acid sequence KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY, with a disulfide bridge between cysteine residues 2 and 7 [3]. The region of $amylin_{20\text{-}29}$ is believed to be very important for the formation of amyloid oligomers/fibrils in an aqueous environment. As indicated in Figure 1, human and primates share similar homology in $amylin_{20\text{-}29,}$ and the synthetic forms of these peptides form amyloid. In contrast, rat and mouse $amylin_{20\text{-}29}$ are identical and do not form amyloid due to the presence of three proline residues. Here, neither rats nor mice spontaneously develop Type 2 diabetes [4].

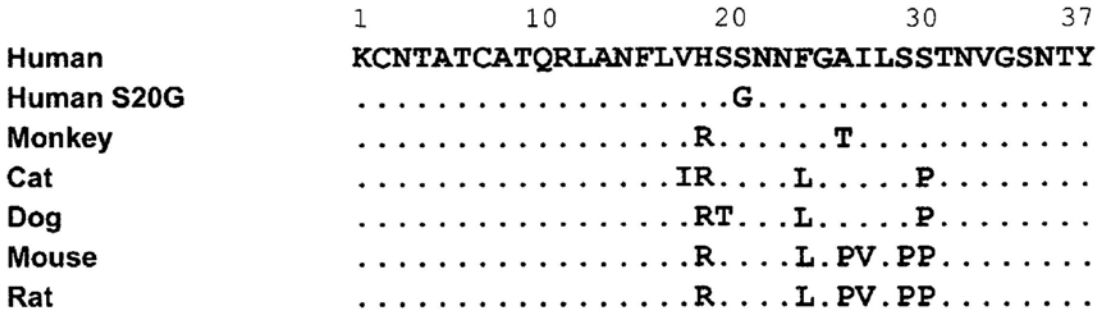

Figure 1 Alignment of amylin ortholog proteins [4].

Amino acid alignment of amylin protein sequences identified in human, human with mutant amylin (S20G), monkey, cat, dog, mouse, and rat. Dots mean conserved residues with human amylin sequence. Red letters mean the amyloidic sequence.

1.1.1 Biology of amylin

1.1.1.1 Gene of amylin

Chromosome location and DNA sequence

Since the pancreatic islets are the main source of full length amylin, the amylin gene is primarily expressed in pancreas, especially in islets [5]. The human amylin gene is located on the short arm of chromosome 12 in the region 12q12.3-p12.1 [6, 7]. Human amylin is encoded by the calcitonin family. The mRNA of human amylin gene is made up of three exons interrupted in the 5'-untranslated region and in the amino-terminal propeptide, by two introns, which are 330 and 4808 base pairs (bp) respectively [6]. The three exons of the human amylin gene are arranged as follows (Figure 2):

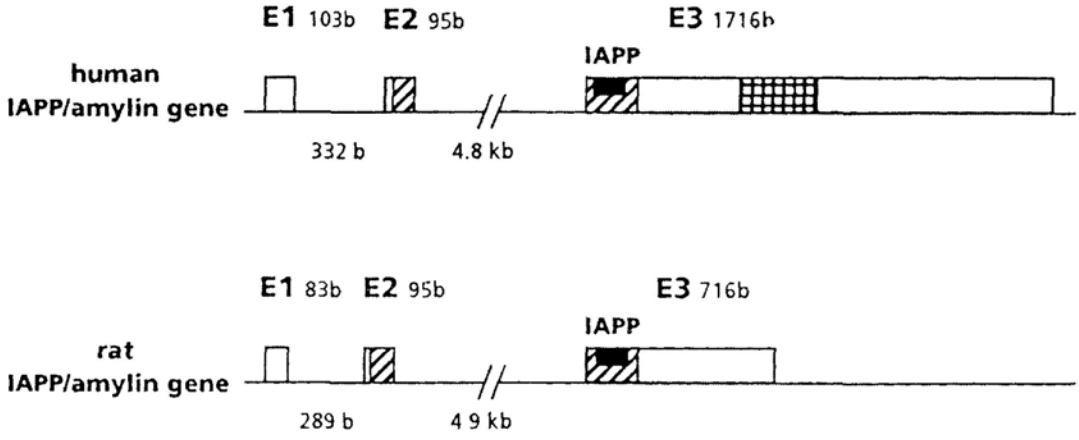

Figure 2. Comparison of gene structure of human/rat amylin.

Exons are represented by numbered boxes. Hatched parts mean protein-encoding regions, while the mature amylin peptides are shown in black. b, bases;

1) Exon 1 (104 bp) encodes most of the 5'-untranslated region of the mRNA;
2) Exon 2 (95 bp) encodes 15 nucleotides of 5'-untranslated region, the putative 22-amino acid signal peptide, and five residues of the amino-terminal propeptide;
3) Exon 3 (1246 bp) encodes the amylin moiety and its processing signals and the 16-amino acid carboxy-terminal propeptide, as well as the 3'-untranslated region of the mRNA (1059 bp) [8].

Regulatory elements of the amylin gene have been characterized in the 5' upstream flanking region within about 1,500 kb of exon 1 [9]. Studies of the human and rat

amylin genes showed similarities and differences in the structure of the 5' upstream sequences in the regulatory elements of these species. For example, the upstream sequence of human gene contains a TATA box and a sequence similar to the rat insulin enhancer [10]. In comparison, the upstream region of the rat amylin gene contains a TATA box, a CCAAT sequence, and a GT element [10]. That means CCAAT sequence and GT element are present in rat and not in human and the TATA box is shared by both species. These sequence differences may lead to different regulation of amylin gene expression in different species.

1.1.1.2 Expression and structure of amylin

Human amylin is composed of 37 amino acid residues and has a theoretical molecular mass of 3,850 Daltons [11]. Amylin is amidated on its carboxyl-terminus with a disulfide bond between the cysteine residues at positions 2 and 7 [12]. The amino acid sequence of amylin derived from islet amyloid in type 2 diabetes is identical to that found in islets of healthy humans and human insulinoma. The amylin structure exhibits close sequence homology among all species in both the amino terminal (residues 1 to 19) and the carboxyl terminal (residues 20 to29) regions. Several studies have shown that human amylin is derived from an 89-amino acid precursor, named as preproamylin. The latter is then processed to a smaller

67-residue precursor, proamylin, and subsequently to the mature amylin (Figure 3). The predicted structure of human proamylin contains small N- and C-terminal putative propeptides with typical dibasic cleavage signals on either side of amylin [8].

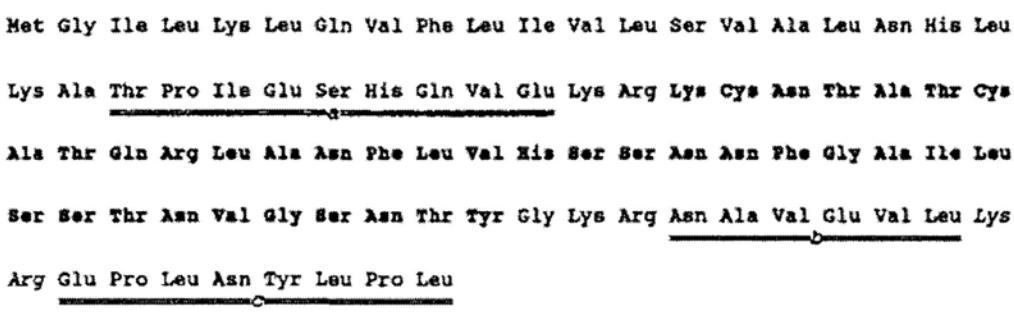

Figure 3. Predicted structure of human Preproamylin.

The sequence of human amylin is shown in bold type. Predicted N- and C-terminal propeptides are underlined.

1.1.1.3 Genetic mutations

Genetic factors are strongly associated with onset of type 2 diabetes in twin and family studies [13] [14]. Some of the target gene mutations include insulin, insulin receptor, insulin receptor substrate, glucokinase, hepatocyte nuclear factor and mitochondria, although these mutations are only found in 5-10% of young people with familial type 2 diabetes [15].

For amylin, the mutation of S20G results in a serine-to-glycine substitution at position 20 in the mature amylin molecule (S20G missense mutation). In *in vitro* studies, compared to the wild type, S20G mutation resulted in the formation of more cytotoxic amyloid which induced apoptosis in transfected COS-1 cells [16]. In type 2 diabetes, the prevalence of S20G mutation has been reported to be 2.6-4.1% in Japanese [17-19] and Chinese from Hong Kong [20, 21], Taiwan [22] and Mainland [21]. All reported cases of mutation S20G were heterozygous. While this mutation is uncommon in Asian populations, even fewer cases have been reported in Caucasians, suggesting that the the causal nature of this mutation with type 2 diabetes may be closely related to ethnicity. Although the heterozygous mutation S20G is more common in diabetic patients than control (2.6 vs. 0.9 %, $p<0.0001$), linkage analysis does not support mutation in or near the amylin gene as a common cause of Type 2 diabetes [23].

In New Zealand Maori, two mutations in the promoter region of the amylin gene (−215 T/G and −132-G/A) and a missense mutation in exon 3 (Q10R) with respective prevalence of 5.4 %, 0.76 % and 0.76 % have been reported in Type 2 diabetes [24]. The presence of the −132 G/A mutation has also been reported in Spanish subjects [25]. Despite their low prevalence, the amylin gene mutations found

predominantly in non-Caucasian populations, might contribute to the complex heterogeneity of Type 2 diabetes in these ethnic groups.

1.1.1.4 Distribution, synthesis and secretion of amylin

Amylin is synthesized in islet β cells, co-stored and co-released with insulin [2, 26]. The hormone is produced through expression of the amylin gene located on chromosome 12, transcribed as an 89-amino acid prepolypeptide, followed by cleavage to form the mature peptide in the islet β cells [1].

Human amylin was first derived from amyloid material in the pancreatic islets [1]. In addition to the pancreatic islets, different cells along the gut, predominantly in the pyloric antrum in rat, mouse, and human, also show amylin-like immunoreactivity [27]. Immunoreactivity was also reported in lesser amounts in the body of stomach, while amylin-reactive cells were found scattered from duodenum to colon in rats [27, 28]. In another histological study of gut amylin, a few amylin-positive cells were found in the basal layer of the mucosa in rats [29].

Amylin-like immunoreactivity is found widely in the central nervous system (CNS). These include a population of small to medium-sized nerve cell bodies in the dorsal

root ganglia, jugular-nodose, trigeminal ganglion and dorsal horn neurons. This reactivity is frequently co-localized with fibers containing calcitonin gene-related peptide (CGRP), substance P, and pituitary adenylate cyclase-activating polypeptide [30]. Specific amylin-like immunoreactivity has also been found in the amygdala and other central brain regions [31].

The osteoblasts have also been shown to secret amylin which may act as a local regulator of bone metabolism [32]. The trachea and lung tissues in the respiratory system also showed amylin immunoreactivity and mRNA expression [33, 34].

Apart from expressing in normal tissue, immunohistological staining of amylin can be found in pancreatic tumors including insulinomas, glucagonomas and PP-omas (pancreatic polypeptide secreting tumors) all stained positive for amylin. However, compared to normal islet cells, tumor cells of pancreatic endocrine neoplasms stain less intensively for amylin [35]. Other researchers had found amylin immunopositivity in islet-cell tumors, medullary thyroid carcinomas and pituitary adenomas [36]. Despite the presence of amylin immunoreactivity in these different sites, their functional significance remains uncertain.

1.1.1.5 Amylin receptors

The calcitonin family consists of 5 members: human amylin, calcitonin, two calcitonin-gene-related peptides (CGRP1 and CGRP2) and adrenomedullin (ADM). There is a 20% sequence homology between amylin, calcitonin and adrenomedullin, and 44% homology between amylin and CGRPs. Receptor activity-modifying proteins (RAMPs) are single-transmembrane proteins that transport the calcitonin receptor-like receptor (CRLR) to the cell surface. The calcitonin receptor-like receptor (CRLR) was identified as a CGRP receptor when coexpressed with RAMP1. Alternatively, when GRLR is co-expressed with RAMP2, it becomes a specific receptor for ADM. Calcitonin receptors (CTR) share 60% homology with the CRLR and predominantly recognize calcitonin in the absence of RAMP. The amylin receptor is recognized when a calcitonin receptor (CTR) is coexpressed with RAMP1. RAMPs are widely distributed with RAMP-1 expressed in uterus, bladder, brain, pancreas, and gastrointestinal tract. RAMP-2 is expressed in the lung, breast, immune system and fetal tissues. RAMP-3 is most abundant in kidney and lung.

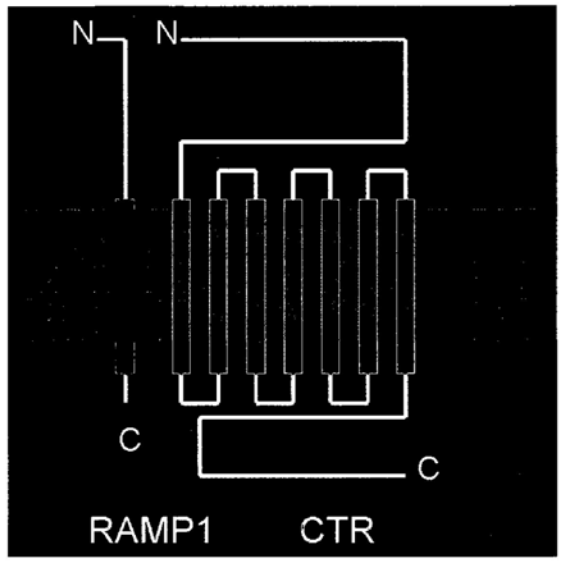

Figure 4. Amylin receptor [37].

An amylin receptor has now been fully characterized. The receptor is found at the cell surface and comprises two components: a seven trans-membrane receptor molecule and an accompanying single trans-membrane molecule called a RAMP. Both molecules must coexist at the cell surface to create a functional amylin receptor .

1.1.1.6 Amylin and insulin

Amylin is secreted in parallel with insulin following stimulation with glucose, arginine, β-hydroxybutyrate and gliclazide [38-40]. Under these conditions, the secretion curve of amylin is similar to that of insulin which includes an acute spike of secretion during the first 5 minutes after stimulation followed by a second phase which extends for at least 30 minutes during glucose stimulation.

In healthy subjects, circulating amylin rises in response to the glucose challenge. There have been few studies on the pathophysiological levels of amylin in human. In one of the studies involving 10 subjects, plasma amylin ranged from a fasting value of 2.03±0.22 pM to a post-glucose challenge value of 3.78±0.39 pM ($p<0.001$). Amylin concentrations were slightly but not significantly higher in the diabetic than nondiabetic subjects before meal ingestion (6.7±0.7 vs.4.8±2.0 pM, $p=0.3$). After meal ingestion, integrated response above basal (378±89 vs. 471±173 pM/3h, $p=0.65$) and postprandial integrated response (1578±194 vs. 1348±455 pM/3h,$p=0.66$) were similar between the diabetic and nondiabetic subjects [41, 42]. In other studies, the amylin to insulin molar ratio was similar at all time points despite high-frequency oscillations but with inter-racial differences in circulating amylin concentrations [43, 44]. In patients with obesity, hypertension, positive family history of insulin

resistance, the circulating amylin levels are increased in line with hyperinsulinaemia [45-47]. Subjects with obesity and impaired glucose tolerance (IGT) have exaggerated amylin although the amylin to insulin ratio remains consistent at all time points. In late stage type 2 diabetes and type 1 diabetes, the secretion of both amylin and insulin becomes deficient, and the response to glucose challenge is negligible which reflects the functional failure of the islet β cells [48].

Amylin is more effective than glucagon in increasing blood glucose by promoting lactate production. It releases lactate from skeletal muscle to supply gluconeogenic substrate to the liver. Amylin is also more potent than glucagon in restoring blood glucose level to resting concentration in fasting rats rendered hypoglycemic by insulin infusion [49]. On the other hand, amylin can slow gastric emptying and reduce satiety which improves blood glucose levels. Given these multiple effects of amylin, co-administration of insulin and pramlintide acetate, a synthetic analog of human amylin, offers better glycemic control in terms of reduced risk of hypoglycemia and less weight gain in both type 1 and type 2 diabetes [50].

Although amylin and insulin are co-secreted by islet β cells, these 2 peptides have different regulatory mechanisms. In experimental studies and under high glucose

condition (e.g. 16.7 mM) stimulated by either dexamethasone or glucose infusion, the relative amount of amylin to insulin secretion was higher compared to fed or fasting conditions. On the other hand, fasting tended to have the opposite effect and decreased the relative amount of amylin to insulin. In these studies, amylin and insulin secretion were generally parallel, consistent with their colocalization in the β cell secretory vesicle and co-release after glucose stimulation. However, the significant differences in the amylin to insulin ratios during low and high glucose conditions suggest that different regulatory mechanisms for amylin and insulin production in the β cell [51]. Similarly, other studies have shown that amylin mRNA, rather than insulin mRNA, was increased in rats treated with dexamethasone [52]. These data suggest that amylin might be a hormone mediated in response to stress.

1.1.2 Physiological actions of amylin

It is widely accepted that amylin, as an endocrine hormone co-secreted with insulin, can regulate fuel metabolism with metabolic, endocrine, and neural consequences, as shown in Figure 5 [53].

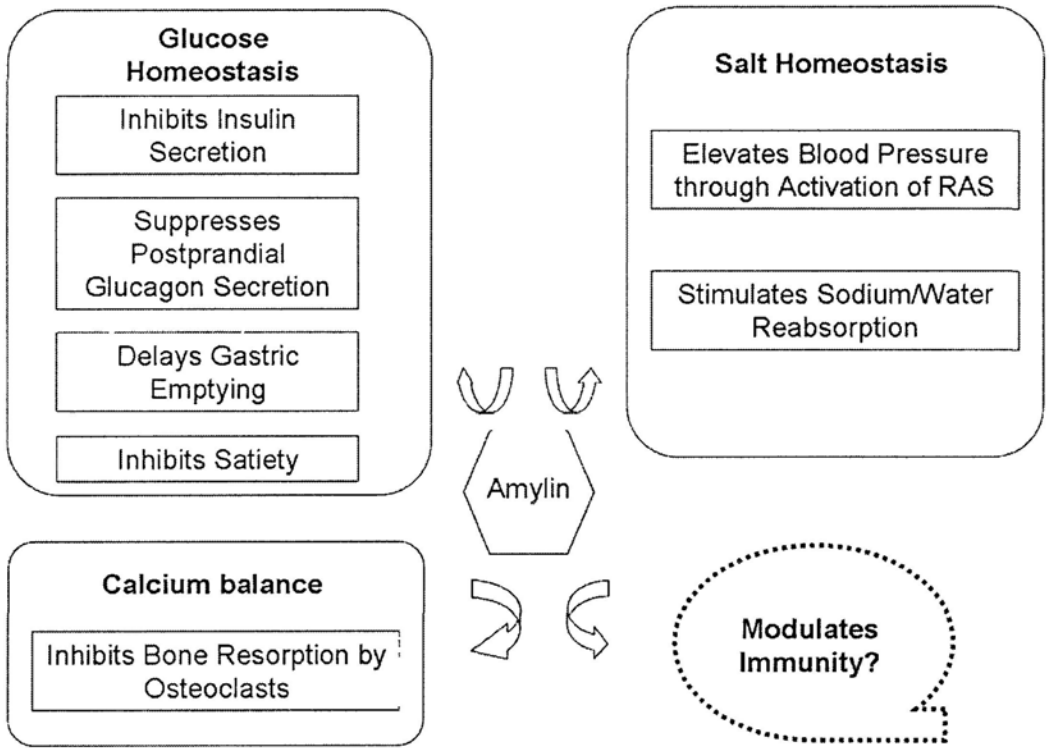

Figure 5. Pluripotent effects of amylin in endocrine, kidney, bone and cardiac vasclular systems.

1.1.2.1 Metabolic and endocrine actions

Amylin, like insulin, is lacking in type 1 diabetes mellitus [48, 54]. Amylin deficiency has been proposed as one of the mechanisms for the perturbed fuel metabolism and hypoglycemia in insulin-treated patients, possibly due to insufficient amylin signal which works in close concert with insulin [53]. Some studies have shown that amylin treatment inhibits insulin secretion from perfused rat pancreases [55-57], isolated pancreatic islets and isolated islet β cells [58]. Consistent with this finding, amylin antagonists such as human amylin$_{8-37}$ or CGRP$_{8-37}$ [59, 60] can augment insulin secretion both *in vitro* and *in vivo* [61, 62]. In addition, amylin-antiserum can potentiate arginine-stimulated or glucose-stimulated insulin secretion from isolated rat islets [63]. In hamster insulinoma cell line HIT-T15, use of amylin-antisense oligonucleotides suppressed synthesis and release of amylin with increased insulin mRNA and content [64]. Studies of male amylin knockout mice subjected to oral or intravenous glucose tolerance tests (OGTT or IVGTT) showed increased plasma insulin concentrations with a rapid plasma glucose disposal compared with the wildtype control group [65]. These results are consistent with the lack of inhibitory effect of amylin on islet β cells which can be reversed with tissue-specific recovery of amylin expression in the islet β cells. In

hIAPP transgenic mice fed with a high fat diet, the high amylin secretion might partly explain the decreased plasma insulin responses and reduced glucose tolerance after glucose loading [66].

Amylin also participates with insulin in the control of glucose homeostasis through delaying gastric emptying, slowing food transit from stomach to small intestine and modulating signals of thirst and satiety in the CNS [67]. Of note, the rate of gastric emptying is a primary determinant of postprandial rise in blood glucose. Here, human and animal models of diabetes both show accelerated rate of gastric emptying [68, 69]. In rat models, subcutaneous administration of amylin slowed gastric emptying rate in both diabetic and nondiabetic rats. The effects of amylin on gastric emptying appear to be mediated by the CNS requiring an intact area postrema. Vagus nerve also appears to be necessary for amylin to take effect [70].

1.1.2.2 Renal actions

Human amylin has been shown to activate plasma renin, stimulate sodium and water reabsorption, elevate blood pressure acutely and may act as a mitogen on proximal tubular cells [71]. The effects of amylin on the renin-angiotensin system (RAS) activity have been demonstrated in normal, hypertensive and diabetic models. For example, amylin injected into human and rat models can elicit a rapid rise in plasma renin activity. In the rat models of hypertension (SHR, the spontaneously hypertensive rat), the density of amylin-binding sites in the kidney was associated with elevation of blood pressure [72].

In the kidney, amylin binds to proximal tubules rather than distal tubules, collecting ducts, or the interstitium [73]. Amylin increased sodium/water reabsorption by 29% compared to baseline when introduced from the peritubular but not the luminal side of the proximal tubules. Ethyl isopropyl amiloride, the competitive inhibitor of the sodium/hydrogen exchanger, can block this effect [73] suggesting that the renal action of amylin may be mediated by this exchanger. Furthermore, the peptide analogue AC187, an amylin receptor antagonist, when introduced intravenously, can

decrease sodium/water reabsorption by 22%. Taken together, these results suggest amylin may play a key role in salt homeostasis.

Amylin also acts as a growth factor in cultured renal cells. It stimulates proliferation of primary cultures of epithelial cells isolated from proximal tubules. This effect can be inhibited by amylin antagonist that inhibits amylin binding [73].

1.1.2.3 Bone actions

Amylin has calcitonin (CT)-like and osteoclast-inhibitory effects. Thus amylin reduces bone resorption and circulating calcium level, although it is 30-40 times less potent than calcitonin in these effects [74]. In some studies, intravenous administration of amylin results in hypocalcemia in rats and rabbits [75]. Amylin may act like a potential paracrine regulator of osteoclast function since amylin has been found to be produced and secreted by an osteoblast cell line [32]. These findings support an important role of amylin, possibly working in concert with CT, in bone metabolism.

1.1.2.4 Vascular actions

Amylin is a potent vasodilator and causes systemic hypotension and tachycardia

when injected intravenously. Amylin (10^{-10}M) can induce relaxation of norepinephrine-precontracted rat aortic rings by more than 50%. This amylin-induced vasodilation is an endothelium-independent process not mediated by nitric oxide (NO) [76]. In addition, amylin may modulate pulmonary vascular tone and induce a dose-dependent and time-reversible endothelial-dependent relaxation of preconstricted pulmonary artery rings. Amylin can also reduce the vascular tone in isolated, perfused and ventilated rat lung [77].

1.1.2.5 Immune modulation

Dysregulated β cell function with hyposecretion of amylin relative to insulin has been described in non-obese diabetic (NOD) mice. The latter is a murine model characterized by autoimmune destruction of islets and deficient β cells, although it is unknown whether amylin hyposecretion is a cause or consequence of the autoimmune β cell destruction. Interestingly, amylin has been suggested as a candidate autoantigen in NOD mouse. An islet-specific CD4+ T-cell clone from these mice recognize a unique autoantigen mapped to the telomeric region of mouse chromosome 6 where the amylin gene resides [78]. Another study has shown that preproamylin 5-13 is a novel HLA class I epitope recognized by cytotoxic T cells from patients with recent-onset type 1 diabetes [79].

1.1.2.5.1 Eosinophils

In the late 90s, an *in vitro* study suggested that human amylin might have immunomodulating effects by augmenting cytokine secretion from eosinophils [80]. Eosinophils are granulocytic cells which can mediate a wide range of acute end-stage inflammatory activities. It may cause extensive cell or tissue damage. Studies have shown that eosinophils are able to release a variety of cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-8 [81]. In this study, human amylin but not rat amylin inhibited the *in vitro* IL-5-mediated survival of cord blood-derived eosinophils (CBEs). Although human amylin alone could not stimulate CBEs to release cytokines, it can increase the release of the GM-CSF by CBEs, the latter being stimulated by the calcium ionophore A23187. In support of its amplifying effects of the eosinophilic inflammatory activities, human amylin can also augment the A23187-induced release of the inflammatory lipid mediator leukotriene C4 by CBEs [80].

1.1.2.5.2 Amylin and neuropeptides including amyloid β (Aβ)

There are several lines of evidence suggesting that human immune response is controlled in a tissue-specific and localized manner by neuropeptides. Indeed, targeted expression of the neuropeptide calcitonin gene-related peptide (CGRP) to the pancreatic β cells prevents autoimmune diabetes in NOD mice [82]. Not only does amylin share 46% amino acid sequence homology with CGRP, it also has a biological activity profile similar to that of CGRP [8]. Of note, a portion of the B-chain of insulin is strongly homologous to these two peptides [37] with the islets of Langerhans synthesizing and secreting both insulin and amylin [2]. Coexpression of receptors for amylin, CGRP and adrenomedullin was found in the pancreatic β cells and all three peptides could inhibit insulin secretion [83]. Therefore, amylin and CGRP, while belonging to the calcitonin family, are also related to the insulin gene superfamily of peptides and may interact with insulin as an antagonist.

The amyloidogenic peptides, amyloid β (Aβ) and human amylin, are the major constituents of amyloid deposits. These two peptides are found in patients with the chronic degenerative disorders, Alzheimer's disease (AD) and type 2 diabetes, respectively. Both Aβ and amylin share a common crossed β-fibril structure although

both peptides have very little sharing in sequence homology. Lipopolysaccharide (LPS)-treated THP-1 (Human acute monocytic leukemia cell line) showed significant increases in mature interleukin (IL)-1β release 48 h following Aβ or human amylin treatment but not with nonfibrillar rat amylin. LPS-stimulated THP-1 cells treated with Aβ or amylin also showed increased release of the proinflammatory cytokines, tumor necrosis factor (TNF)-α and IL-6, as well as the chemokines IL-8 and macrophage inflammatory protein-1α and -1β. These studies suggest that fibrillar amyloid peptides such as amylin and Aβ can induce signal transduction pathways that initiate an inflammatory response [84]. In support of the immunomodulating role of amylin, in various animal models of inflammation, amylin has been found to be active against mouse ear oedema induced by croton oil and acetic acid-induced peritonitis in the rat [85].

1.1.3 Transgenic animal models of human amylin (hIAPP)

Islet amyloid has long been implicated in the pathogenesis of type 2 diabetes. The islet β cell-secreted amylin was found to be the main component of islet amyloid deposits. Since pancreatic biopsies are ethically unacceptable, animal models including hIAPP transgenic mice, rats, or pigs were generated to study the relationship between type 2 diabetes and islet amyloidosis.

1.1.3.1 Transgenic rodent models overexpressing hIAPP

To address the relationship between islet amyloid and type 2 diabetes, different colonies of transgenic mice expressing the gene for human amylin have been created by a number of groups [86-89]. Some colonies do not develop diabetes spontaneously and islet amyloid deposition occurs rarely even in very old mice [89, 90]. Various strategies including increased dietary fat [90] or treatment with steroids or hormones [86] or by co-expression of obesity genes [91, 92] were used to induce amyloid deposition and diabetes in these animals. Jason J *et al* developed a homozygous hIAPP transgenic mouse model which spontaneously developed diabetes mellitus by 8 weeks of age. However, amylin derived amyloid deposits were found in only a minority of islets at approximately 20 weeks of age [93]. These findings suggest that amylin oligomers rather than fibrils may cause diabetes.

The hIAPP transgenic mice used in our study were a kind gift from Dr. Steven E Kahn's group (Seattle, University of Washington). These mice were originally produced and maintained on a C57BL/6J $\times$DBA/2J (F1) genetic background. The transgene was composed of a fragment of hIAPP cDNA linked to the rat insulin II promoter. The β cells of these transgenic mice produce and secrete amylin in

amounts 2-3 times greater than the native (mouse) peptide. The plasma concentration of insulin and glucose were similar between transgenic and control mice [88, 94]. However, islet amyloid developed in these mice when they were fed with diet containing moderate amounts of fat (9% instead of 4.5% wt/wt) or interbred with mice carrying genetic mutations (A^{vy}/a or ob/ob) associated with β cell dysfunction [91, 92].Consistent with data published by Dr. Kahn [88], we also did not find islet amyloid in these mice up to 12 month old and that none of these mice developed diabetes when fed by normal chow (see later chapters).

1.1.4 Amylin in diabetes

Amylin deficiency and diminished amylin response to a meal challenge have been reported in advanced stages of both type 1 and type 2 diabetes [95, 96].

1.1.4.1 Amylin in type 1 diabetes

Autoimmune diabetes in both humans and mice exhibits 3 clearly distinguishable stages: (1) a silent insulitis phase which is accompanied by circulating autoantibodies targeted to various pancreatic constituents; (2) a clinical phase of insulitis characterized by infiltration of autoreactive T lymphocytes and other inflammatory cells into the islets and (3) an overt diabetes phase with extensive

destruction of ß-cells, deficient insulin production and hyperglycemia. Phase 1 of silent insulitis can persist for long period of time, suggesting that immunoregulatory control mechanisms are able to keep autoreactive T cells in check before metabolic decompensation [97].

Insulin-deficient animal models showed reduction or absence of amylin, irrespective of whether insulin deficiency was induced chemically using streptozotocin [98-101] or by autoimmune β-cell destruction such as BB rats [54]. Several studies have shown hyposecretion of amylin relative to insulin in NOD mice, a murine model with autoimmune destruction of islet β-cells [102]. The molar ratio of amylin/insulin secretion declined at 6-9 weeks and continued to decline with advancing insulitis in NOD mouse. In the early prediabetic phase, relative hypersecretion of insulin in relation to amylin might occur [102]. In type 1 diabetic patients, both pancreatic amylin content [103] and plasma amylin concentrations were low (e.g. 0.7 pM) [42] or undetectable [104], even after nutrient stimulation [105] [106]. Children with type 1 diabetes also had absent amylin secretion [107]. Taken together, type 1 diabetes is characterized by pancreatic β-cell deficit with both amylin and insulin deficiencies.

1.1.4.2 Amylin in type 2 diabetes

Type 2 diabetes is characterized by up to 60% reduction in β cell mass and islet amyloid formation [108, 109]. Compared to age-matched control and based on autopsy findings, type 2 diabetic cases had increased frequency and larger areas of amyloid deposit per islet [110]. Amylin is the major component of islet amyloid although it remains unclear whether this is due to deposition of mature human amylin fibrils [111-113] or their oligomeric precursors [114, 115].

There are plausible pathogenetic roles of amylin in type 2 diabetes: 1) Increased amylin secretion may inhibit insulin secretion and increase insulin resistance in skeletal muscle and liver. 2) Islet amyloid deposits may form a barrier which physically disrupts transport of glucose into β cells and prevents trafficking of insulin from β cells into the islet capillaries. 3) The extracellular islet amyloid deposits may cause physical damage to β cells with loss of biological functions [116].

Secretion of both amylin and insulin is elevated in human subjects with insulin resistance but decreased in patients with islet failure [117, 118]. However, in type 2 diabetes, there is relative hyposecretion of amylin compared to insulin [105]. While

the physiological significance of this relative deficiency is not clear, maintaining a fixed ratio of amylin to insulin (approximately 1:20) provides more precise blood glucose maintenance in both type 1 and type 2 diabetes compared to insulin alone [74].

1.2 Immune modulation

Our immune system is developed to attack not only exogenous pathogenic micro-organisms such as bacteria, viruses, fungi and parasites but also, endogenous pathogens such as tumors. There are 2 main types of immune response. The **innate immune response** is mediated mainly by immune cells and cytokines which defend the host against foreign organisms in a non-specific manner. Phagocytes including macrophages or dendritic cells (DC, see later) act as antigen presenting cells (APC). These cells are activated by binding of foreign substances to Toll-like receptor (TLR) expressed on their cell surfaces. Apart from causing cytokine release, these activated APCs can modulate the activity of the regulatory T (Treg) and effector T (Teff) lymphocytes which belong to the adaptive immune system. The **adaptive immune response** is mediated mainly through B lymphocytes and T lymphocytes. These are highly specificialized cells which recognize a particular pathogen (or antigen) with

memory function to prevent or mitigate the severity of recurring disease. The main function of B cells is to protect the host by producing antibodies that identify and neutralize foreign objects like bacteria and viruses. T cells have a wider range of activities which include the control of B lymphocyte development and antibody production. Another group of T lymphocytes interacts with phagocytic cells to help them destroy engulfed pathogens. A third set of T lymphocytes recognizes and destroys virus-infected cells.

1.2.1 Phenotypic characterization of T regulatory cells

CD is an antigen which represents 'Cluster of Differentiation' protein'. CD3 forms part of the T cell receptor (TCR) complex expressed by a mature T lymphocyte. Thus, all T lymphocytes are CD3 positive cells. CD4 (cluster of differentiation 4) is a glycoprotein expressed on the surface of T helper (Th) cells, Treg cells, monocytes, macrophages, and dendritic cells (DC). CD25, the interleukin-2 receptor alpha chain, is widely used to define CD4+ Treg cells. For instance, Stephens, L. A *et al* indicated that CD25 was a very specific marker for activated CD4 (+) Treg cells in rats in *in vitro* and *in vivo* functional studies [119]. However, more recent research suggests that Treg cells are defined by expression of the forkhead family transcription factor Foxp3 (forkhead box p3). Expression of Foxp3 is required for

development of Treg cells and appears to control a genetic program specifying the fate of this cell lineage. The Treg cells belong to the cell lineage which expresses the MHC class II antigen with the majority of the Foxp3-expressing Treg cells also expressing CD4 and CD25.

1.2.2 Dendritic cells, toll-like receptors and immune regulation

Dendritic cells (DC) are phagocytic cells present in tissues in close contact with the external environment, mainly the skin and inner mucosal lining of the nose, stomach and intestines. They are very important in the process of antigen presentation and serve as a link between the innate and adaptive immune systems. TLRs are critical components of the innate immune system to detect microbial antigens, activate APC and induce adaptive immune responses. The innate system uses pathogen recognition receptors (PRRs) to identify a limited set of conserved molecules present on foreign microbes. Among these PRRs, toll-like receptors (TLRs) are the most important set. TLRs recognize distinct molecular patterns characteristic of different classes of pathogenic organisms. For example, TLR2 recognizes peptidoglycan found in Gram-positive bacteria, while TLR4 recognizes lipopolysaccharide (LPS) [120]. Although the primary objective of TLR expression on DCs is to activate innate immune inflammatory responses, TLRs also participate

in shaping subsequent adaptive immune responses through their expression on the DCs that control T-cell activation.

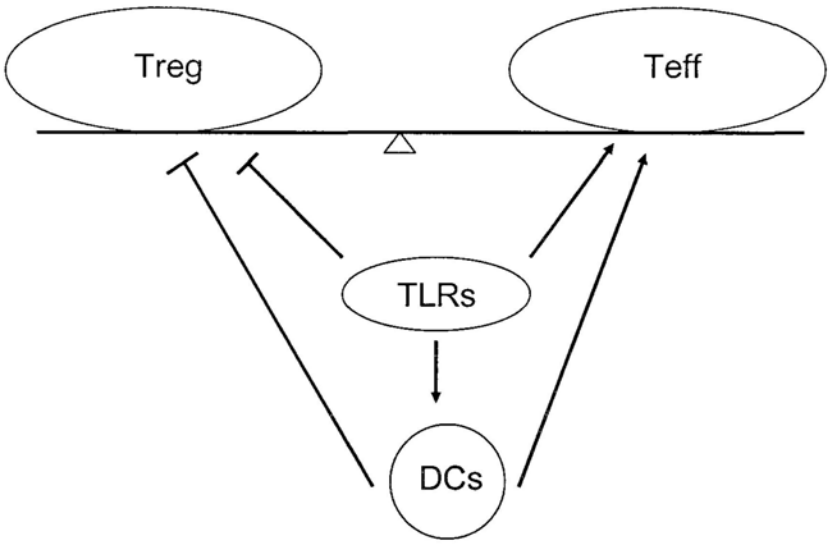

Figure 6 Relationship between Treg cells, Teff cells and dendritic cells.
Toll-like receptors (TLR), expressed on antigen presenting cells (APC) such as macrophages, recognize foreign particles e.g. virus and bacteria and cause direct cytotoxic responses. On the other hand, TLRs expressed on dendritc cells (DC) can modulate the development of regulatory T (Treg) and effector (Teff) cells and balance their 'cytotoxic' (Teff) and 'self-recognizing' (Treg) activity. Proliferation of Teff cells is usually regulated by Treg cells so that suppressed function of Treg cells can lead to the proliferation of Teff cells.

1.2.3 Innate immunity and Treg cells in inflammatory diseases

The innate immunity enables the host to respond to foreign organisms rapidly without the need of prior adaptive responses [121]. Innate immune effector mechanisms are implicated in many inflammatory diseases. For example, activated monocytes and macrophages which secrete a large amount of TNF-α, play a central

role in inflammatory responses in the joints of patients with rheumatoid arthritis (RA). Interestingly, there is an abundance of CD4+CD25+ Treg cells capable of suppressing proliferation of CD4+ Teff cells in the inflamed joints of patients with RA [122]. In addition, CD4+CD25+ Treg cells can inhibit innate immune pathology in the murine models of inflammatory bowel disease (IBD) triggered by bacterial infection with Helicobacter hepaticus (*H. hepaticus*) [123]. Other immune-suppressive cytokines such as TGF-β and IL-10 produced by CD4+CD25+ Treg cells are also important in controlling innate immune pathology. For example, *H. hepaticus* infection can trigger the development of intestinal inflammation mediated by cells of the innate immune system and pro-inflammatory cytokines. There is characterized by infiltration of neutrophils and monocytic cells in the intestinal lamina propria and spleen [124]. Transferred CD4+CD25+ Treg cells can completely suppress the activation of these innate immune responses in the intestine and spleen [124].

1.2.4 T reg cells in autoimmunity

While these immune responses are developed primarily to destroy foreign antigens such as pathogens, the destructive processes often involve both pathogens and host. Thus, it is logical to assume that there are regulatory mechanisms which maintain

immunohomeostasis to prevent self harm, so called 'immune tolerance'. This is mainly achieved through clonal deletion of destruction of most self-reactive T cells in the thymus [125, 126]. In normal subjects, there are potentially hazardous self-reactive lymphocytes in the peripheral blood stream. In recent years, CD4+CD25+ Treg cells have been identified as a key player in maintaining immunological homeostasis and conferring self-tolerance [127, 128] [129, 130]. These cells are actively involved in the negative control of inflammation, organ rejection and tumor immunity as evidenced by their ability to suppress immune responses directed against tumor or non-host antigens. One of the strongest evidence comes from the onset of polyautoimmune syndrome in some mouse strains that underwent thymectomy three days after birth [131, 132]. In addition, PVG.RT1^{u} adult rats develop thyroiditis and insulin-dependent diabetes following thymectomy and sublethal irradiation [133]. In these cases, autoimmunity can be prevented by administration of selected Treg cells from nonthymectomized animals. In NOD mice which is a mouse model of type 1 diabetes, thymectomy [134] or administration of cyclophosphamide [135] at 3-week of age can accelerate the onset of diabetes. However, diabetes can be inhibited in the immuno-incompetent hosts when administrated with Treg cells. In addition, induction of Treg cells can nonspecifically inhibit onset of diabetes after some infections, such as mycobacteria

[136], viruses [137] and parasites [138]. In the same vein, enriched CD4+CD25+ Treg cells from normal mice can suppress allergy and induce tolerance to organ grafts in graft-versus-host disease after bone marrow transplantation [139]. This evidence strongly supports the role of Treg cells in the control of autoimmune disease and inflammatory diseases (Figure 6). Based on this premise, it can be argued that reduction of CD4+CD25+ Treg cells or attenuation of their suppressive activity of 'self destruction' can elicit immune responses such as autoimmunity, tumor immunity, microbial immunity and allergy. By inference, increased number of Treg cells or augmentation of the suppressive activity of Treg cells may induce transplantation tolerance, maintain feto-maternal tolerance as well as prevent autoimmunity, allergy and cancer.

1.2.5 TLR and autoimmunity

TLR may directly or indirectly regulate the immunosuppressive function of Treg cells in autoimmune and infectious diseases [140, 141]. Recent studies have shown that TLR2-deficient mice have reduced number of Treg cells [142]. Similarly, engagement of TLR-4 or TLR-9 on freshly isolated mouse splenic DCs can significantly abrogate the immuno-suppressive function of CD4+CD25+ Treg cells [143]. Activation of TLRs expressed by macrophages can lead to release of various

cytokines such as IL-6, TNF-α, etc [144]. On the other hand, engagement of TLRs expressed on DC is required for DC maturation, secretion of cytokines and antigen presenting ability which can indirectly control the immunosuppression function of Treg cells [145]. Taken together, TLR may play an important role in immune regulation by modulating the function of Treg cells, both directly and indirectly (Figure 7).

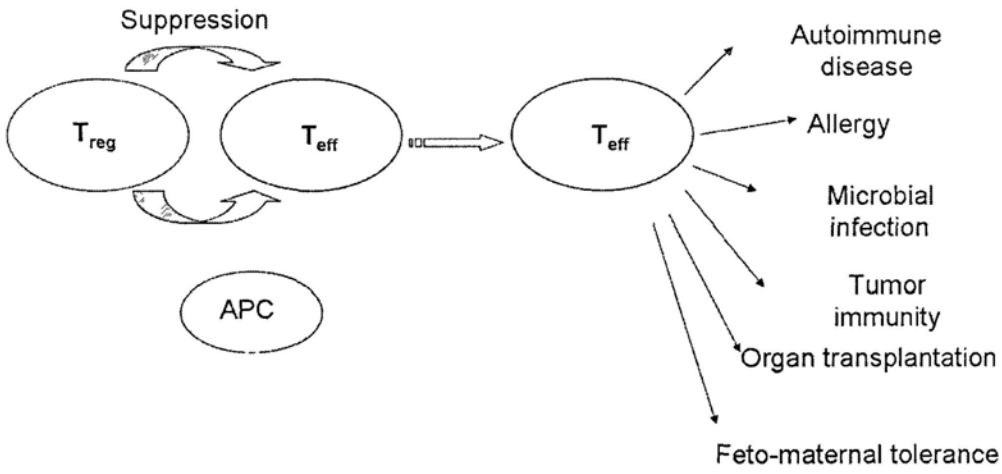

Figure 7. Peripheral tolerance induced by Treg by suppressing Teff cells. Expression of toll like receptors (TLRs) on some dendritic cells (DC) can shape the adaptive immune responses by modulating the activity of regulatory T cells (Treg) and effector T (Teff) cells The balance between the activity of the Treg cells (which confer self recognition) and Teff cells (which activate targeted immune responses) can lead to various clinical conditions including autoimmunity, tumor immunity, organ rejection, allergy and maternal-fetal recognition.

1.2.6 Immune modulating cytokines in autoimmune diabetes

Type 1 diabetes is caused by autoimmunity-mediated β cell destruction. Both inflammatory mediators such as cytokines and activated autoreactive T cells may

contribute to β cell destruction in type 1 diabetes.

1.2.6.1 Transforming growth factor-β (TGF-β)

In the spontaneously diabetic NOD mice model, Tregs have been shown to suppress T cell immunity in a TGF-β dependent manner. The immunoregulatory role of TGF-β was first elucidated after induction of oral tolerance to different autoantigens such as myelin basic protein in Experimental Allergic Encephalomyelitis (EAE) [146] and insulin in NOD mouse [147]. Transgenic expression of porcine TGF-β1 in pancreatic insulin-secreting β cells [148] and glucagon-secreting α cells [149] has been shown to protect NOD mice against onset of diabetes. In addition, transgenic expression of TGF-β in islet-infiltrating Treg cells can provide similar protection by suppressing anti-islet pathological T cells [150]. In an adoptive transfer experiment involving prediabetic mice, the protective effect conferred by Tregs against spontaneous diabetes was abolished by anti-TGF-β treatment [151].

1.2.6.2 Tumour necrosis factor-α (TNF-α)

TNF-α is produced predominantly by activated macrophages but also expressed by lymphocytes, natural killer cells, mast cells, endothelial cells, fibroblasts, and microglial cells [152]. It is a cytokine with pleiotropic effects implicated in many

inflammatory diseases. Studies of NOD mice, a typical model of type 1 diabetes, have demonstrated the protective effects of TNF-α against autoimmunity. Injection of recombinant TNF-α resulted in suppression of insulitis and prevention of spontaneous diabetes in NOD mice [153, 154]. The administration of TNF-α not only protected against spontaneously developing diabetes, but also adoptively transferred diabetes. The latter involved the intravenous injection of spleen cells from acute diabetic mice into irradiated recipient mice which then developed diabetes [154]. In addition, TNF-α expression in the pancreas can prevent onset of type 1 diabetes in the NOD mice by abrogating development of islet specific pathogenic Teff cells [154]. Apart from type 1 diabetes, immune dysregulation of TNF-α has been found in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [155]. Mice lacking TNF-α [156] or those lacking TNF-receptor 1 (TNFR1) [157] develop collagen-induced arthritis (CIA), with much lower frequency compared to wild type mice. Taken together, TNF-α can be considered as an anti-inflammatory cytokine in autoimmune disorders such as Type 1 diabetes and RA.

1.2.6.3 Interleukins and other cytokines

Type 1 diabetes is characterized by dysregulation of immune system. Here,

pro-inflammatory cytokines such as IL-6 secreted by CD8+ cytotoxic cells are considered pathogenic, while anti-inflammatory cytokines such as TGF-β secrected by Treg cells are protective. However, many of these cytokines have pleiotropic effects which can possess both pro and anti-inflammatory effects depending on the triggering events and clinical course [158, 159].

Amongst these cytokines, IL-6 is considered a pro-inflammatory cytokine implicated in many immunological diseases. It is a multifunctional cytokine which can regulate immune response, acute phase response and inflammation. Acute phase response consists of local vasodilation and migration of macrophages with release of cytokines (e.g. TNF-α and IL-6), platelet activation, leukocyte infiltration culminating in cell death. This is then followed by cellular repair and fibrous formation, often mediated by other cytokines (e.g. TGF-β) as part of a healing process [160]. In both naturally-occurring and experimentally-induced autoimmune diseases such as RA, increased amount of IL-6 can be found in synovial fluids from affected patients [161]. IL-6 is also found to be produced by immune cells that infiltrate the islets of Langerhans of NOD females at all ages [162]. Depletion of IL-6 by neutralizing antibodies significantly reduced the incidence of type 1 diabetes in NOD mice [163] and/or BB rats, both of which are type 1 diabetes animal models

[164]. Other autoimmune diseases where IL-6 may play pathogenetic roles include chronic inflammatory proliferative disease (CIPD), B cell lymphoma, systemic lupus erythematosus (SLE), Castleman's disease and plasmacytoma/multiple myeloma [160].

1.2.7 Dysregulation of immunity in diabetes

1.2.7.1 Defects of Treg cells in type 1 diabetes

Type 1 diabetes is characterized by progressive and selective destruction of insulin-producing pancreatic β cells by autoreactive T lymphocytes. Although the pathogenesis of autoimmune type 1 diabetes has been studied extensively, the exact mechanisms in the initiation and progression of β cell destruction remain unknown.

In the NOD mice, the first sign of activation of autoreactive T cell occurs only at 3-4 weeks of age, heralded by the intra-islet inflammatory infiltration followed by islet-cell destruction and overt diabetes by the age of 3-5 months. The delayed onset of autoimmune processes and pre-diabetic phase may be due to CD4+ Treg cells countering the anti-islet immune response [165]

Other researchers have reported that NOD mice might have a generalized defect in

their ability to generate effective numbers of Treg cells. The percentage of Treg cells in NOD is approximately half that of other autoimmune resistant strains of mice, [166, 167]. The working model in the NOD system is that Treg cells actively suppress the anti-islet response by Teff cells but ultimately this suppression is insufficient to maintain immune homeostasis [166].

Treg cells are also present in human. Human Treg cells share similar features with that of mouse models [168, 169]. In human, the central immune organs such as the thymus play important roles in the maturation of T lymphocytes. Newly diagnosed type 1 diabetic patients have reduced Treg cells in the periphery but the *in vitro* suppressive ability of these Treg cells on cytotoxic T cells has not been tested [170].

1.2.7.2 Association between Toll-like receptors and diabetes

Toll-like receptors (TLRs) are expressed on APC such as macrophages and recognize molecular patterns relating to a variety of microbial infections. Stimulation through TLRs leads to activation of APC culminating in production of inflammatory cytokines and type 1 interferons (IFNs) which include IFN-α and IFN-β. Activation of TLRs can also modulate the immune responses mediated by Treg and Teff cells. In this connection, inhibition of TLR-4 can block inflammatory

pathways with prolonged islet allograft survival [171].

1.2.7.3 Linkage between metabolic and immune systems

Apart from microbial agents, TLRs, especially TLR-4, is also a sensor for endogenous lipids and free fatty acids, thus linking metabolic with immune systems [172]. Of note, TLR-4-deficient mice became obese due to overeating suggesting activation of TLR-4 may influence appetite and energy balance. Furthermore, there is an increased expression of TLR-4 in fat tissue from obese and diabetic mice. As such, many proinflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α, monocyte chemo-attractant protein (MCP-1) and macrophage migration inhibitory factor, which are closely linked to insulin resistance, are expressed by white adipose tissue [173]. In adipocytes, long chain free fatty acids and LPS can activate TLR-4 signaling pathway, leading to the expression of inflammatory mediators such as IL-1 and IL-6 which can decrease peripheral glucose uptake and glycogen synthesis as well as promote gluconeogenesis [174].

1.2.7.4 Inflammatory cytokines in type 1 and type 2 diabetes

Both autoimmue type 1 diabetes and diabetic complications share common features of inflammation and oxidative stress. C reactive protein (CRP), vascular cell adhesion molecule-1 and nitrotyrosine are increased in diabetic patients with microvascular complications [175]. Similarly, increased release of interleukin (IL)-1β and superoxide anions by monocytes have been reported in type 1 diabetes [175]. In normal subjects, increased inflammatory markers predict type 2 diabetes [176], suggesting that inflammation may occur early during IGT. In a prospective, nested case-control study of healthy, middle-aged women in the United States, inflammatory markers, IL-6 and CRP within the highest quartiles predicted new onset of type 2 diabetes over a 4-year period with a relative risk of 7.5 for IL-6 (95% confidence interval [CI], 3.7-15.4) and 15.7 for CRP (95% CI, 6.5-37.9) compared to the lowest quartile [177]. These findings are similar to those in the Monitoring of Trends and Determinants in Cardiovascular Diseases study of healthy, middle-aged men, in whom CRP concentrations in the highest quartile was associated with increased risk of type 2 diabetes over a 7-year period [178]. Taken together, these data suggest close linkages between innate immunity, autoimmunity, obesity, insulin resistance and type 2 diabetes although the exact nature of these associations

remains to be elucidated.

1.2.7.5 Pancreatic lymph nodes and diabetes

Type 1 diabetes results from autoimmune destruction of pancreatic islet cells which is mediated by immune cells such as T cells and APC. The first stage of disease, known as insulitis, is marked by leukocyte invasion of the pancreatic islets. This is followed by the second stage characterized by overt diabetes, with massive destruction of islet β cells and loss of glucose homeostasis [179]. Pancreatic lymph nodes (PLNs) and to a lesser extent, the spleen, are the first two organs to be infiltrated by leukocytes [180]. β cell antigens are transported specifically to PLNs where they trigger reactive T cells to invade the islets [181]. Here, PLNs are required for priming of T cells cytotoxic to β cell, at least in NOD mice. Excision of PLNs at 3 weeks protected mice against development of insulin autoantibodies (IAAs), insulitis, and diabetes almost completely [182]. Experimental studies have shown that expanded T cells from PLNs of type 1 diabetic subjects recognize an insulin epitope [183], suggesting that insulin may be an autoantigen. Simiarly, over expression of insulin messenger RNA in the PLNs of NOD mice during early age is associated with islet autoimmunity [184]. On the other hand, oral administration of insulin can induce the presence of Treg cells in the pancreas and corresponding

draining lymph nodes of NOD mice. This microenvironment favors the secretion of IL-4 which can suppress the activity of Th1 (T helper cells) which are autoreactive T cell clones, thereby conferring protection against autoimmune diabetes [185]

1.3 Questions and hypothesis

1.3.1 Rationale

In my extensive review of literature, there are several lines of evidence suggesting an important immunomodulating role of amylin and the intimate relationships between innate and adaptive immunity in the pathogenesis of type 1 and type 2 diabetes which have formed the premise of my thesis:

1. Hyposecretion of amylin relative to insulin has been reported in Type 1 diabetes and advanced type 2 diabetes.
2. Defective CD4+CD25+ Treg cells have been reported in patients with type 1 diabetes and NOD mice.
3. Chronic inflammation characterized by increased release and action of proinflammatory cytokines are implicated in insulin resistance and type 2 diabetes.
4. There are close linkage between, metabolic, innate and adaptive immune

systems in the activation of various cytokines and autoreactive T and B cells.

5. Review of literature suggests pluriopotent effects of amylin which may have modulating effects on immune function

6. During the examination of the pancreas of hIAPP mice, I observed marked reduction in PLN which is known to play a key role in autoimmunity.

1.3.2 Working hypothesis

Based on these premises, I hypothesize that amylin may modulate autoimmune responses, in part mediated by the innate immune system, by suppressing TLR and increasing Treg cells which are known to suppress the cytotoxic activity of autoreactive T cells (Figure 8).

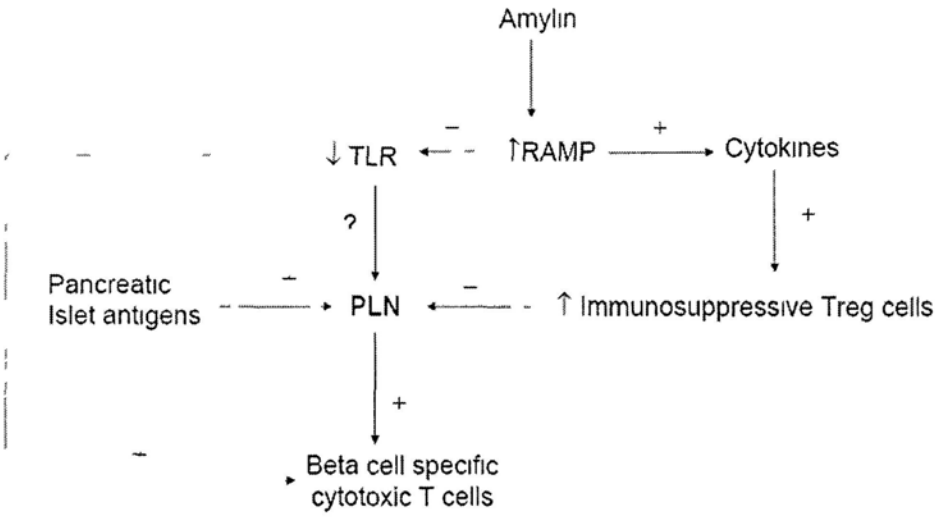

Figure 8 Working hypothesis.
Human amylin may cause upregulation of the receptor activity modifying proteins (RAMPs) to modulate both the innate and adaptive immune response including 1) release of cytokines; 2) expansion of Treg cells and 3) suppressed TLR expression with an overall consequence of reduced formation of pancreatic lymph nodes (PLNs) where autoreactive cytotoxic T cells are primed and developed to initiate the onset of insulitis and β cell destruction.

1.3.3 Research questions

In this study, I used hIAPP transgenic mice to investigate the possible effects of amylin on metabolic and immune responses and asked the following questions:

1. What are the age-related changes in plasma levels of glucose, amylin and insulin in hIAPP transgenic mice?
2. What are the morphological changes of PLN in hIAPP transgenic mice?
3. What are the effects of human amylin on CD4+CD25+ Treg cells?
4. What is the changed expression level of toll-like receptor-4 (TLR-4) in the spleen of hIAPP transgenic mice?
5. What are the expression levels of immune-modulating cytokines such as TNF-α, TGF-β, and IL-6 in the spleen of hIAPP transgenic mice?
6. What are the expression levels of amylin receptor activity modifying proteins (RAMPs) in pancreas and spleen of hIAPP transgenic mice?

2

Materials and Methods

2.1 Animal Studies

2.1.1 hIAPP transgenic mice model as a model for type 1 diabetes

Several hIAPP transgenic mice strains have been developed to study the role of islet amyloid formation in the pathogenesis of type 2 diabetes. However, due to differences in genetic background strains and dietary intake, these transgenic mice can have different phenotypes. In order to investigate the role of amylin metabolism and amyloid deposits in human type 2 diabetes, some researchers cross-bred hIAPP transgenic mice maintained on FVB or C57BL/6 genetic background with mice carrying genetic mutation (A^{VY}/a or ob/ob) known to be associated with β cell dysfunction, obesity and insulin resistance [91, 92]. Other researchers employed a hIAPP homozygous transgenic mouse model characterized by progressive β-cell loss due to increased β-cell apoptosis [186], a model which closely resembles the islet pathology in humans with Type 2 diabetes [108]. In my experiment, I used the mice which originated from Steven Kahn's laboratory, which were generated by intercrossing C57BL/6J female mice with the hIAPP transgene with DBA/2J wild-type male mice. Table 7 summaries the metabolic features of hIAPP transgenic mice model developed by different research groups.

Group Leader	Background	Phenotype	Diet	Genetic
Garth J.S.Cooper	FVB/N	Develop diabetes	Diet 86 with 3% fat	Hemı/homozygous
Steven E. Kahn	C57BL/6J*DBA/2J	Develop diabetes with islet amyloid	9% high fat diet	Hemizygous
Perter C. Butler	FVB/N	Develop diabetes spontaneously	Diet 5001 with 4.5% fat	Homozygous
Ours	C57BL/6J*DBA/2J	Do not develop diabetes	Diet 5001 with 4.5% fat	Hemizygous

Table 7. hIAPP transgenic mice model developed by different research group.

2.1.2 Breeding and feeding of hIAPP mice

2.1.2.1 Breeding of hIAPP mice

Five hemizygous transgenic mice expressing human amylin in their pancreatic β-cells were kindly given to our laboratory by Dr. Steven Kahn at the VA Puget Sound Health Care System, Seattle, WA, USA to facilitate this project. Breeding was conducted in the Laboratory Animal Services Centre at the Chinese University of Hong Kong. The hIAPP transgenic mice and their nontransgenic littermates were generated by breeding hIAPP transgenic C57BL/6 female mice with DBA/2J wild-type male mice (Figure 9) [88]. The transgene consists of a fragment of the hIAPP cDNA linked to the rat insulin II promoter. Transgenic status was determined by polymerase chain reaction (PCR) of genomic DNA using primers directed against the hIAPP transgene, as previously described [187].

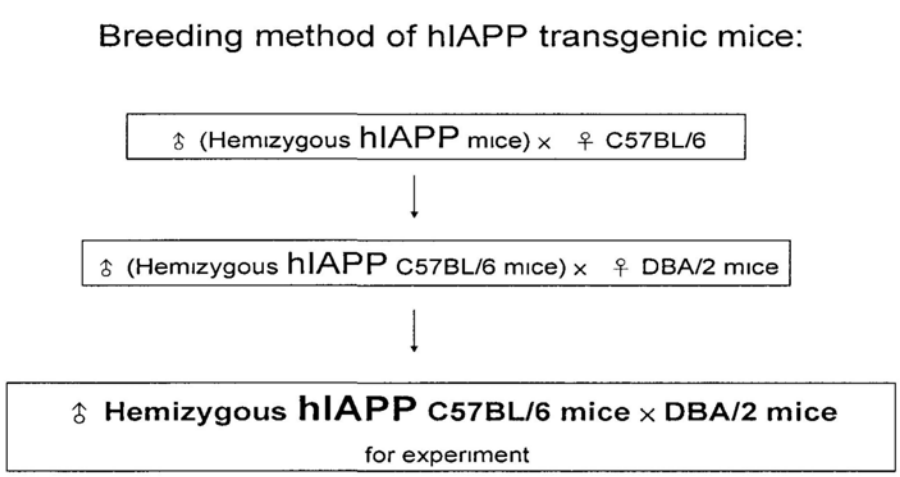

Figure 9: Breeding method of hIAPP transgenic mice.

2.1.2.2 Housing and feeding of hIAPP transgenic mice

Mice were housed on a 12-h light to dark cycle at 22°C, having free access to water and fed with a standard normal-fat laboratory rodent diet (5001 Rodent Diet, LabDiet, St Louis, MO) containing 12% calories from fat (4.5% fat by weight), 60% calories from carbohydrates, and 28% calories from protein (23% protein by weight). This diet was different from the moderate-fat diet used in previous studies to induce islet amyloid deposition, primarily in male mice [90].

2.1.3 Ethics approval

The study was approved by the Animal Research Ethics Committee of the Chinese University of Hong Kong. All experiments were conducted in accordance to the Animals (Control of Experiments) Ordinance of the Department of Health of the Hong Kong SAR Government.

2.1.4 Genotyping by PCR

The transgene consisted of a fragment of a hIAPP cDNA linked to the rat insulin II promoter. Transgenic status was determined by the polymerase chain reaction (PCR) of genomic DNA using primers directed against the hIAPP transgene, as previously described [187]. Briefly, the sense primer was 5'-CTG AAG CTG GTA TTT CTC A-3' and the anti-sense primer was 5'-AGA TGA GAG AAT GGC ACC AAA-3'. The reaction mixture also included primers for the β2-microglobulin gene, which was used as an internal control for successful amplification. The sense primer for β2-microglobulin was 5'-CAC CGG AGA ATG GGA AGC CGA A-3', and the anti-sense primer was TCC ACA CAG ATG GAG CGT CCA G-3'. The hIAPP allele resulted in a 182-bp and β2-microglobulin allele in a 295-bp band. The thermal cycle reaction consisted of 94°C for 30s, followed by 35 cycles of 60°C (30s) and 72°C (1 min). Gel electrophoresis showed that the β2 internal control band (295 bp) was present in all samples, whereas the hIAPP band (182 bp) was present only in samples from hIAPP transgenic mice. A total of 196 bred mice were genotyped, and 108 (55%) animals carried the human amylin transgene. In this study, 24 male mice (12 hIAPP transgenic mice and 12 nontransgenic littermates) were studied up to 12 to 18 months of age.

2.1.5 Animal research protocols

Protocol for hIAPP mice:

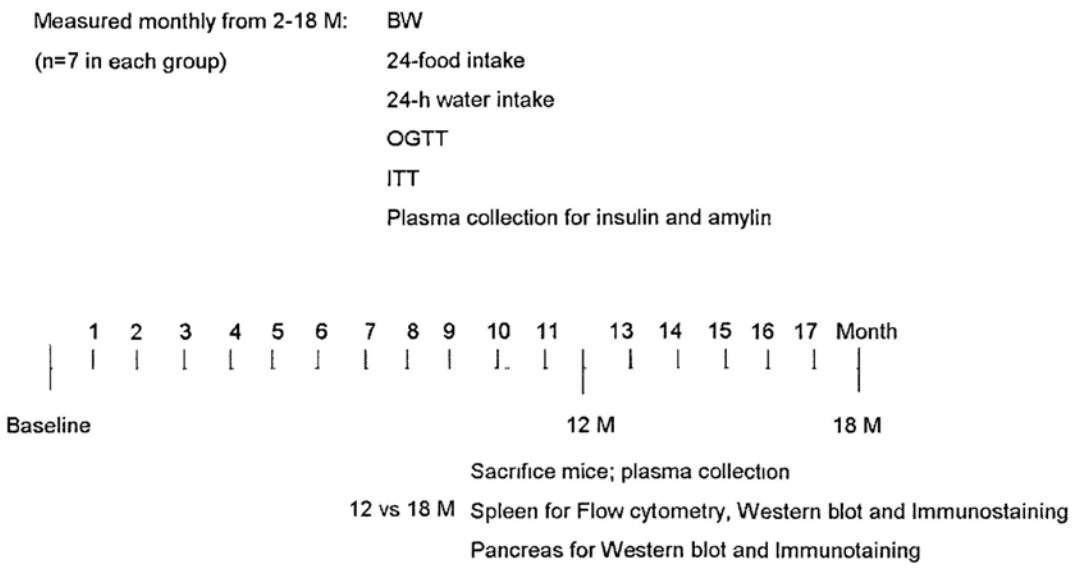

Figure 10. Study protocol of hIAPP mice between 2 and 18 months of age.

During a 18-month study period, body weight, daily food intake, daily water intake, fasting blood glucose (FBG) and blood glucose during oral glucose tolerance test (OGTT) were measured monthly (see Table 1 for detailed procedures). Retro-orbital blood extraction was performed for measurement of plasma insulin and amylin monthly between 2 and 18 months of age. At age of 12 or 18 months, transgenic mice and non-transgenic littermates were sacrificed at each time point. Pancreas and half of the spleen were kept in liquid nitrogen for protein analysis or fixed with 10%

formaldehyde solution for histological examinations. Another half of the spleen was collected to make a single splenocyte suspension for preparation of supernatant of splenocyte culture for flow cytometry.

2.1.6 Metabolic and biochemical studies

2.1.6.1 Body weight, food and water intake

Transgenic mice and non-transgenic mice were kept in cages separately and before each weekly procedure, bedding was changed prior to measurement. On the day of procedure, 24 hour food and water intake were measured. Body weight was measured after 8 hours of fasting before administration of oral glucose tolerance test (OGTT)

Time	Works
	Mice were fasted for 8 hours with free access to water
8:00 am	Bedding was changed before OGTT administration
(preparation)	Prepare 30% glucose solution by dissolving 9 g monohydrate glucose in 30ml of water
	Measure FBG
	Measure BW
4:00 pm	Gavage mice with 0.5 mL 30% glucose solution per mice (equivalent to 150 mg glucose)
	Keep mice in a quiet environment
4:30 pm	Measure 30 min blood glucose
5:30 pm	Measure 60 min blood glucose
6:00 pm	Measure 120 min blood glucose and feed mice with food

Table 1: Procedure of OGTT test.

2.1.6.2 Blood glucose

Between the age of 2 and 18 months, all mice underwent testing monthly. After at least 8 hours of fasting, blood glucose was measured followed by administration of OGTT using 150 mg of glucose in a 30% glucose solution (9 g monohydrate glucose dissolved in 30ml of water) to all mice. Blood glucose was measured at 0, 10, 20, 30, 60, and 120 min after administration. For insulin tolerance test (administered at least 2 days after OGTT to avoid the effect of fasting), an insulin dose of 0.5U/mL/kg BW was injected intra-peritoneally after 4-h fasting. Blood glucose was measured using the glucose meter from tail blood at 0, 10, 20, 30, 60, and 120 min after insulin administration. All blood glucose values were measured using the same glucose meter (Onetouch Ultra, LifeScan, Milpitas, CA, USA) throughout the 18-month study period to avoid inter-machine variabilities.

2.1.6.3 Plasma collection

After 8 hours of fasting, blood was collected from retro-orbital site of all mice using a capillary tube filled with 6% EDTA as anti-coagulant. The EDTA solution was added to the collection tube to achieve a ratio of blood: anticoagulant solution of 1:19. After the blood was collected into the tube, it was mixed well and put on ice

immediately. The blood was then centrifuged at 3000 rpm for 20 min at room temperature. The plasma was then separated and stored at -80°C for future use.

2.1.7 Measurement of plasma insulin

Fasting plasma insulin concentration was measured by enzyme immunoassay using a rat specific insulin ELISA kit (Catalog no.: 10-1124-01, MERCODIA, Sweden).

2.1.7.1 Principles

Mercodia rat insulin ELISA is a solid phase two-site enzyme immunoassay based on the direct sandwich technique. Two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacted with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microtitration well. A simple washing step removed unbound enzyme-labelled antibody. The bound conjugate was detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction was stopped by adding acid to give a colorimetric endpoint which was then read spectrophotometrically by an ELISA analyzer.

2.1.7.2 Procedures

Briefly, enzyme conjugate and substrate 3, 3′, 5, 5'-TMB were added into calibrator 0-5.5 μg/L and plasma samples. After incubation and wash, insulin concentrations were read at the absorbance of 450 nm by the ELISA Analyzer (μQuant, Bio-Tek Instruments Inc, Winooski, VT). Plasma insulin concentrations were expressed as pmol/L. Sensitivity and specificity of the kit are listed in appendix 1.

2.1.8 Measurement of human plasma amylin

Plasma amylin was measured by a monoclonal antibody-based sandwich radioimmunoassay using Linco human amylin ELISA kit (Catalog no.: EZHA-52K, MI). The capture antibody detects the intact disulfide bond between positions 2 and 7 of amylin with a lowest detection limit of 1 pM in 50 μL plasma sample.

2.1.8.1 Principles

The human Amylin ELISA kit is a monoclonal antibody-based sandwich immunoassay for determining amylin levels in human plasma. The capture antibody recognizes amylin (alternatively known as amylin acid or deamidated amylin) and is complexed with streptavidin-alkaline phosphatise. The substrate, 4-methylumbelliferyl phosphate (MUP), is applied to the completed sandwich and the fluorescent signal, monitored at 355 nm/460 nm, is proportional to the amount of amylin present in the sample.

2.1.8.2 Procedures

Briefly, detection conjugate and substrate ESS-MUP solution were added into standard and plasma samples. After incubation and wash, the plate was then put on a fluorescent plate reader (SpectraMAX Gemini, Molecular Devices) with an excitation/emission wavelength of 355 nm/460 nm. Plasma amylin concentrations were expressed as pmol/L. The lowest level of human amylin that can be detected by this assay in plasma sample is 1 pM/50 μL. This kit is also suitable for measurement of rat amylin and feline plasma, however, the precise percentage of cross-reactivity between human and non-human amylin has not been determined.

2.1.9 Measurement of plasma mouse TGF-ß

Mouse protein concentrations of TGF-β in spleen supernatants were measured using splenocytes purified from transgenic mice (n=7) and their nontransgenic littermates (n=7) at 12 months of age. TGF-β concentration in splenocyte supernatants was measured by ELISA (R&D Systems, Minneapolis, MN).

2.1.9.1 Principles

This assay employs a quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for TGF-ß 1 was pre-coated onto a microplate. Standards, controls and samples were pipetted into the wells and any TGF-ß 1 present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for TGF-ß 1 was added to the wells to sandwich the TGF-ß 1 immobilized during the first incubation. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The color intensity was in proportion to the amount of TGF-ß 1 bound in the initial step.

2.1.9.2 Procedures

Briefly, standards or samples (each in triplicate) were added to each well in a 96-well plate and incubated for 2 hours at room temperature. Standard curves were made according to the known amount of TGF-β in standard solutions provided by the manufacturer. TGF-ß1 conjugated antibodies were added to each well and incubated for 2 hours at room temperature. Finally, substrate solution was added to each well and incubated for 30 min at room temperature. The optical density of each well after 30 min was determined using a microplate reader (μQuant, Bio-Tek Instruments Inc, Winooski, VT) set to 450 nm.

2.1.10 Histological studies

2.1.10.1 Tissue processing

Mice were sacrificed at age of 12 or 18 months. The whole pancreas was fixed in 10% neutral formaldehyde and embedded in paraffin. Serial cross-sections (4 μm) were cut perpendicular to the long axis of the pancreas and spleen. Sections were stained with periodic acid-Schiff (PAS) for light microscopy in order to examine PLNs. Presence or absence of PLNs was determined based on at least six serial sections of the pancreas.

2.1.10.2 Periodic Acid Schiff (PAS) staining

The Periodic Acid Schiff (PAS) stain is used to demonstrate glycogen and mucopolysaccharide components in tissue samples detected by light microscopy. The preferred fixative is neutral buffered formalin. The histochemical technique is based on the principle of periodic acid oxidation of the glucose residues. The aldehyde products then react with the Schiff reagent to give rise to a purple-magenta color. A suitable basic stain is often used as a counterstain. Substances that can be demonstrated using the PAS stain include glycogen, mucins, collagen, reticulum, basement membranes, glomerular mesangium, vascular wall, fibrin, thyroid colloid, amyloid, and a number of secretions or tissue constituents. In this study, PAS staining of pancreatic sections obtained from 12 and 18 months can provide a basic overview of pancreatic structure including presence of PLNs.

2.1.10.3 Immunofluorescence staining

Double immunofluorescence staining was performed on serial pancreatic tissue sections using 1) rabbit anti-RAMP1 antiserum (1:1000; Alpha Diagnostics International, San Antonio, TX) and mouse anti-insulin antibody (1:1000; Invitrogen-Zymed, Carlsbad, CA), 2) rabbit anti-TGF-β antiserum (1:100; Santa

Cruz, CA), 3) mouse anti-amylin (1:400; AbD Serotec, Raleigh, NC), and 4) rabbit anti-TNF-α antiserum (1:100; Abcam, Cambridge, MA). Frozen tissue sections of the spleen were either single-labeled with rat anti-mouse CD25 antibody (IL-2 receptor, α chain, p55, BD Biosciences Pharmingen, San Diego, CA) or double-stained with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 antibody (RM4-5, rat IgG2a, BD Biosciences Pharmingen) and R-PE rat anti-mouse CD8a (BD Biosciences Pharmingen). Tissue slides were blocked with 1% BSA for 30 min before treatment with primary antibodies for 2 hours at room temperature. For negative control, the primary antibody was replaced by 5% normal rabbit serum. Immunofluorescence was detected using appropriate secondary antibodies (dilution, 1:200) conjugated with Alexa 488 (green) or Alexa 568 (red), and cell nuclei were counterstained with DAPI (1:200, Invitrogen Corp, CA). Slides were mounted with an anti-fading reagent, ProLong (Molecular Probes, Eugene, OR), stored in the dark at 4 °C, and examined within 1-3 days.

2.1.10.4 Light and immunofluorescence microscopy

Stained slides were examined using a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Hamburg, Germany). Representative images were automatically taken using a digital spot camera (Version 3.1 for Windows 95/98/NT, Diagnostic Instruments Inc, Sterling Heights, Michigan, USA). The original magnifications were ×200.

Immunofluorescence techniques can localize antigens of distinct molecules in tissue sections. Subtle differences at the molecular level can be detected on microscopy using specific antibody. The latter is bound by a readily identifiable label, usually in the form of a fluorochrome (e.g. fluorescein or rhodamine), which can absorb radiation and turn the molecule into an 'excited state' with electron redistribution and emission of radiation of a different wavelength. Two methods of immunofluorescence techniques have been extensively developed and applied since 1941. One is called direct technique, where the antibody is conjugated directly with the fluorochrome and used to detect the target antigen in the slide when examined under ultraviolet light microscopy. The other is called indirect (sandwich) technique which involves interaction between antigen and antibody. After a washing step, a secondary antibody conjugated to a fluorochrome is allowed to react. After further

washing step, fluorescence can indicate the presence of the target antigen. In my study, both direct and indirect immunofluorescence techniques have been used.

2.1.10.5 The total pancreatic lymph node (PLN) area

Quantitiative image analysis of the total PLN area was performed using the automatic Nikon Integrated Biological Imaging system (Nikon, Tokyo, Japan). Morphometric data were expressed as a pixel ratio to the target area examined. For each sample, one section stained for PAS at objective magnification ×20 was selected randomly to determine the number of PLNs and the total PLN area. The total PLN area was determined by measurements of PLN area containing lymphocytes surrounded by adipose tissue, adjacent to or enclosed by pancreas parenchyma using image analysis software (MetaMorph 4.0 image acquisition program for windows). A mean total PLN area was therefore determined. Pancreatic sections were evaluated by the examiner who was not aware of the origin of the sections. Here, most the sections were prepared by the technical staff and read blindly by myself or Dr. HL Zhao followed by uncoding to indicate whether they belonged to the transgenic or non-transgenic mice.

2.2 *Ex vivo* Studies: Primary mouse spleen cell culture and collection of supernatant of splenocyte

2.2.1 Preparation of single splenocytes suspension

Single splenocytes suspension was freshly prepared by grinding the spleen tissues with the plunger of a 5-ml disposable syringe at the time of sacrificing the mice at 12 month of age for fluorescence activated-cell sorter (FACS) analysis and detection of cytokines. Splenocytes were treated with an ammonium chloride-based hemolysis buffer (BD biosciences pharmingen) to remove red blood cells according to the protocol. Then, splenocytes were washed for 3 times with an adjusted cell concentration to 1×10^7 cells /mL using cell counter.

2.2.2 Preparation of splenocyte supernatant

After the single splenocyte suspension was freshly prepared according to the previous protocol, the splenocytes were counted and plated at 10^7 cells/mL/well in round-bottom 24-well microplates containing RPMI 1640 medium supplemented with 10% fetal calf serum and 100 U/ml penicillin/streptomycin. Then, the primary cultures were maintained at 37°C and 5% CO_2 After 48-h incubation, supernatants were collected and stored at -80°C for measurement of cytokines.

2.2.3 Preparation of synthesized human amylin and mouse amylin solution

The full-length peptide (Human amylin$_{1-37}$ peptides and rat amylin) were synthesized using t-boc chemistry and purified using reverse phase HPLC by Dr. James I. Elliott (Keck Biotechnology Center, Yale University, New Haven, CT). The lyophilized peptide was pretreated with a 1:1 mixture of trifluoroacetic acid/ hexafluoroisopropanol (HFIP and TFA) [188]. The dissolved protein was transferred to a new tube and subjected to a gentle stream of N_2 for 5-10 min to evaporate the HFIP/TFA. The lyophilized peptides were dissolved in 0.5% acetic acid to prepare a 50 nM stock solution. The stock solution was diluted with the culture medium to obtain the desired final protein concentrations and put on ice for at least 30 min to allow complete dissolution of proteins. All protein solutions were prepared freshly before use.

2.2.4 Flow cytometry

2.2.4.1 Principles

Flow cytometry is operated in accordance to the basic laws of physics including fluidics, optics, and electronics etc. Flow cytometry is a system for sensing cells or particles as they move in a liquid stream through a laser (light amplification by stimulated emission of radiation) or light beam past a sensing area. The relative

light-scattering and color-discriminated fluorescence of the microscopic particles is measured. Analysis and differentiation of the cells is based on size, granularity, and whether the cell is carrying fluorescent molecules in the form of either antibodies or dyes. When the cell passes through the laser beam, light is scattered in all directions. Light scattered in the forward direction at low angles (0.5–10°) from the axis is proportional to the square of the radius of a sphere and size of the cell or particle. Light can also enter the cell and be reflected and refracted by the nucleus and other contents of the cell. Thus, the 90° light (right-angled, side) scatter may be considered proportional to the granularity of the cell. The cells may be labeled with fluorochrome-linked antibodies or stained with fluorescent membrane, cytoplasmic, or nuclear dyes. Thus, flow cytometry can be used to measure different cell types, presence of membrane receptors and antigens, membrane potential, pH, enzyme activity, and DNA content.

2.2.4.2 Reagents

Lysis Buffer (cat. no. 555899) was purchased from BD Biosciences Pharmingen (San Diego, CA). Heat inactivated FCS (cat. no. 16140), RPMI medium 1640 (cat. no. 22400) and penicillin-streptomycin (cat. no. 15140) were purchased from Gibco

Lab (Scotland, UK). Table 1 lists the detailed information of various antibodies used in my experiments.

Ab	Species	Dilution	Company	Cat. No.	Fluorescence
CD3	rat anti-mouse	1:5	BD	555276	PE-cy5
CD4	rat anti-mouse	1:5	BD	553650	FITC-conjugated
CD8a	rat anti-mouse	1:5	BD	553032	PE conjugated
CD25	rat anti-mouse	1:5	BD	553075	R-PE-conjugated
CD16/CD32	rat anti-mouse	1:5	BD	553142	-

Table 2. Information of fluorescence antibodies in Flow Cytometry.

2.2.4.3 Procedures

Splenic CD4+CD25+ Treg cells were counted in transgenic mice (n=12) and their nontransgenic littermates (n=12) at 12 months of age using flow cytometry. *Ex vivo* study was performed to investigate whether treatment with synthesized human amylin would stimulate induction of CD4+CD25+ Treg cells from the splenocytes of nontransgenic littermates. In my initial experiments, purified splenocytes were incubated *ex vivo* with 10, 100, or 1000 nmol/L hIAPP or rat amylin (Keck Biotechnology Center, Yale University, New Haven, CT) for 24, 48, or 72 hours. For optimal results, purified splenocytes were treated with 10 nmol/L human amylin or rat amylin for 24 hours. For fluorescence-activated cell sorting analysis, suspensions of

dissociated splenocytes were prepared as previously described. For immunofluorescence staining and flow cytometry, splenocytes were incubated with 2.4G2 to block FcRs and then incubated with an optimal concentration of fluorochrome-labeled monoclonal antibodies (5* dilution) for 30 min at 4°C in the dark. Cells were washed three times and resuspended in PBS with 1% FCS. At least 10,000 cells were assayed using FASCalibur flow cytometry (Becton Dickinson, Mountain View, CA), and data were analyzed with CellQuest software (Becton Dickinson). Lymphocytes were gated based on forward and side light scatter and expression of CD3. Then gated lymphocytes were analyzed for expression of CD4 and CD25. All spleen lymphocytes were gated according to the expression of CD3.

2.2.5 Measurement of mouse TGF-β and TNF-α in the splenic supernatant using ELISA

Protein concentrations of TGF-β and TNF-α in spleen supernatants were measured using splenocytes purified from transgenic mice (n=7) and their nontransgenic littermates (n=7) at 12 months of age. These cytokines were also examined in splenocytes of age-matched nontransgenic littermates (n=7) before and after treatment with synthetic, full-length human amylin$_{1-37}$ peptides. The peptides were incubated with cells at the concentration of 10 nmol/L for 48 hours. Mouse TGF-β

concentration in splenocyte supernatants was measured by ELISA (R&D Systems, Minneapolis, MN) and determined by the ELISA Analyzer (μQuant, Bio-Tek Instruments Inc, Winooski, VT).

2.2.6 Measurement of Th1 and Th2 cytokines in the splenic supernatant using cytometric bead array (CBA)

For detection of mouse Th1/Th2 cytokines, isolated splenocytes were cultured for 48 hours at 37°C with 10% FCS in RPMI 1640 medium. All experiments were performed in duplicate. Collected supernatant samples were analyzed for GM-CSF, IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17 and TNF-α using a commercially available cytometric bead array (CBA) reagent kits (BMS820FF, Bender MedSystems, Vienna, Austria). Samples were analyzed using BD CellQuestTM software and BDTM CBA Software for a multi-fluorescence BD fluorescence-activated cell sorter (FACS CaliburTM) Flow Cytometer according to the manufacturer's instructions.

2.2.6.1 Procedures and principles

Briefly, beads were coated with antibodies with specific reactivity to each of the analytes in the multiplex system. The beads can be differentiated by their sizes and distinct spectral addresses. Then a mixture of coated beads for each analyte to be measured was incubated with the samples or standard mixture. The analytes present in the sample were bound to the antibodies linked to the fluorescent beads. A

biotin-conjugated second antibody mixture was added and the specific antibodies bound to the analytes were captured by the first antibodies. Finally, Streptavidin-Phycoerythrin was added which was bound to the biotin conjugate which emitted fluorescent signals for detection and quantitation.

2.2.7 Western blot assays

2.2.7.1 Principles

Western blot is a useful and sensitive tool to detect protein in concentrations ranging from ng/ml to pg/ml in serum, urine or culture supernatant. Western blot method involves the use of a high-quality antibody directed against a desired protein. First, proteins were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by size. Second, proteins were transferred from SDS-gel to a nitrocellulose membrane or PVDF membrane and blocked with milk to prevent any non-specific binding of antibodies to the surface of membrane. Third, the primary antibody was incubated with the membrane. Fourth, after washing the membrane 3 times, the membrane was incubated with the secondary antibody (an antibody-enzyme conjugate, e.g., horseradish peroxidase (HRP)). Finally, the protein bands were visualized with the dye and results were read accordingly.

2.2.7.2 Reagents

Reagents used for Western blot in my study are shown in Table 3.

Ab.	Species	Company	Cat. No.	Dilution	MW (KD)	Second Ab
RAMP1	Rabbit	Alpha-Dianostic	RAMP11-A	1:200	13	1:2000
RAMP2	Rabbit	Alpha-Dianostic	RAMP21-A	1:200	20	1:2000
RAMP-3	Rabbit	Alpha-Dianostic	RAMP31-A	1:200	40	1:2000
TNF-α	Rabbit	Abcam	Ab6671	1:100	26	1:2000
TGF-ß	Rabbit	Santa-cruz	Sc-146	1:200	25	1:2000
ß-actin	Rabbit	Abcam	Ab8227	1:10,000	47	1:10,000
TLR-4	Rabbit	Abcam	Ab47093	1:250	75-80	1:10,000

Table 3. Information of antibodies used in Western blot.

2.2.7.3 Procedures

Western blot assays were performed to detect protein expression of RAMPs, TNF-α, TGF-ß, and TLR-4 in the spleen and RAMPs in pancreas. Fresh spleen and pancreas tissues were homogenized in 0.15 M NaCL, 0.05 M Tris-HCl (pH 7.4), 0.5% sodium deoxycholate, 1% NP-40, 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA plus complete protease inhibitor cocktail (Catalog# 11697498001, Roche Diagnostics, Mannheim, Germany). After measuring protein concentrations with Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL), the extracts were boiled at

100°C for 10 minutes, separated on SDS-PAGE gels (Bio-rad, Hercules, CA) and transferred to polyvinylidine difluoride membranes. Equal loading of proteins (50 μg) was confirmed by Coomassie staining and subsequent β-actin immunoblotting. Membranes were blocked in 10% non-fat milk in Tris-buffered saline with 0.05% Tween (TBST) and incubated with primary antibody diluted in TBST for 1 hour at room temperature. After incubation with horseradish peroxidise-conjugated goat anti-mouse or goat anti-rabbit antibody (1:10000; Abcam, Cambridge, UK) for 1 hour at room temperature, the protein bands were visualized using ECL chemiluminescence reagent from Amersham-Pharmacia (Piscataway, NJ).

To ensure equal loading of proteins, membranes were incubated and probed with a rabbit anti-β-actin antibody (Abcam, Cambridge, CA) with dilution of 1:10,000, which recognizes the β-actin protein at approximately 43 kD. Signals were quantitated by densitometry and corrected for the β-actin signal, using the Kodak Digital Image station 440CF and the ID Image Analysis software program.

2.3 Human studies: human peripheral blood mononuclear cells (PBMC) treated with human amylin

2.3.1 Procedures

2.3.1.1 Preperation of human PBMC

Peripheral blood mononuclear cells (PBMC) from blood donors were prepared by centrifugation of peripheral EDTA blood using density gradient (Ficoll-Paque Plus, GE Healthcare Bio-Sciences Corp, NJ, USA). After the red blood cell lysis step and washing step, PBMC (1×10^6 cells/ml) were treated with or without human amylin at 10 nM at 37°C for 24 hours. PHA (1 μg/ml) and LPS (2 μg/ml) were used to stimulate lymphocyte counts.

2.3.1.2 Flow-cytometric analysis of FoxP3+CD4+,CD25+ Treg cells

After treatment, PBMCs were stained for surface staining reagent: CD4 and CD25 for 20 min at room temperature. After a washing step, PBMC were fixed with BD Foxp3 Buffer set at 50:1 ratio for 10 min at room temperature (BD Biosciences Corp, CA, USA). The fixed PBMCs were permeabilized for 30 min using BD Perm Buffer. Cells were then washed twice and stained for intracellular staining reagent Foxp3 for

30 min at room temperature. Finally, PBMC were washed twice and resuspended with 2% FBS stain buffer.

Mouse IgG isotypic antibodies were used to normalize the background signal (BD Pharmingen). Lymphocytes were differentially analyzed by excluding monocytes using their forward and side-scatter properties. Their expression (%) was calculated based on the percentage of Foxp3+ T cells in CD4+ T cells.

2.4 Statistics

Data are expressed as mean ± standard error and absolute number or percentage (%). Differences in means were compared using independent student *t*-tests (SPSS, Statistics Package for the Social Sciences 13.0 for Windows XP, SPSS Inc., Chicago, IL). Association between two sets of parametric data was evaluated using the Pearson correlation coefficient. Data with skewed distribution (amylin and insulin) were log transformed before analysis. A two-tailed *p*-value less than 0.05 was considered significant.

3

Results

3.1 Genotyping results of transgenic mice over-expressing human islet amyloid polypeptide

Polymerase chain reaction (PCR) was performed using mouse genomic DNA to identify human islet amyloid polypeptide (hIAPP) transgene. PCR gel showed that β2 internal control band (295 bp) was present in all samples, whereas hIAPP band (182 bp) was present only in samples from hIAPP transgenic mice (Figure 11). The transgenic rate was 53.3% (80/150), which was lower than 80% in male hIAPP transgenic mice published by Dr. Steven Kahn [88].

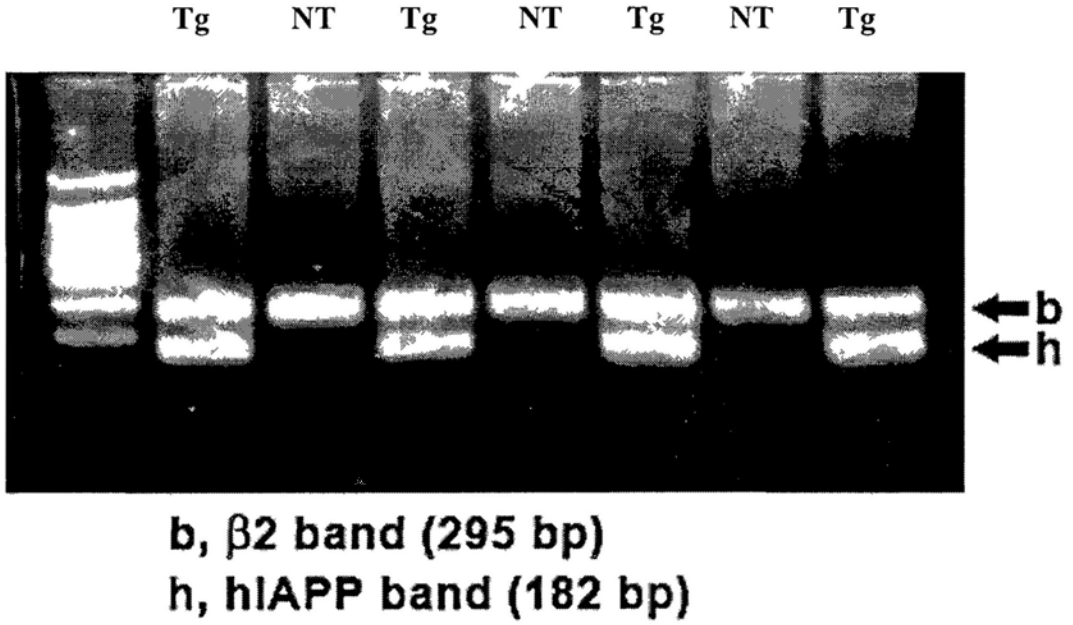

Figure 11: Genotyping result.
Hemizygous hIAPP transgenic mice were genotyped using the PCR reaction of tail DNA. In the PCR gel, hIAPP band (182 bp) was present only in samples from hIAPP transgenic mice. The internal control was the β2 macroglobin band (295 bp). Tg means hIAPP transgenic mice; NT means non-transgenic littermates.

3.2 Plasma insulin and amylin levels of transgenic mice over-expressing hIAPP

ELISA assays using mouse specific kits were performed to measure fasting plasma amylin (Figure 12) and insulin (Figure 13) concentrations in hIAPP transgenic mice and their nontransgenic littermates at 3, 6, 9, 12 and 18 month old. Both insulin and amylin levels were log transformed (based 10) before analysis and expressed as geometric mean in the figures.

On average, transgenic mice had lower fasting plasma insulin and substantially higher amylin concentration than the nontransgenic littermates although differences were significant only at 12 month and 18 month. Consequently, plasma insulin to amylin ratio were markedly lower (all $p<0.001$) in hIAPP transgenic mice than their nontransgenic littermates (Figure 14). This finding was consistent with previous reports [66] indicating that amylin over-expression may suppress insulin secretion from pancreatic β-cells in male hIAPP transgenic mice.

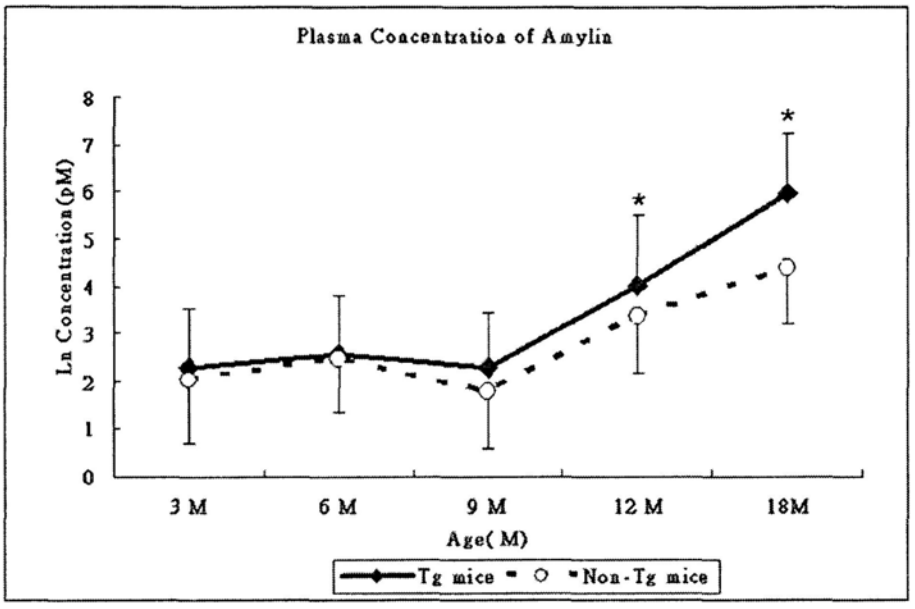

Figure 12. Plasma amylin concentration in hIAPP transgenic and non-transgenic mice .

ELISA assay was performed to measure plasma amylin concentrations at 3, 6, 9, 12 and 18 months of age. The transgenic mice showed higher level of plasma amylin level from 3 months to 18 months old, although the difference was significant only at 12 and 18 month old. Data were mean ± SE, *$p<0.05$.

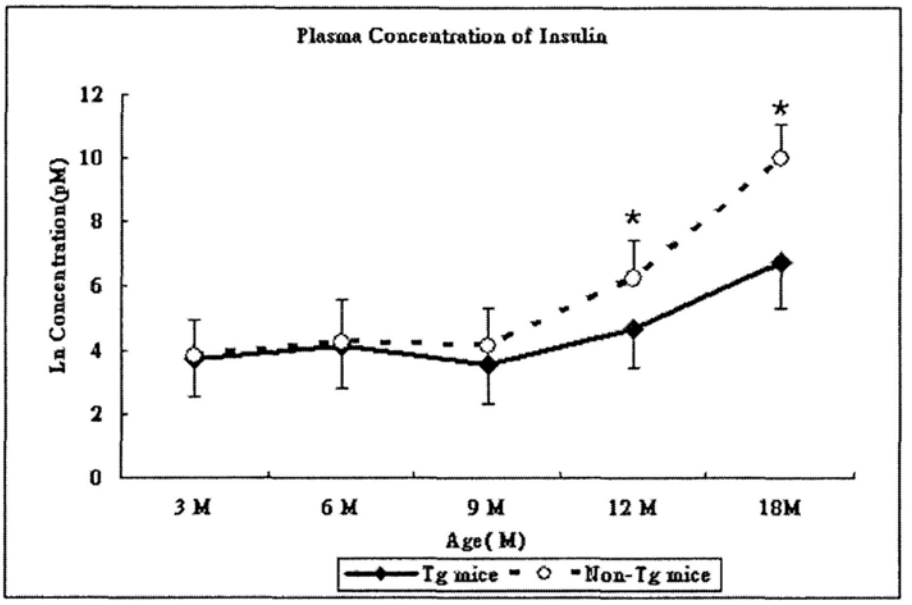

Figure 13 Plasma insulin concentration in hIAPP transgenic and non-transgenic mice.

ELISA assay was performed to measure plasma insulin concentrations at 3, 6, 9, 12 and 18 months of age. The transgenic mice showed significantly lower level of insulin compared with the non-transgenic group although the difference was significant only at 12 and 18 month old. Data are mean ± SE, *$p<0.05$.

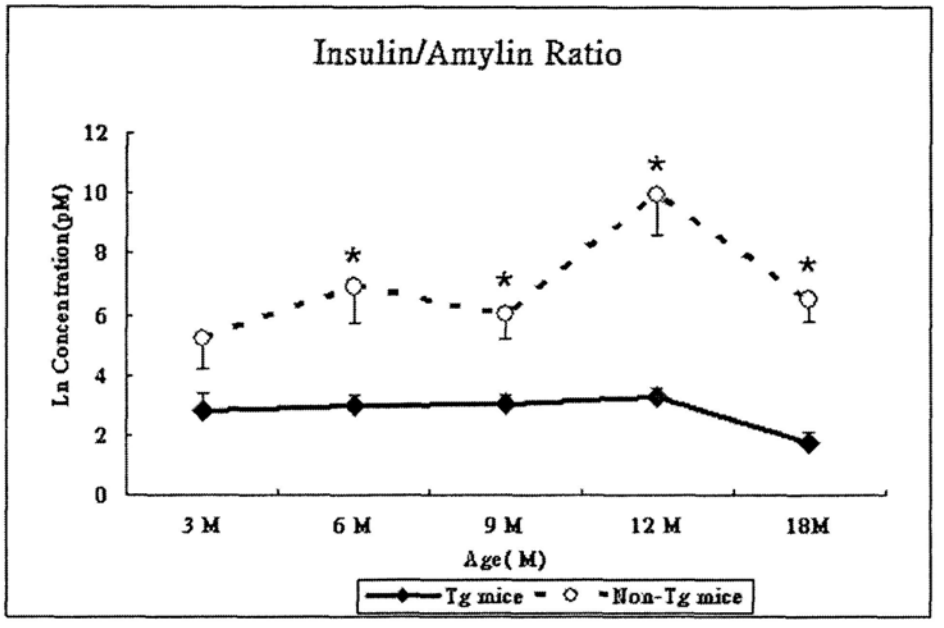

Figure 14. Plasma insulin to amylin ratio in hIAPP transgenic and non-transgenic mice..

Insulin to amylin ratio was calculated. hIAPP transgenic mice showed significantly lower insulin to amylin ratio at all ages compared to their nontransgenic littermates except at 3 month. Data are mean ± SE, * $P<0.05$.

	Mice	3M	6M	9M	12M	18M
Insulin	**Tg**	3.72±1.05	4.13±1.10	3.57±1.08	4.70±1.10*	6.74±1.13*
	Non-Tg	3.80±1.04	4.27±1.07	4.16±1.06	6.24±1.14	10.00±1.04
Amylin	**Tg**	2.30±1.09	2.56±1.10	2.27±1.06	4.02±1.14*	5.99±1.04*
	Non-Tg	2.01±1.09	2.48±1.05	1.79±1.10	3.36±1.07	4.37±1.05
Insulin/Amylin	**Tg**	2.81±0.56	3.00±0.33*	3.04±0.29*	3.29±0.31*	1.78±0.30*
	Non-Tg	5.22±0.99	6.91±1.19	6.04±0.85	9.93±1.34	6.51±0.71

Table 4. Plasma levels of insulin and amylin and insulin to amylin ratio in transgenic (Tg) and non-transgenic hIAPP mice.
Plasma levels of insulin, amylin and insulin to amylin ratio in the mice at 3, 6, 9, 12 and 18 months of age. Data are mean ± SE. *p<0.05. M, month.

3.3 General metabolic characteristics of hIAPP transgenic mice

There was no significant difference in mean body weight, 24-h food consumption, daily water intake and blood glucose levels between hIAPP transgenic mice and their nontransgenic littermates up to 18 months of ages (all $p>0.1$).

3.3.1 Body weight

Both the hIAPP transgenic mice and their non-transgenic littermates showed a steady increase in body weight during the 15-month period of observation from 3 month onward, especially between 3 and 6 months followed by a smaller weight gain thereafter. In the transgenic mice, body weight increased from 26.7±0.6g to 46±2.1g ($p<0.001$). The respective figures in the non-transgenic mice were 25.5±0.9g and 47.9±1.3g ($p<0.001$). The change in body weight between the 2 groups of animals were similar (all $p>0.1$) (Figure 15).

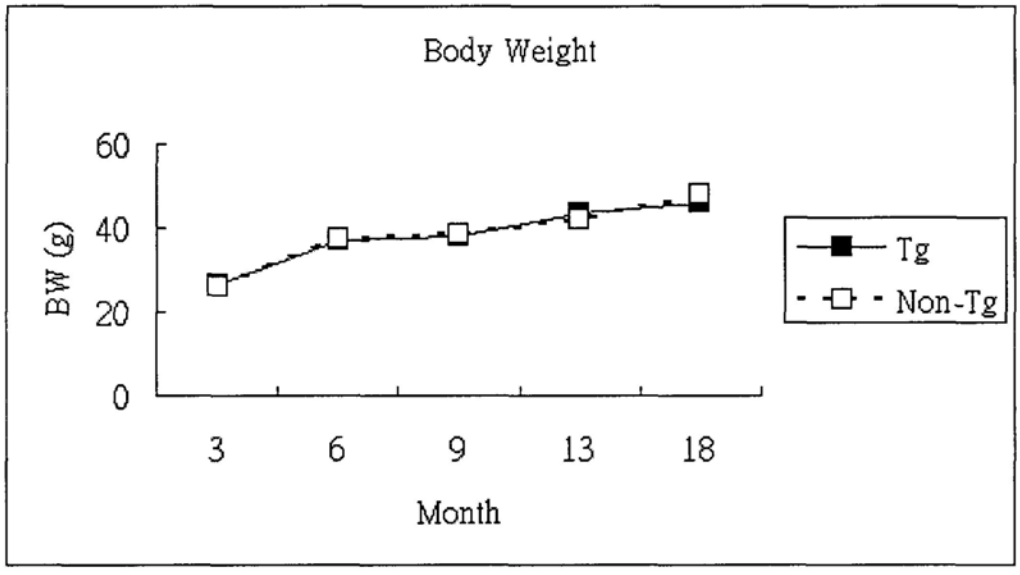

Figure 15. Body weight in hIAPP transgenic and non-transgenic mice.
Body weight was measured in transgenic mice and non-transgenic littermates. There was no significant difference between the two groups of animals from 3 to 18 months of age. Data are mean ± SE.

3.3.2 Daily food intake

Daily food intake was similar in the transgenic mice and non-transgenic littermates. Food intake increased from 5.2±0.2g at 2 month to 7. 7±0.6g at 18 month in the transgenic mice. The respective figures were 5.1±0.2g and 6.4±0.4g in the non-transgenic mice (all $p>0.05$) (Figure 16).

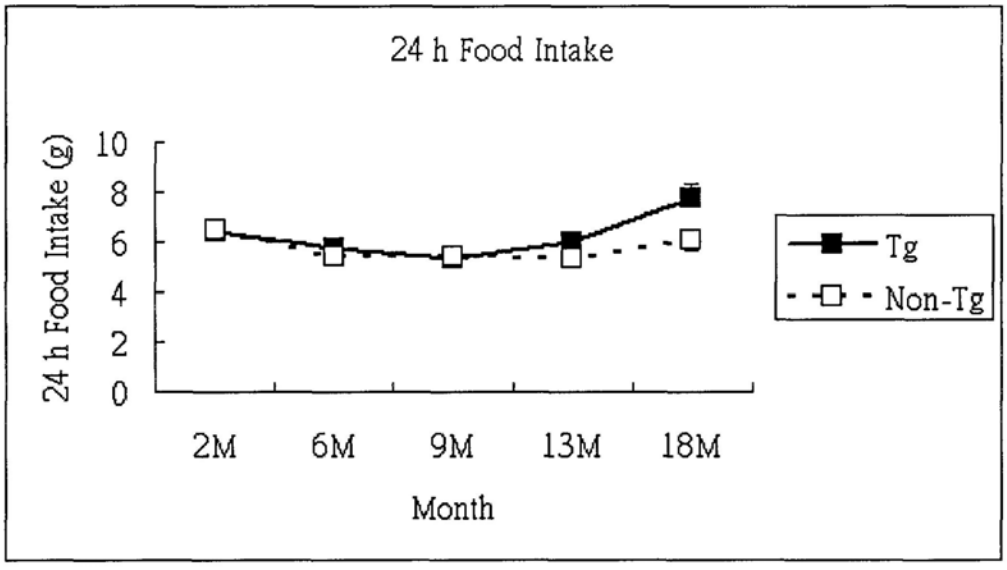

Figure 16. Daily food intake in hIAPP transgenic and non-transgenic mice. Daily food intake was measured in the transgenic mice and non-transgenic littermates. There was no significant difference between the two groups from 3 to 18 month old. Data are mean ± SE.

3.3.3 Daily water intake

Daily water intake was similar between the transgenic mice (6.6±0.5 mL to 9.4±1.0 mL) and non-transgenic littermates (7±0.6 mL to 10.6±1.4 mL) (all $p>0.1$) from 3 to 18 months of age (all $p>0.05$) (Figure 17).

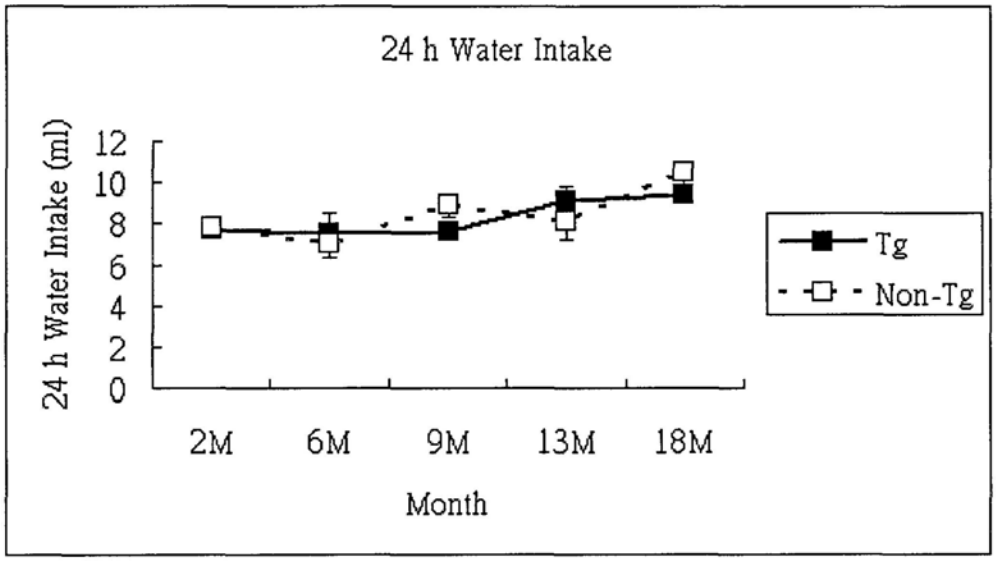

Figure 17. Daily water intake between hIAPP transgenic and non-transgenic mice.
Daily water intake was measured in the transgenic mice and non-transgenic littermates. There was no significant difference between the two groups. Data are mean ± SE.

3.4 Blood glucose levels

3.4.1 Blood glucose levels at fasting and postload conditions

Blood glucose levels after 8 hours of fasting and during OGTT tests were measured at 3, 6, 12, 18 months of age. Both groups of animals had similar blood glucose levels (Figure 18). Area under blood glucose concentration versus time curve (AUC) was similar between hIAPP transgenic mice and their nontransgenic littermates at different ages: month 3: 26.4±0.8 vs. 27.5±1.8 mmol/L; month 6: 27.1±2.9 vs. 25.0±1.5 mmol/L; month 9: 21.5±0.4 vs. 22.9±0.8 mmol/L and month 12: 22.4±0.8 vs. 21.4±0.8 mmol/L (all $p>0.1$). Hence, over-expression of hIAPP did not significantly impact glucose homeostasis in male transgenic mice fed on normal chow.

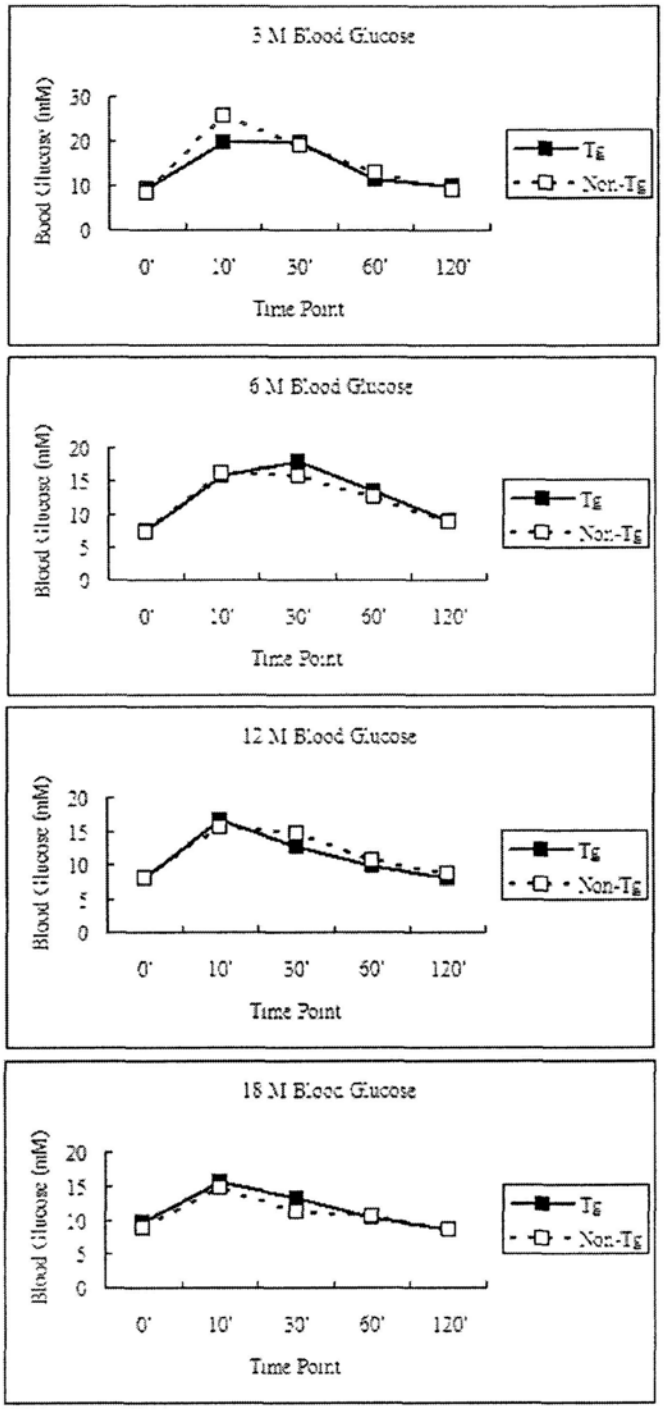

Figure 18. Blood glucose levels during oral glucose tolerance test (OGTT) at multiple time points in hIAPP transgenic and nontransgenic mice.
Blood were sampled from hIAPP transgenic mice and their nontransgenic littermates at different time points during OGTT on a monthly basis between 3 and 18 months of ages. Blood glucose levels and area under the curve (AUC) were similar between hIAPP transgenic mice and their non-transgenic littermates up to 18 months of age. Data are mean± SE.

3.4.2 Blood glucose change pattern

The longitudinal change in fasting (Figure 19) and 2h post-load blood glucose (Figure 20) levels were similar between hIAPP transgenic mice and their nontransgenic littermates up to 18 months of age.

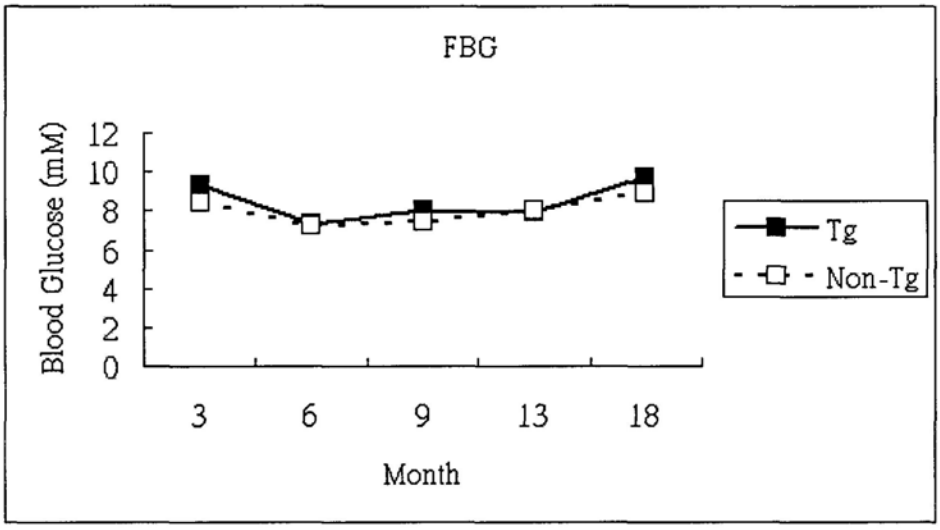

Figure 19. Fasting blood glucose levels in hIAPP transgenic and nontransgenic mice.
Fasting blood glucose levels of hIAPP transgenic mice and their nontransgenic littermates were measured on monthly basis between 3 and 18 months of ages. There was no significant difference in fasting blood glucose between the transgenic mice and nontransgenic littermates over the 15-month period. Data are mean ±SE.

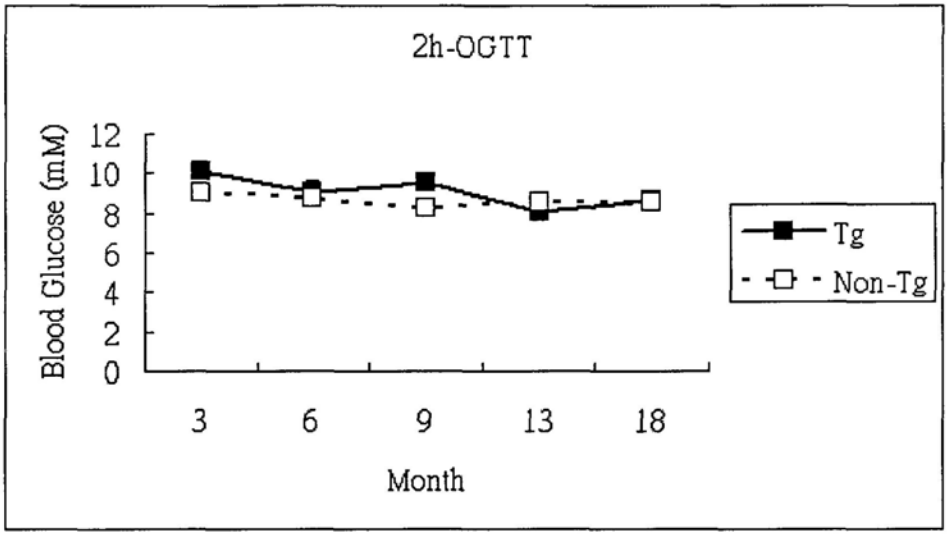

Figure 20. Post OGTT 2-h blood glucose levels in hIAPP transgenic and nontransgenic mice.

Post OGTT 2-h blood glucose levels of hIAPP transgenic mice and their nontransgenic littermates were measured on a monthly basis between 3 and 18 months of ages. There was no significant difference in post OGTT 2-h blood glucose between the transgenic mice and nontransgenic littermates. Data are mean ± SE.

3.4.3 Insulin tolerance test

Blood glucose levels were measured during insulin tolerance test (ITT) performed at 6, 12 and 18 months of age (Figure 21). Area under the curve (AUC) blood glucose were lower in hIAPP transgenic mice than their nontransgenic littermates at 6 month (11.1±1.7 vs. 12.3±1.0 mmol/L), 12 month (15.3±0.8 vs. 16.8±1.9 mmol/L) and 18 month (14.8±0.9 vs. 17.6±0.5, mmol/L), reaching significance at 18 months of age ($p=0.02$).

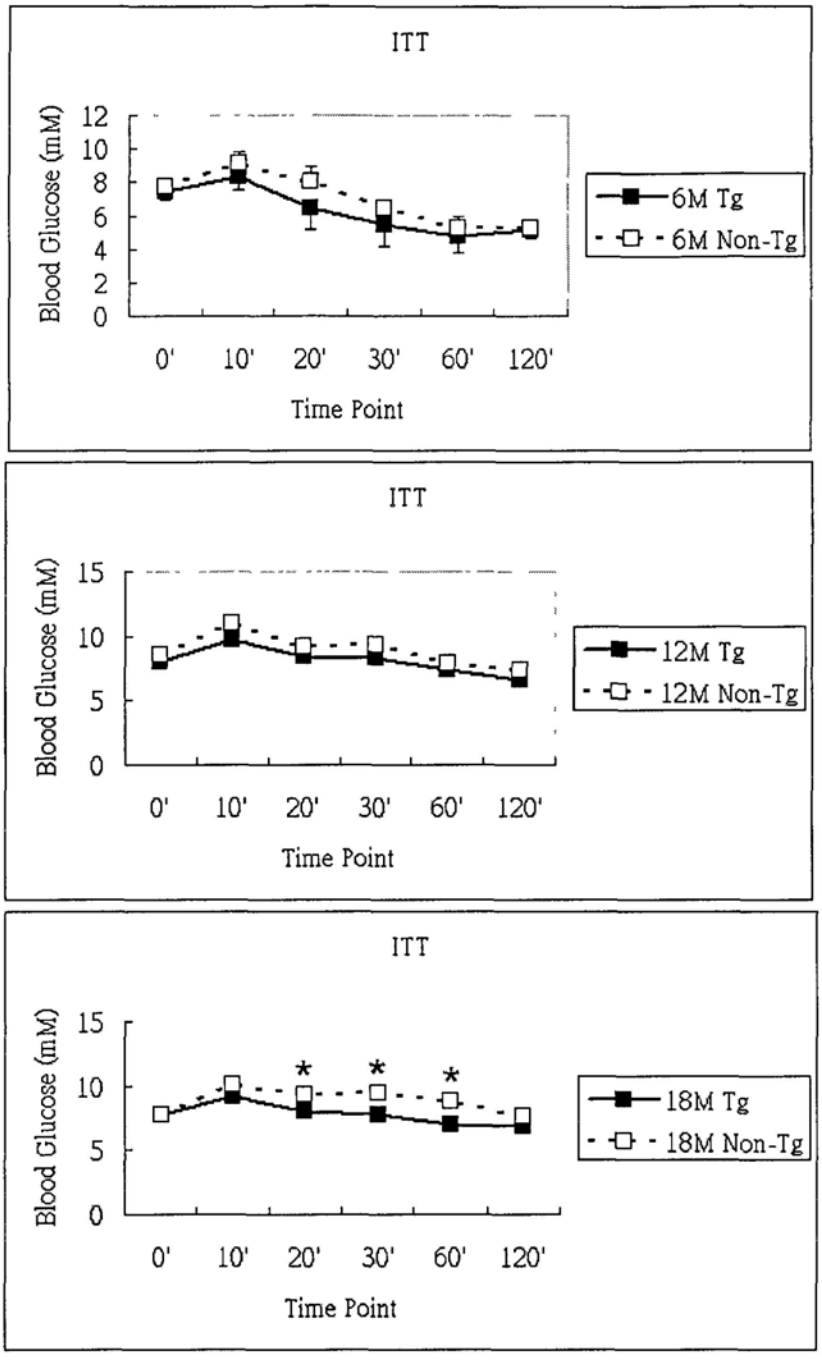

Figure 21. Blood glucose levels of insulin tolerance test (ITT) in hIAPP transgenic and nontransgenic mice.

Detailed ITT was performed at 6, 12 and 18 months of age. Blood glucose levels and area under the curve (AUC) were lower in hIAPP transgenic mice than their nontransgenic littermates at 18 months of age ($p=0.02$). Data are mean±SE.

3.4.4 HOMA-IR

Insulin resistance was assessed by homeostasis model assessment of insulin resistance index (HOMA-IR, Figure 22). HOMA-IR is an empirical mathematical formula based on fasting plasma glucose and insulin levels, developed as a surrogate measurement of *in vivo* insulin sensitivity:

HOMA-IR = fasting plasma insulin (μIU/mL) × fasting plasma glucose (mmol/L) × 22.5

The hIAPP transgenic mice had lower HOMA-IR than non-transgenic littermates at 12 month (17.8±4.0 vs. 49.1±12.3, p=0.001) and 18-month (32.1±6.1 vs. 81.7±7.1, p=0.0001).

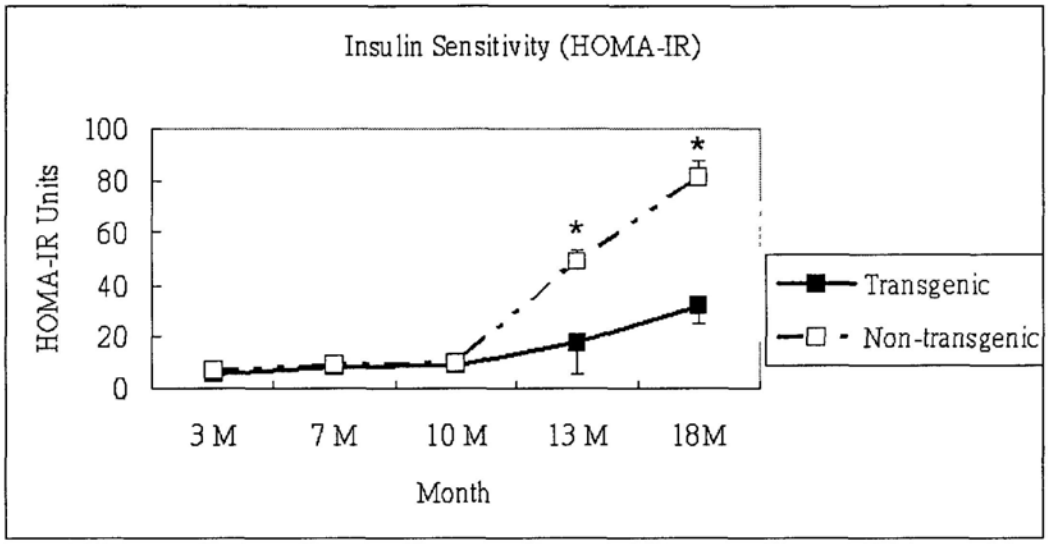

Figure 22. Homeostasis model assessment of insulin resistance (HOMA-IR) in hIAPP transgenic and nontransgenic mice.

HOMA-IR was calculated at 3, 6, 9, 12 and 18 months of age. HOMA-IR was lower in hIAPP transgenic mice than their nontransgenic littermates at 12 and 18 months of age. Data are mean±SE.

3.5 Immune modulation by hIAPP

3.5.1 Diminished pancreatic lymph nodes in hIAPP transgenic mice

Pancreatic lymph nodes (PLNs) drain lymph from the pancreas into the pancreaticosplenic lymph nodes. In NOD mice, PLNs are required to prime T cells reactive against β-cells [182]. On light microscopy of cross-sections of the pancreas, PLNs were localized mainly within the adipose capsule surrounding the pancreas (Figure 23). PLNs were found in 100% (12 PLNs in 12 mice) of nontransgenic littermates and 41.7% (5 PLNs in 12 mice) of hIAPP transgenic mice at 12 months of age. These findings suggest an association between reduction in PLNs and enhanced hIAPP expression in some hIAPP transgenic mice ($p = 0.005$, relative risk = 0.417, 95% confidence interval [CI] = 0.213 - 0.814). The average number of PLNs was 2 (10 PLNs in 5 mice) per transgenic mouse compared to 3.25 (39 PLNs in 12 mice) per nontransgenic mouse ($p = 0.006$ by nonparametric Mann-Whitney U test).

The hIAPP transgenic mice exhibited a smaller total area of PLNs ($85,700 \pm 40,400$ μm^2) than their nontransgenic littermates ($418,000 \pm 131,000$ μm^2) ($p = 0.041$). The average cross-sectional PLN area in the hIAPP mice was as low as 20.5% of the PLN

area in the littermate controls, suggesting that even when they were present, hIAPP transgenic PLNs were abnormally small. These findings suggest that human amylin overexpression might suppress PLN formation (Figure 23).

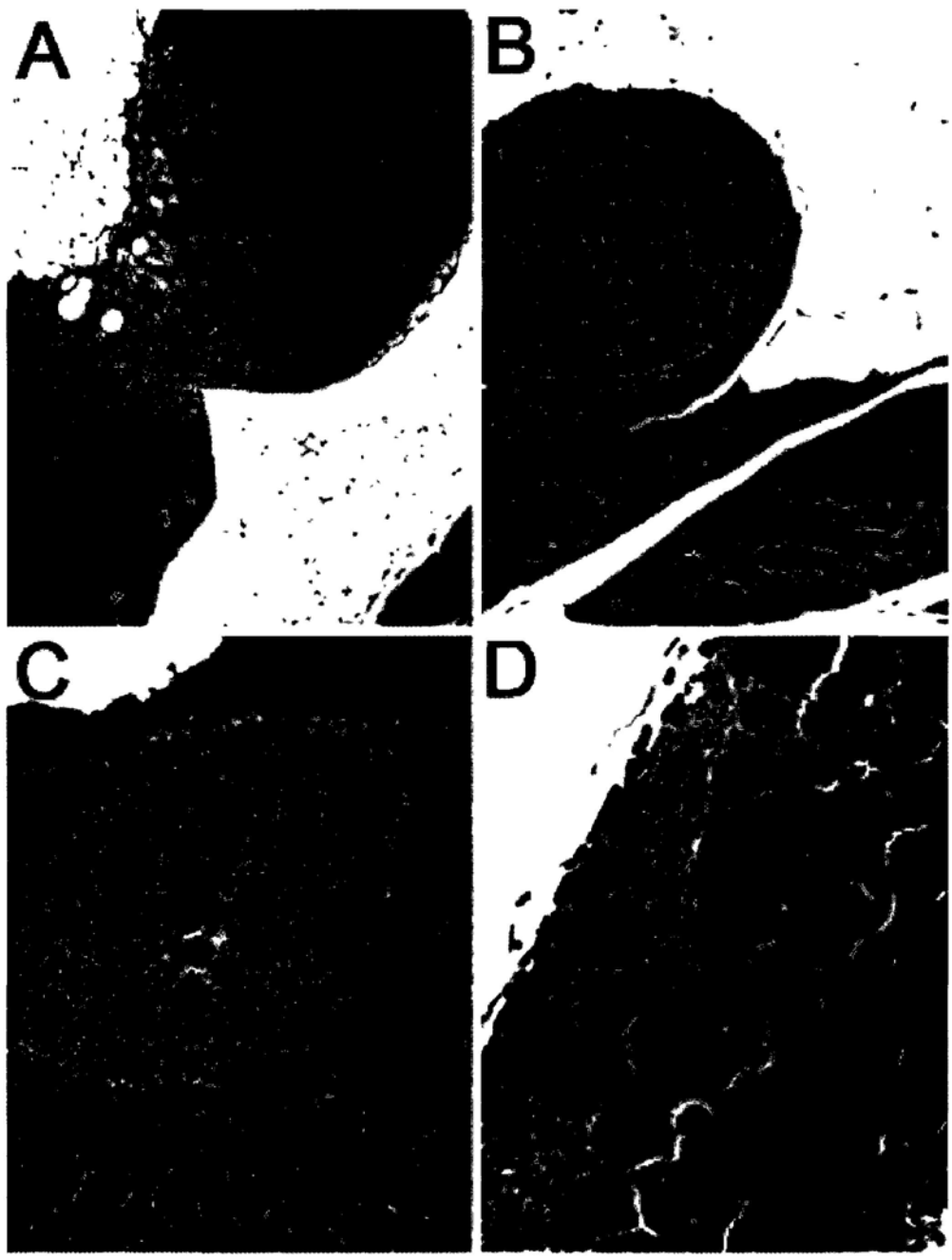

Figure 23. Pancreatic lymph nodes and lymphocyte aggregates in hIAPP transgenic and nontransgenic mice.

Periodic acid Schiff (PAS) staining was performed on pancreatic tissue sections of nontransgenic littermates (panels A-C) and hIAPP transgenic mice (panel D). A, Two pancreatic lymph nodes in the adipose capsule of the pancreas (× 50). B, One pancreatic lymph node adjacent to pancreas parenchyma (× 100). C, One pancreatic lymph node enclosed by pancreas parenchyma (× 100). D, A small patch of pancreatic lymphocyte aggregates under the capsule despite magnification by 2-fold compared to the same site in the non-transgenic mice (× 200).

3.5.2 Induction of splenic Treg cells by hIAPP

3.5.2.1 *In vivo* expansion of splenic Treg cells in hIAPP transgenic mice

T lymphocytes play a central role in cell-mediated immunity. Naturally occurring CD4+CD25+ Treg cells are essential for the maintenance of immunological tolerance. We applied flow cytometry to splenocytes isolated from transgenic mice (n=7) and their nontransgenic littermates (n=7) at 12 and 18 months of age to investigate whether the suppression of PLN formation resulted from changes in CD4+CD25+ Treg cells. As shown in Figure 24 and Table 5, the percentage of CD4+CD25+ Treg cells was higher in hIAPP transgenic mice than their nontransgenic littermates at age of 12 month (3.5±0.2% vs. 2.8±0.3%, $p = 0.039$) and 18 month (2.2±0.1 vs. 1.9±0.1, $p=0.044$), suggesting that enhanced hIAPP expression *in vivo* induced CD4+CD25+ Treg cells.

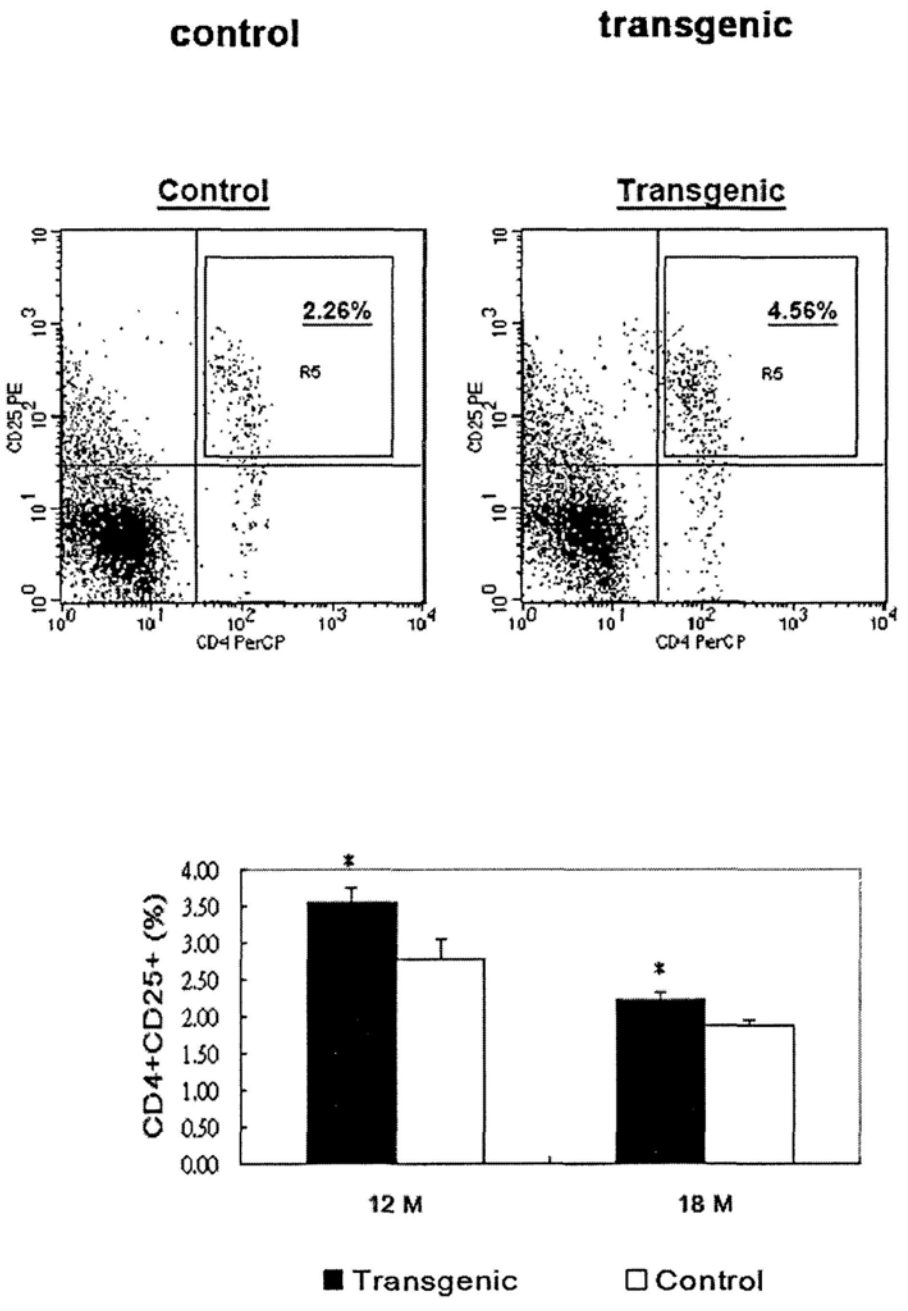

Figure 24. CD4+CD25+ Treg cells in splenocytes detected by flow cytometry in hIAPP transgenic and nontransgenic mice. .

Flow cytometry of CD4+CD25+ Treg cells was performed using splenocytes freshly purified from hIAPP transgenic mice (transgenic, n=7) and their nontransgenic littermates (control, n=7) at 12 and 18 months of age. Lymphocytes were gated according to the expression of CD3.

3.5.2.2 Spleen CD4+ and CD8+ T subsets

Other splenic T lymphocyte subsets, including CD3+CD4+ helper T cells, and CD3+CD8+ cytotoxic T cells were also quantitated by flow cytometry. As shown in Figure 25, and Table 5, the percentage of CD4+ T cells, CD8+ T cells of splenic CD3+ cells and the ratio of CD4+ to CD8+ cells were similar between the two groups at 12 month and 18 month (Figure 25).

T subsets	Mice	12 month	18 month
CD4+CD25+ T cells	Tg	3.54±0.20 (P=0.039)	2.21±0.11 (P=0.040)
	Non-Tg	2.79±0.69	1.87±0.10
CD4+ T cells	Tg	67.07±1.21 (P=0.882)	37.46±1.60 (P=0.051)
	Non-Tg	67.34±1.32	42.39±1.61
CD8+ T cells	Tg	11.64±0.80 (P=0.440)	45.34±0.71 (P=0.100)
	Non-Tg	10.90±0.48	42.57±1.38
CD4+/CD8+ ratio	Tg	5.96±0.47 (P=0.632)	0.84±0.04 (P=0.105)
	Non-Tg	6.24±0.34	1.00±0.08

Table 5. Percentage of CD4+ T cells, CD8+ T cells, CD4+/CD8+ ratio in transgenic (Tg) and non-transgenic (non-Tg) hIAPP (Tg) and mice detected by flow cytometry. Data are mean ± SE

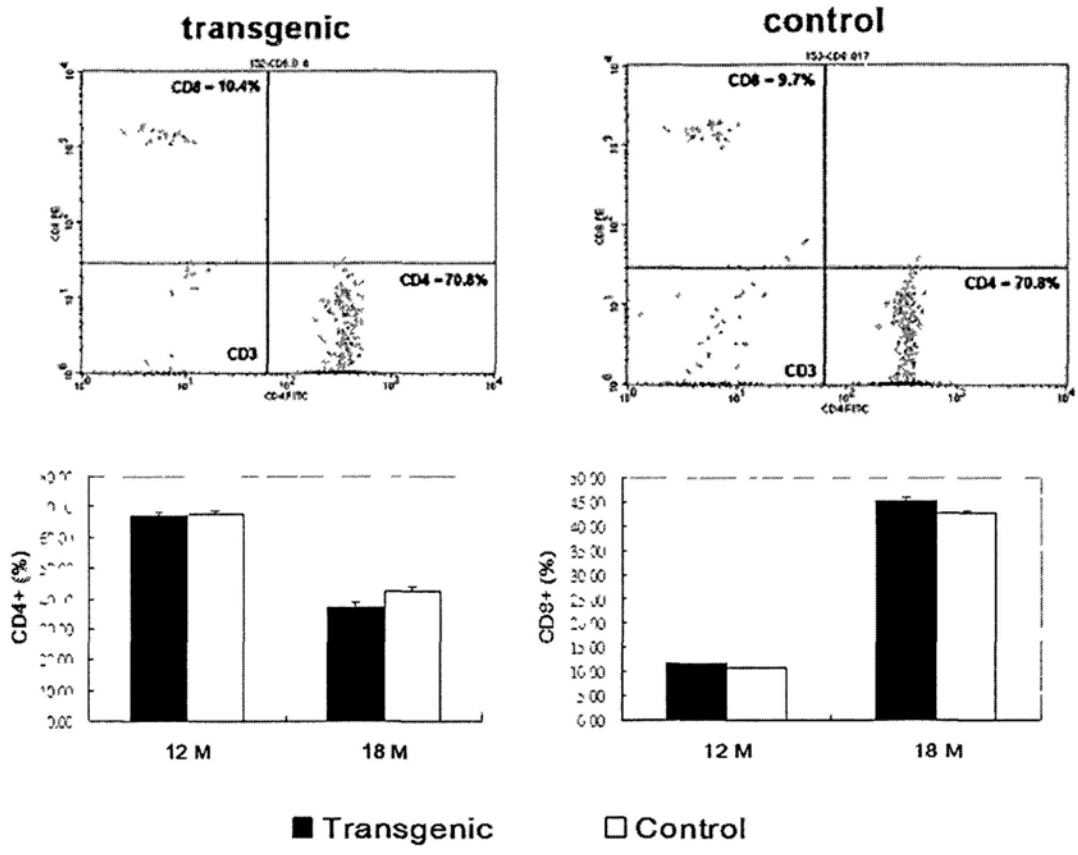

Figure 25. CD4+ and CD8+ T cells of the spleen measured by flow cytometry in hIAPP transgenic and nontransgenic mice.

Flow cytometry of CD4+ and CD8+ T cells was performed using splenocytes freshly purified from hIAPP transgenic mice (transgenic, n=7) and their nontransgenic littermates (control, n=7) at 12 and 18 months of age. Lymphocytes were gated according to the expression of CD3.

Histological examination (Figure 26) by immunofluorescence microscopy using cell surface marker (CD4+, CD8+ and CD25+) were performed on the splenocytes of transgenic mice (n=7) and their nontransgenic littermates (n=7) at 12 month of age. Immunostaining showed a higher number of CD4+CD25+ Treg cells in the hIAPP transgenic mice than their nontransgenic littermates. A similar immunoreactivity of CD4+ and CD8+ T lymphocytes was detected in the splenocytes of both strains of mice.

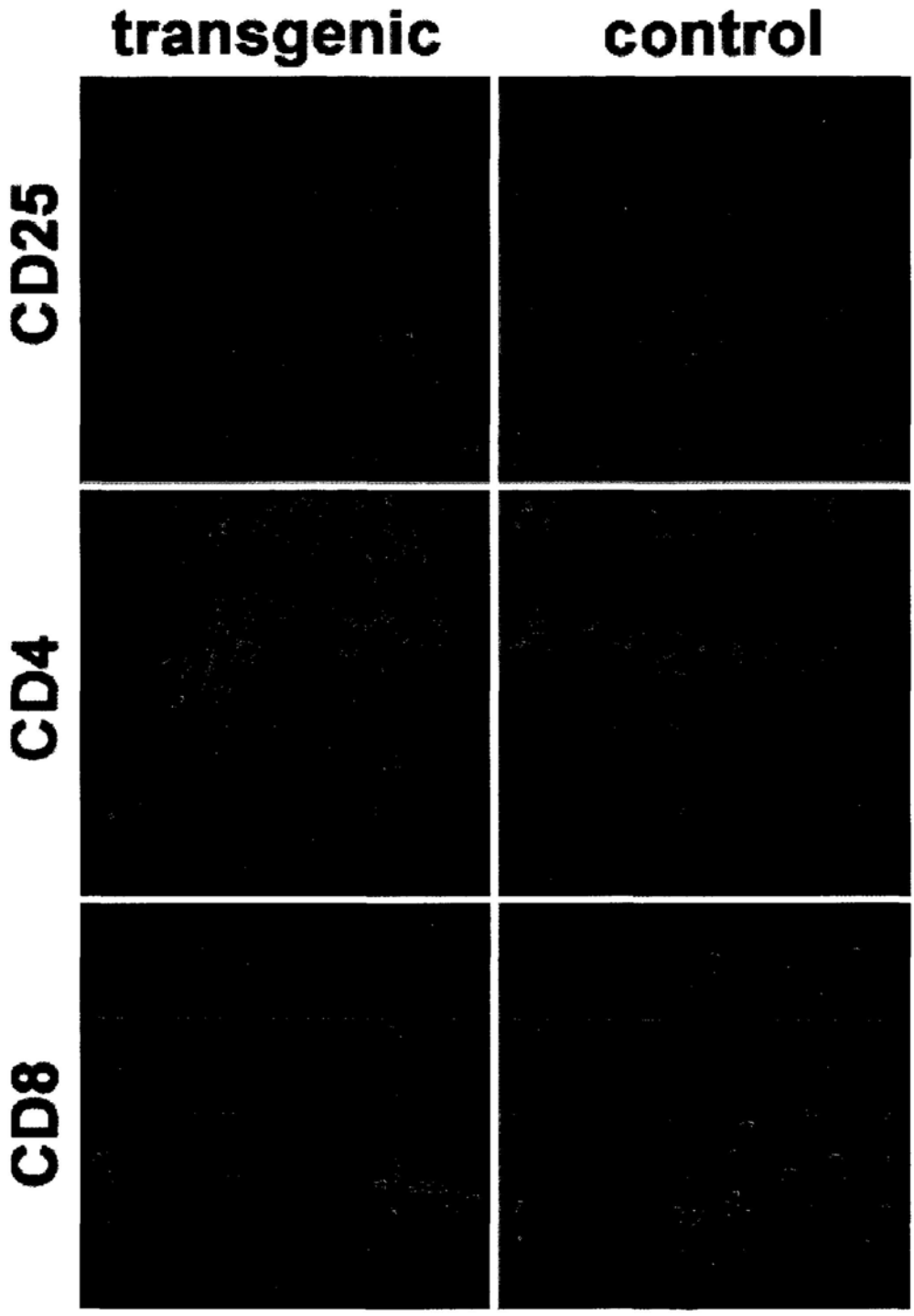

Figure 26. CD4+CD8+ Treg cells and other T lymphocyte subsets shown by immunofluorescence microscopy in hIAPP transgenic and nontransgenic mice. Frozen tissue sections of the splenocytes from transgenic mice (transgenic) and their nontransgenic littermates (control) at 12 months of age were labeled for CD4+CD25+ Treg cells (red membrane) and double-stained for CD4+ (green membrane) and CD8+ (red membrane) T cells. Original magnification, × 200.

3.5.3 *Ex vivo* expansion of splenic Treg cells induced by synthesized hIAPP

Ex vivo study was performed to investigate whether hIAPP treatment could stimulate the expansion of CD4+CD25+ Treg cell population from the splenocytes of nontransgenic mice. Dose-response relationship was performed to seek the optimal amylin concentration (Figure 27). The results demonstrated that 10nM human amylin can stimulate the expansion of Treg cells to the greatest degree. Thus, splenocytes purified from seven control littermates were incubated *ex vivo* with synthesized hIAPP or mouse amylin at a concentration of 10 nmol/L. Flow cytometry demonstrated that human amylin treatment significantly increased the percentage of CD4+CD25+ regulatory T cells, from 2.90 ± 0.16% to 3.69 ± 0.25% (paired student *t*-test $p = 0.011$), or an average increase of 25.4% (Figure 28). These data are consistent with the *in vivo* finding of the higher percentage of CD4+CD25+ regulatory T cells (3.53 ± 0.20 %) in hIAPP transgenic mice (n=7) than their nontransgenic littermates (n=7) (2.78 ± 0.25%). In contrast, the mouse amylin treatment had no effect on the percentage of CD4+CD25+ Treg cells [3.04±0.27% vs. 2.89±0.37% (p=0.426)]. Taken together, these observations strongly suggest that hIAPP overexpression induced splenic CD4+CD25+ Treg cells as confirmed by immunohistochemical examination, *in vivo* and *ex vivo* studies using flow cytometry.

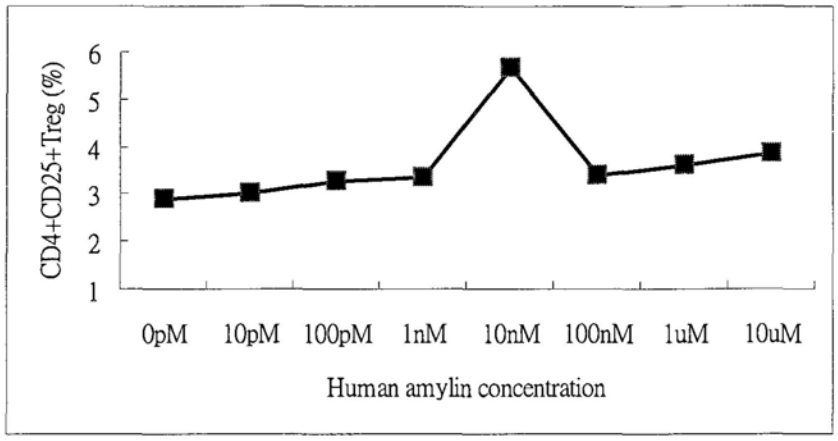

Figure 27. *Ex vivo* induction of CD4+CD25+ Treg cells using synthetic hIAPP in cultured splenocytes from hIAPP nontransgenic mice in different dosages.
Flow cytometry was used to measure the percentage of CD4+CD25+ Treg cells, before and after treatment with different dosage of human amylin for 48 hours. It was demonstrated that 10 nM is the optimal concentration.

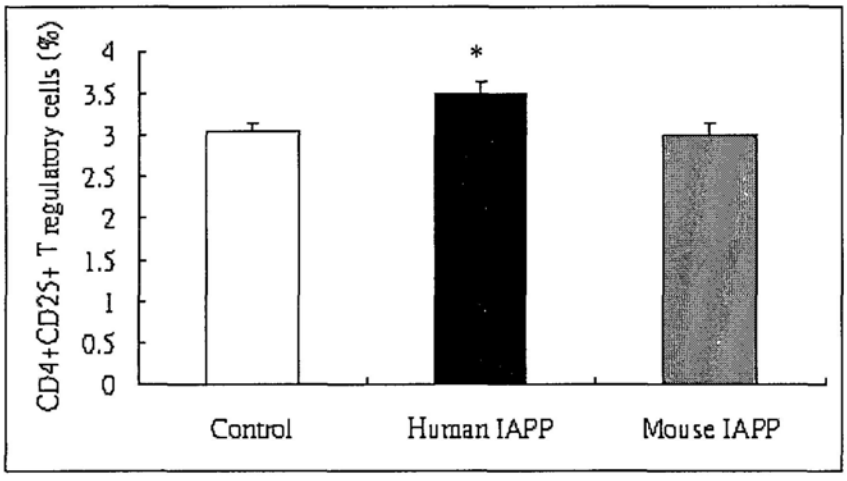

Figure 28. *Ex vivo* induction of CD4+CD25+ Treg cells using synthetic hIAPP in cultured splenocytes from hIAPP nontransgenic mice.
Flow cytometry was used to measure the percentage of CD4+CD25+ Treg cells, before and after treatment with 10 nmol/L human amylin for 48 hours. The splenocytes were isolated from nontransgenic littermates (n=7) at 12 months of age. T lymphocytes were gated according to the expression of CD3. *Paired student *t*-test p=0.011.

3.5.4 *Ex vivo* expansion of human Foxp3 CD4+CD25+ Treg cells induced by synthetic hIAPP

Ex vivo study was performed to investigate whether hIAPP treatment could stimulate the expansion of CD4+CD25+ Treg cell population from human peripheral blood mononuclear cells (PBMC). PBMC purified from ten blood donors were incubated *ex vivo* with synthetic hIAPP or mouse amylin at the concentration of 10 nmol/L. Flow cytometry demonstrated that hIAPP treatment significantly increased the percentage of CD4+CD25+ Treg cells, from 3.06 ± 0.12% to 4.31 ± 0.31% (paired student *t*-test $p = 0.002$) (Figure 29). These data are consistent with the *in vivo* finding of higher percentage of CD4+CD25+ Treg T cells (3.53 ± 0.20%) in hIAPP transgenic mice (n=7) compared to 2.78 ± 0.25% in their nontransgenic littermates (n=7). Treatment with mouse amylin treatment slightly increased the percentage of CD4+CD25+ Treg from 3.06±0.12% to 3.83±0.36% (p=0.08), but short of significance. These findings strongly suggest that hIAPP overexpression induced Foxp3+CD4+CD25+ Treg cells in human PBMC using flow cytometry.

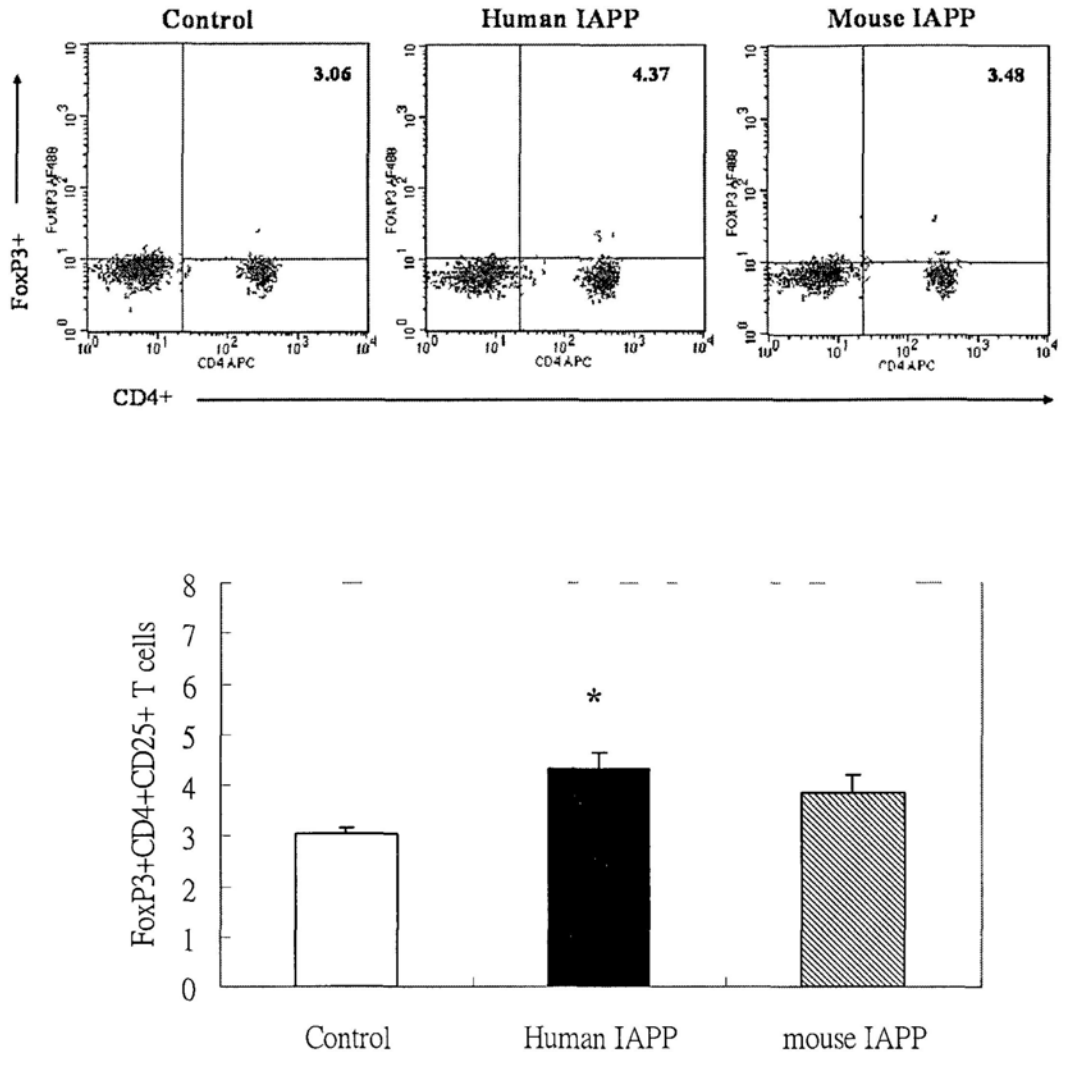

Figure 29. *Ex vivo* induction of Foxp3+CD4+CD25+ Treg T cells from human peripheral blood mononuclear cells (PBMC) using synthetic human and mouse amylin.

Flow cytometry was used to measure the percentage of Foxp3+CD4+CD25+ Treg cells, before and after treatment of 10 nmol/L hIAPP for 48 hours. The human PBMC were isolated from blood donors (n=10). Lymphocytes were gated according to the expression of CD4. *Paired student *t*-test p=0.002.

3.5.5 Changes in immune cytokines

3.5.5.1 Upregulation of mouse TGF-β by hIAPP

The differentiation, expansion, and function of CD4+CD25+ Treg cells are regulated by TGF-β [149, 150, 189-192]. I therefore examined TGF-β protein levels and its secretion in the splenocytes using ELISA (Figure 30A and 30B) and Western blot (Figure 30C). As measured by ELISA, TGF-β protein concentration in the spleen supernatant of transgenic mice (n=7) was higher (2476.1 ± 42.5 pg/ml) than their nontransgenic littermates (2238.1 ± 51.5 pg/ml) ($p = 0.002$) (Figure 30A). Western blot showed that TGF-β protein expression in splenocytes was 50.5% higher in transgenic mice than nontransgenic littermates ($p = 0.002$) (Figure 30C).

Immunofluorescence microscopy also revealed higher TGF-β expression in the PLN (Figure 32A) and pancreas (Figure 32B, upper panel) of the transgenic mice than non-transgenic mice. In *ex vivo* study, incubation of the splenocytes from nontransgenic littermates (n=7) with 10 nmol/L hIAPP resulted in a numerical increase of 2.5% in the TGF-β protein concentration ($p = 0.273$; Figure 30B). Taken together, these data strongly suggested that enhanced hIAPP expression might stimulate TGF-β protein expression in the spleen and pancreas to increase the expansion of CD4+CD25+ Treg lymphocytes in hIAPP transgenic mice.

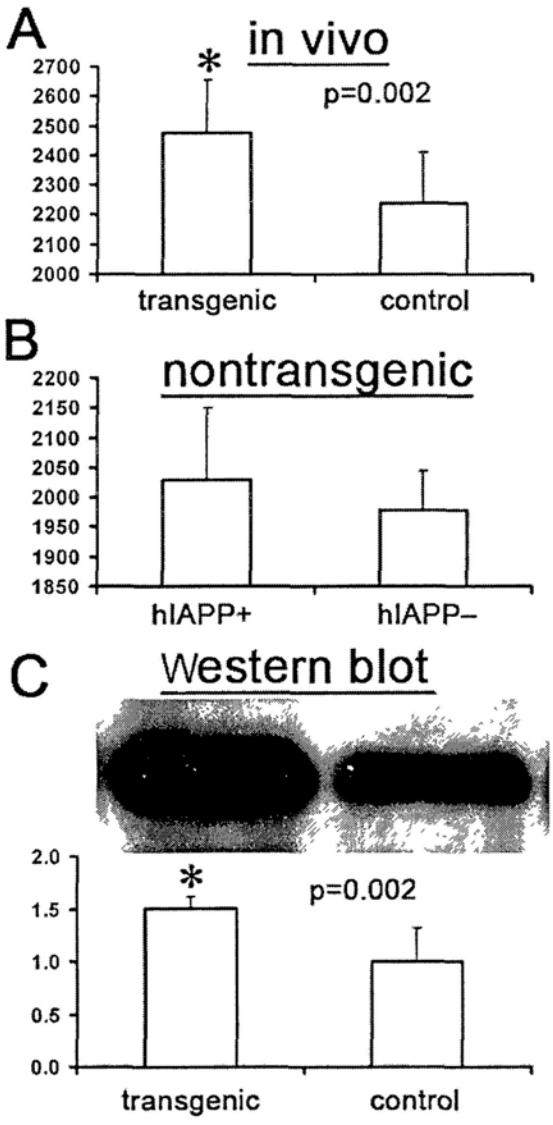

Figure 30. ***In vivo*** **and** ***ex vivo*** **data on association between human amylin and TGF-β protein expression. .**

A, ELISA assays detected TGF-β protein concentrations (pg/ml) in spleen supernatants from transgenic mice (n=7) and nontransgenic littermates (n=7) at 12 months of age. B, Splenocytes were purified from nontransgenic littermates (n=7) at 12 months of age. TGF-β protein concentrations (pg/ml) in the cultured splenocyte supernatants before and after treatment with 10 nmol/L hIAPP were measured by ELISA. C, Equal amounts of tissue lysates (50 μg) from the spleens of hIAPP transgenic mice (transgenic, n=7) and their nontransgenic littermates (control, n=7) at 12 months of age were subjected to SDS-PAGE and immunoblotted for TGF-β. Pixel intensity was normalized to the levels of β-actin, and a value of 1 was assigned to the TGF-β levels in nontransgenic littermates. Data are expressed as mean ± SD, * $p < 0.01$ *vs.* control.

3.5.5.2 Upregulation of TNF-α by hIAPP

Local expression of transgenic TNF-α in islets prevents autoimmune type 1 diabetes in NOD mice by reducing the development of autoreactive, islet-specific T cells, which are normally suppressed by CD4+CD25+ Treg cells [193]. Using immunofluorescence microscopy, TNF-α staining in the PLN (Figure 32A) and pancreatic islet cells (Figure 32B, lower panel) was higher in transgenic mice than their nontransgenic littermates. Using fluorescent bead immunoassay (Figure A; Table 6), there was higher concentrations of TNF-α in the splenocyte supernatants of transgenic mice than their nontransgenic littermates. On Western blot, splenic TNF-α levels were two-fold higher in hIAPP transgenic mice than controls ($p = 0.003$) (Figure 31C). In *ex vivo* experiments, incubation of supernatant of splenocytes cultured from nontransgenic littermates with 10 nmol/L hIAPP increased TNF-α concentration by 14.5% (Figure 31B) but short of significance ($p = 0.12$). Taken together, these data indicate that increased human amylin expression might stimulate expression of the cytokines, TNF-α and TGF-β, to modulate autoimmunity and innate immunity in hIAPP transgenic mice.

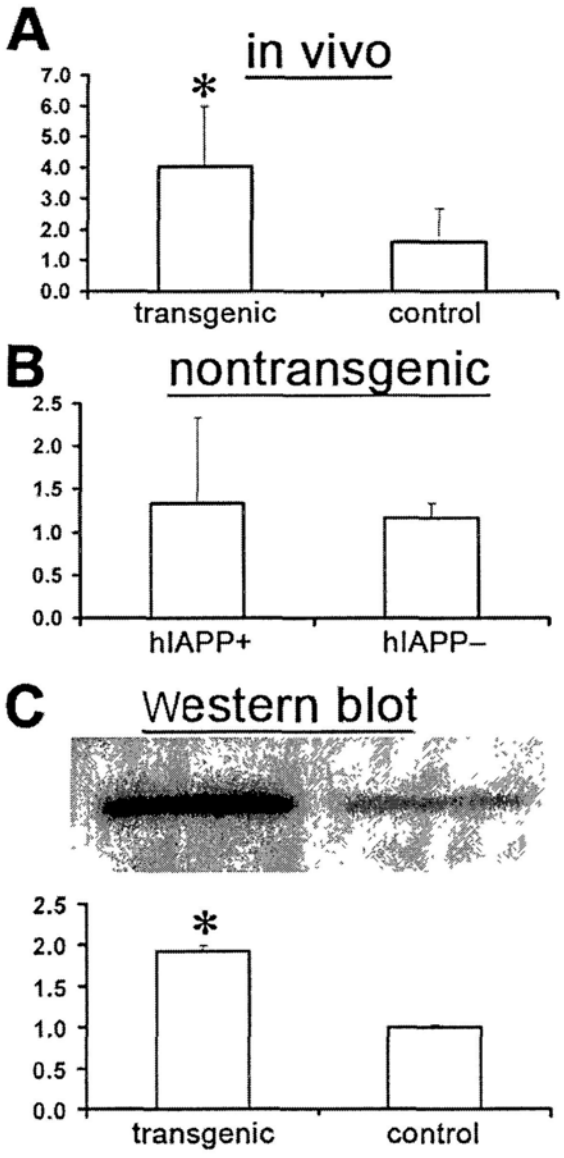

Figure 31. ***In vivo*** **and** ***ex vivo*** **data on associations between hIAPP and TNF-α protein expression.**

A, Cytometric bead assays detected TNF-α protein concentration in spleen supernatants of transgenic mice (n=7) and their nontransgenic littermates (n=7) at 12 months of age. B, Splenocytes were purified from nontransgenic littermates (n=7) at 12 months of age. TNF-α protein concentrations in the cultured splenocyte supernatants before and after treatment with 10 nmol/L hIAPP were measured by cytometric bead assay. C, Equal amounts of tissue lysates (50 μg) of the spleen from hIAPP transgenic mice (transgenic, n=7) and their nontransgenic littermates (control, n=7) at 12 months of age were subjected to SDS-PAGE and immunoblotted for TNF-α. Pixel intensity was normalized to the level of β-actin, and a value of 1 was assigned to the TGF-β levels in nontransgenic littermates. Data are expressed as mean ± SD, * $p < 0.05$ *vs.* control.

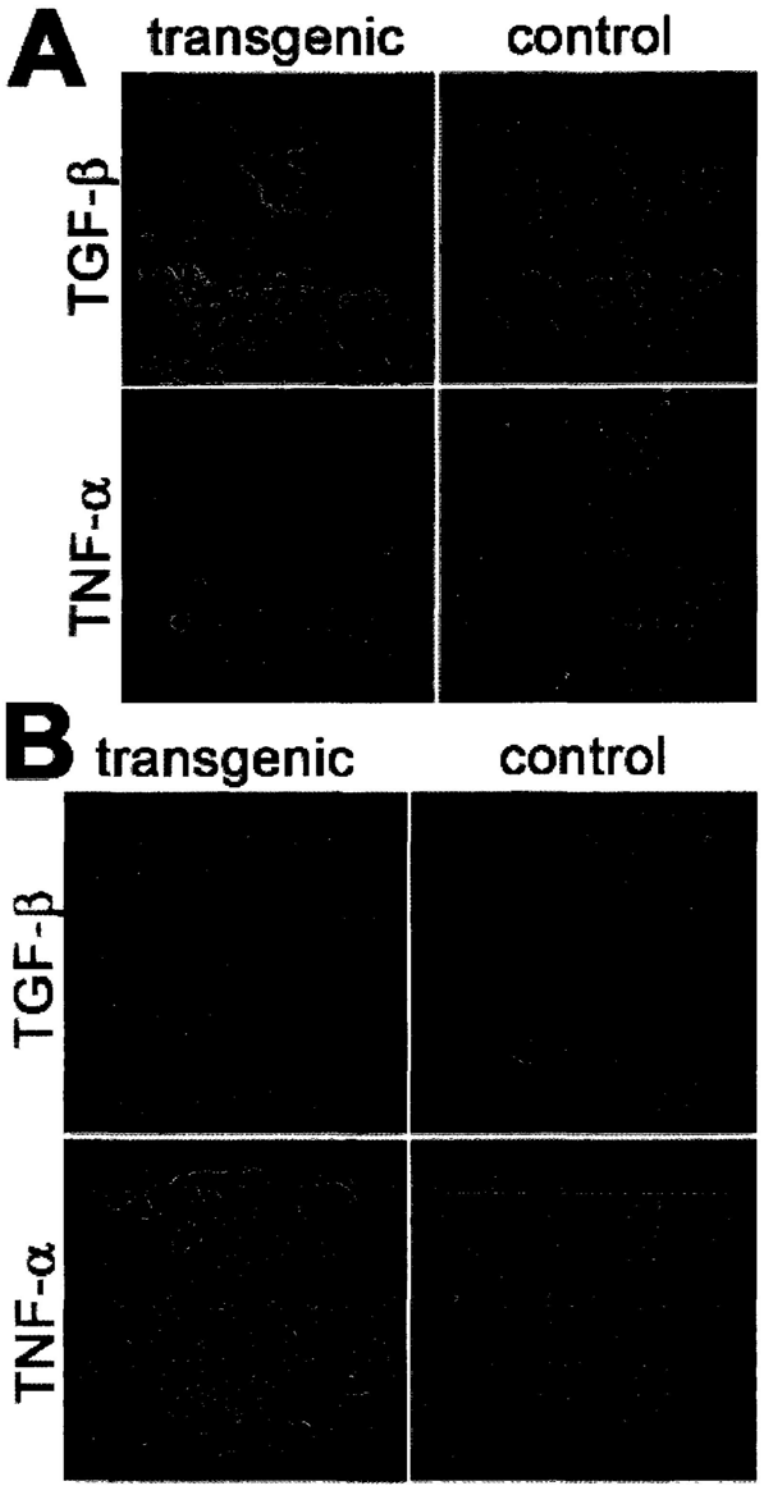

Figure 32. Expression of TGF-β and TNF-α in the pancreas of hIAPP transgenic and nontransgenic mice.

Tissue sections of the pancreas from transgenic mice (transgenic) and their nontransgenic littermates (control) at 12 months of age were stained with TGF-β or TNF-α. Immunofluorescence microscopy showed substantially higher expression of TGF-β and TNF-α in the pancreatic lymph nodes (panel A) and pancreatic islets (panel B) of transgenic mice. Original magnification, × 200.

3.5.5.3 Regulation of other cytokines by hIAPP

Type 1 diabetes is a T-cell-mediated autoimmune disease, resulting in destruction of the insulin-producing β cells in the pancreas. Disease progression is thought to involve the action of T-cells, particularly those producing Th1-type cytokines. The latter also induce proinflammatory responses responsible for killing intracellular parasites and perpetuating autoimmune responses [194]. To identify the possible roles of hIAPP on cytokine expression, splenocytes supernatants from hIAPP transgenic mice and nontransgenic littermates were analyzed for the production of a panel of cytokines during a 48h period. Specifically, multiplexed cytokine detection was used to measure levels of GM-CSF, IFN-γ,IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17 and TNF-α using a commercially available cytometric bead array (CBA) reagent kit. In the supernatants from the hIAPP transgenic mice, there were increased level of TNF-α (4.0±1.9 v.s. 1.6±1.1, p=0.009, pg/ml) but reduced level of IL-6 (318.0±69.1 vs. 516.7±165.4, p=0.028, pg/ml). The levels of other cytokines were similar between the 2 groups (Table 6).

Cytokines	hIAPP transgenic mice, (n=19)	Nontransgenic control mice, (n=11)
TNF-α	4.0±1.9*	1.6±1.1
IL-4	17.5±8.6	11.6±1.3
IL-17	92.7±26.9	87.3±17.3
IL-1α	223.3±18.3	257.8±22.0
IL-2	101.4±9.0	110.8±11.7
IL-6	318.0±69.1*	516.7±165.4
IL-10	42.2±7.4	59.6±27.8
IFN-γ	1891.3±549.2	2403.2±815.6
GM-CSF	44.6±10.3	55.3±6.5

Table 6. Cytokine concentrations in splenocyte supernatant of hIAPP transgenic and nontransgenic mice.
Note: Data are mean±SE. All mice were 12 months of age. *$p<0.05$
Units: pg/ml

3.5.6 Increased tissue expression of amylin receptors in hIAPP transgenic mice

3.5.6.1 RAMPs in spleen and pancreas

Human amylin is structurally similar to calcitonin gene-related peptide [8]. Calcitonin receptors dimerize with receptor activity-modifying proteins (RAMPs) to generate high-affinity hIAPP receptors. Therefore, RAMP expression in the spleen and pancreas of transgenic and control mice were examined by immunofluorescence microscopy and Western blot. On average, the levels of RAMP1, RAMP2, and RAMP3 were higher in the spleen tissue of the transgenic mice than control mice by, respectively, 221.1% (Figure 33A), 165.5% (Figure 33B), and 147.9% (Figure 33C) ($p < 0.05$ in all cases). All three RAMPs were immunolocalized to the spleen; RAMP2 was the most abundant protein, and RAMP3 was the least abundant. RAMP1, but not RAMP2 or RAMP3, was detected in the pancreas and hIAPP transgenic mice showed 2.8-fold higher RAMP1 levels in the pancreas than did littermate controls (Figure 33D). No protein expression of RAMP-2 or RAMP-3 was observed in the pancreas. Immunofluorescence microscopy confirmed higher RAMP1 levels in adipocytes of the peri-pancreatic fat capsule, T lymphocytes of the PLN, exocrine acinar cells, and endocrine islet cells in transgenic mice (Figure 34). These results suggest that enhanced RAMP protein expression in the spleen,

pancreas and PLN may form hIAPP receptor complexes for human amylin signaling to induce immunity via the expansion of CD4+CD25+ Treg cells.

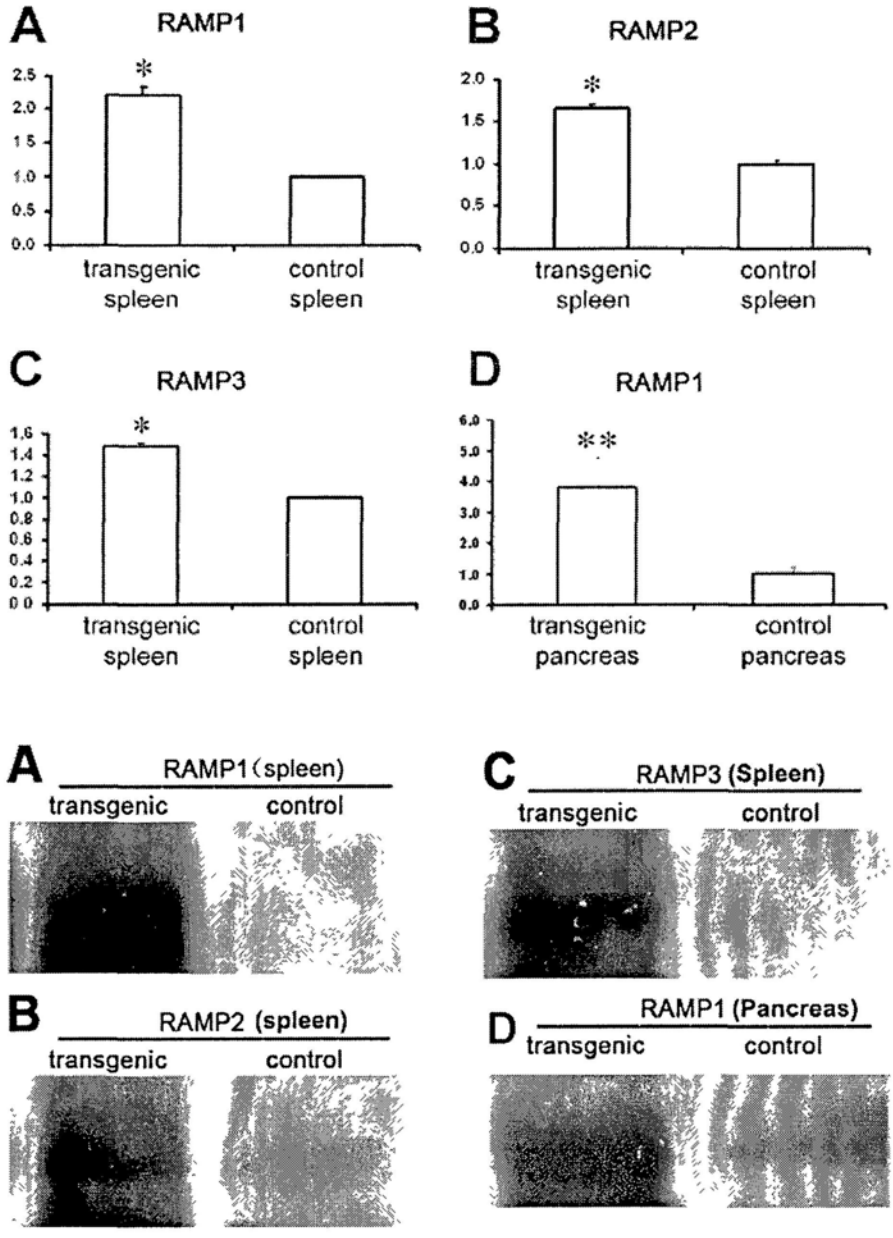

Figure 33. RAMP levels in spleen and pancreas measured by Western blot in hIAPP transgenic and nontransgenic mice.

Equal amounts of tissue extracts (50 µg) of the spleen from hIAPP transgenic mice (n=7) and their nontransgenic littermates (control, n=7) at 12 months of age were subjected to SDS-PAGE and immunoblotted for RAMP1 (A), RAMP2 (B), and RAMP3 (C). Tissue extracts (50 µg) of the pancreas from hIAPP transgenic mice (n=7) and their nontransgenic littermates (control, n=7) at 12 months of age were subjected to SDS-PAGE and immunoblotted for RAMP1 (D). Pixel intensity was normalized to the level of ß-actin, and a value of 1 was assigned to the respective RAMP levels in nontransgenic littermates. Data are expressed as mean ± SD, * $p < 0.05$ vs. control, ** $p = 0.002$ vs. control.

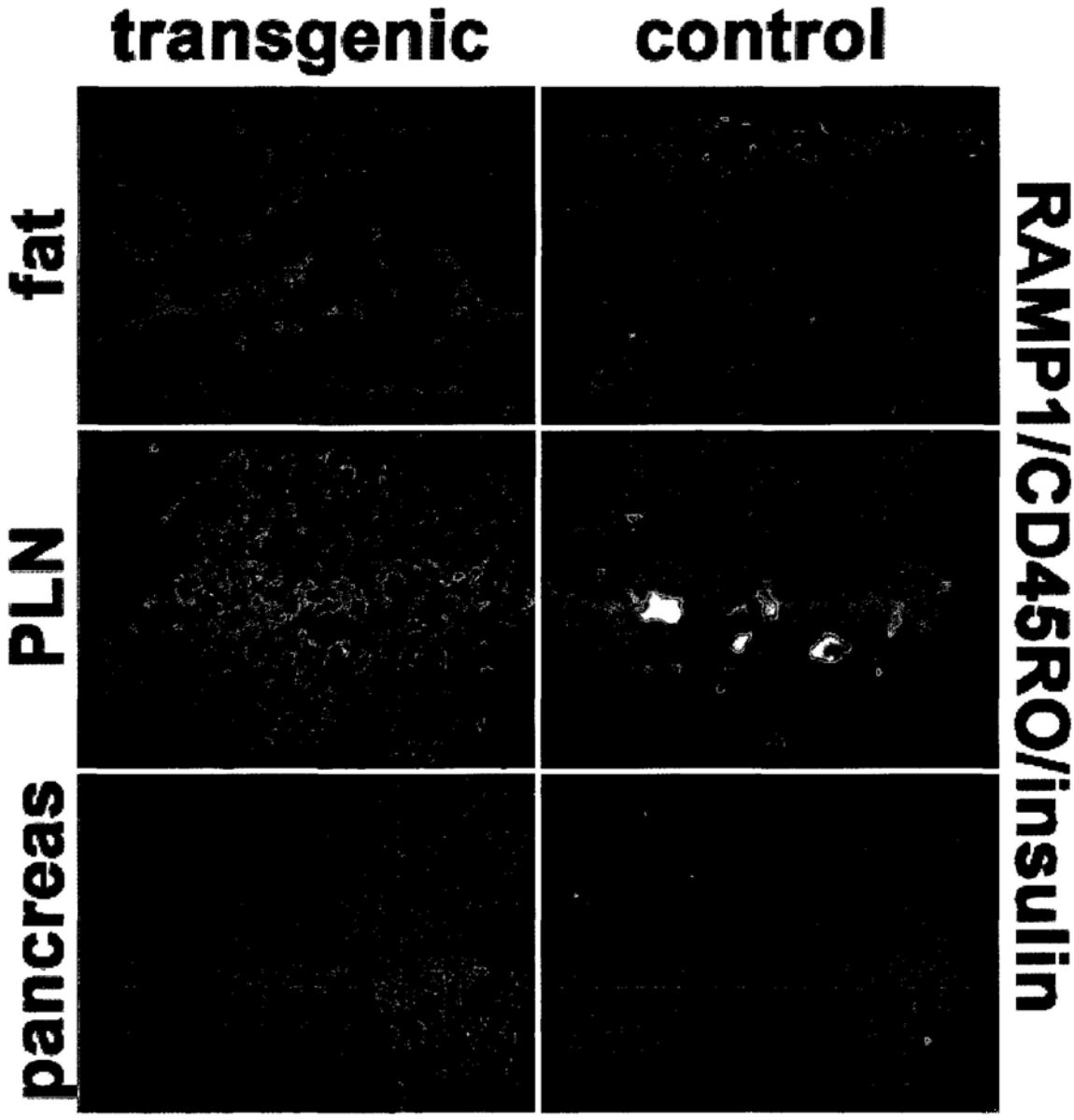

Figure 34. Expression of RAMP1 in the pancreas assessed by immunofluorescence microscopy in hIAPP transgenic and nontransgenic mice. Tissue sections of the pancreas from transgenic and nontransgenic mice at 12 months of age were double-stained for RAMP1 (red), activated T cell marker CD45RO (green) or insulin (green). RAMP1 immunoreactivity (red) was stronger in hIAPP transgenic mice than control littermates in the following regions: the peri-pancreatic adipose tissue, pancreatic lymph node (PLN), exocrine acinus (left, red), and endocrine islet (right, green and red). Original magnification, × 200.

3.5.6.2 Decreased expression of toll-like receptor (TLR)-4

Subsets of T lymphocytes may express TLR which are involved in the control of innate and adaptive immune responses. Expression of TLR may counter the 'immunosuppressive' function of Treg cells to ensure the mounting of a pathological T cell response, if required [140].

Protein expression of TLR-4 was examined using Western blot to explore the association between CD4+CD25+ Treg cells and suppression of PLN. There was lower expression of TLR-4 in transgenic mice at 12 month of age than non-transgenic littermates (Figure 35). This result suggest that reduced expression of TLR-4 might upregulate the function of Treg cells with suppression of PLN.

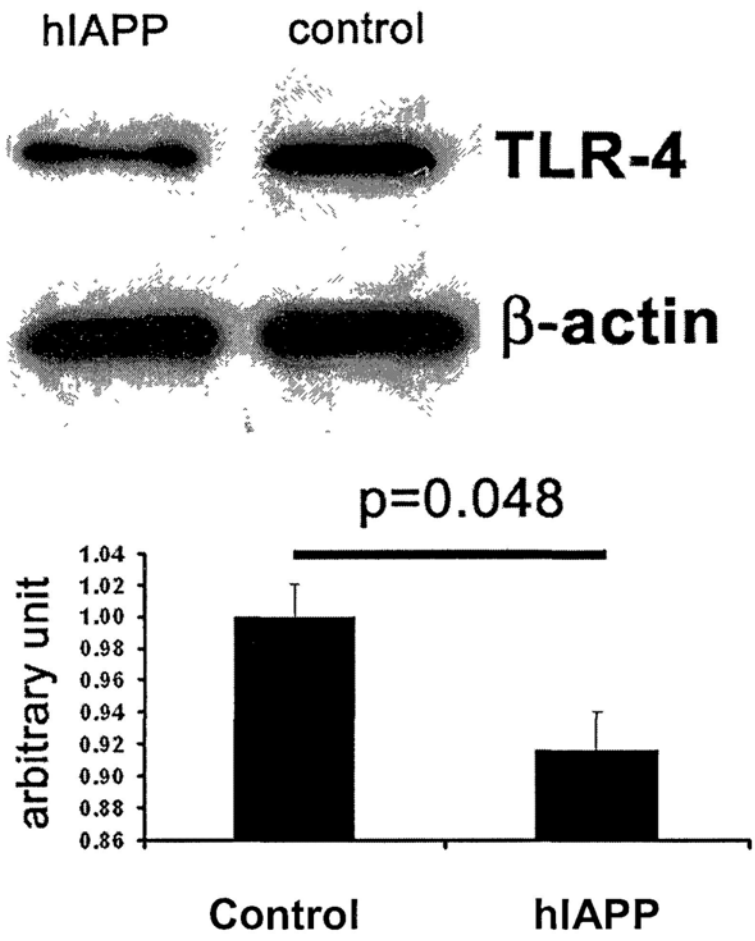

Figure 35. Splenic expression of toll-like receptor (TLR)-4 detected by Western blot in hIAPP transgenic and nontransgenic mice.

Equal amounts of tissue extracts (50 μg) of the spleen tissue from hIAPP transgenic mice (n=7) and their nontransgenic littermates (control, n=7) at 12 months of age were subjected to SDS-PAGE and immunoblotted for TLR-4. Data are expressed as mean ± SD.

4

CHAPTER 4: Discussion

4.1 Use of hIAPP mice as a model of type 1 diabetes

The role of amylin in glucose metabolism remains controversial and relatively under-researched despite its co-secretion with insulin. In this thesis, I originally planned to use hIAPP mice as a model of type 2 diabetes to study the role of amylin toxicity, As discussed in Chapter 3, different hIAPP transgenic animals have been developed which show different phenotypes depending on the genetic background and diet. Previous *in vitro* study by Cooper and coworkers indicated that pancreatic amylin could cause insulin resistance in skeletal muscle with inhibited glycogen synthesis [195]. Twenty years later, the same research group reported spontaneous development of diabetes in hemizygous human IAPP transgenic mice maintained on a FVB/N genetic background known to be associated with insulin resistance. However, these mice did not have islet amyloid or peripheral insulin resistance [196].

Due to these inconsistent findings, I followed the natural aging of these transgenic mice fed on normal diet. In my detailed profiling studies, I did not find any difference between the hIAPP transgenic mice and their non-transgenic littermates in blood glucose profile. Somewhat counter-intuitively, from 12 months onward to 18

months of age, hIAPP transgenic mice exhibited better insulin sensitivity profile than non-transgenic mice. Importantly and surendipitously, I discovered the marked difference in size and number of PLNs between the 2 groups of mice suggesting possible immunomodulating and protective role of human amylin against autoimmune type 1 diabetes.

4.2 Immuno-modulation through amylin signaling

Despite the importance of autoimmunity and innate immunity in both type 1 and type 2 diabetes and that amyloid formation is a hallmark of type 2 diabetes, the immunoregulatory role of human amylin has not been fully explored. In this study and for the first time, in these transgenic hIAPP mice fed on normal chow, I observed 1) over-expression of hIAPP and upregulated RAMPs in the pancreas and spleen; 2) reduced number and size of PLNs; 3) increased expression of CD4+CD25+ Treg cells and 4) increased TGF-β and TNF-α but reduced TLR-4 and proinflammatory cytokine IL-6 levels expression. These findings were further confirmed by *ex vivo* experiments where synthetic human but not rat amylin induced Treg cells and to some extent increased TNF-α and TGF-β expression in splenocytes supernatant isolated from hIAPP nontransgenic mice and PBMCs in healthy blood donors.

These findings are compatible with my overall hypothesis inferred from current knowledge that human amylin, acting through RAMP, may stimulate expansion of CD4+CD25+ Treg cells to suppress formation of PLN formation, an effect possibly mediated by TGF-β and TNF-α. *Ex vivo* administration of amylin also reduced expression of IL-6, a proinflammatory cytokine secreted by dendritic cells (DCs), possibly through suppression of TLR receptor. This series of events can theoretically prevent the onset of insulitis, characterized by infiltration of destructive lymphocytes and phagocytes, in the presence of triggering antigens or events. Here, probably due to the lack of exogenous antigen, apart from diminution of PLN in the hIAPP transgenic mice, both groups of mice had similar histopathological changes. Taken together, these novel results suggest that human amylin may prevent the onset of autoimmune-mediated type 1 diabetes, partly through innate immune mechanisms.

Islet β-cell deficit and dysfunction are hallmarks of both type 1 and type 2 diabetes [108, 186]. Hyposecretion of amylin relative to insulin has been described in NOD mice with autoimmune destruction of islet β-cells [102]. While type 1 diabetic patients had very low or undetectable amylin level, patients with type 2 diabetes also show basal hyposecretion of hIAPP relative to insulin [105]. In advanced stages of type 1 and type 2 diabetes, there is marked amylin deficiency and diminished amylin

response to a meal challenge [42, 197]. It is widely accepted that amylin, as a neuroendocrine hormone, may complement the effects of insulin to maintain glucose homeostasis by suppressing postprandial glucagon secretion [198], slowing the rate of gastric emptying and food transit from stomach to small intestine [166] and suppressing food intake [199]. Moreover, apart from modifying feeding behaviors, amylin can also promote renal water and sodium reabsorption and possibly induce peripheral insulin resistance [8]. Together with its highly conserved nature, amylin appears to have major biological roles although given its pluripotent effects on multipe systems, the net effects of amylin on blood glucose will depend on many prevailing and interacting factors.

4.3 Amylin can suppress pancreatic lymph nodes

In this thesis, the most noticeable finding was the marked diminution of the PLN size in the transgenic mice, suggesting a possible immunomodulating role of hIAPP. Based on this finding and literature review, I postulate that amylin might play a role in autoimmune type 1 diabetes since PLN is the major site where immune cells are primed and inflammatory cytokines activated to mediate autoimmune destruction. Current knowledge also suggests that β cell antigens are taken up by dendritic cells (DCs) in the islets to be transported to the draining PLNs, where antigens are

processed and presented to circulating CD4+ T cells. The activated CD4+ T cells then recruit and activate both specific and non-specific inflammatory cells to cause the inflammatory insulitis infiltrates and eventually β cell destruction [200] [181].

In NOD mice, excision of panLNs (panLNx) at 3 week protected the mice against insulitis and development of diabetes. These interventional findings support the notion that PLNs are site of initial priming of β-cell autoreactive T cells and are a perequisite for the initiation and activation of diabetogenic tendency through autoimmunity [182]. Another evidence supporting the role of the draining lymph nodes as a hideout for pathogenic T cells is the low frequency of pathogenic T cells in the spleens of people with diabetes and that the pathogenic T cells may indeed emerge from the lymph nodes draining from the pancreas. In NOD mice, antigens are presented by the DC in the PLN to activate autoimmune response by inducing cytotoxic Teff cells and other immune cells (Figure 36) [201]. Interestingly, subcutaneous injection of streptozotocin induced time- and dose-related popliteal lymph node enlargement and alteration in lymphocyte subsets in mice [202], a finding that parallels the selective impairment of amylin secretion after streptozotocin treatment [203]. In my study, I found that over-expression of hIAPP can decrease the PLN area and total numbers of PLN where the process of

destructive autoimmunity initiates. These 'surendipitous' findings suggest a possible causal link between enhanced human amylin expression and PLN suppression and that PLNs may be targets for preventing or treating immunity-associated diabetes by supplementing human amylin.

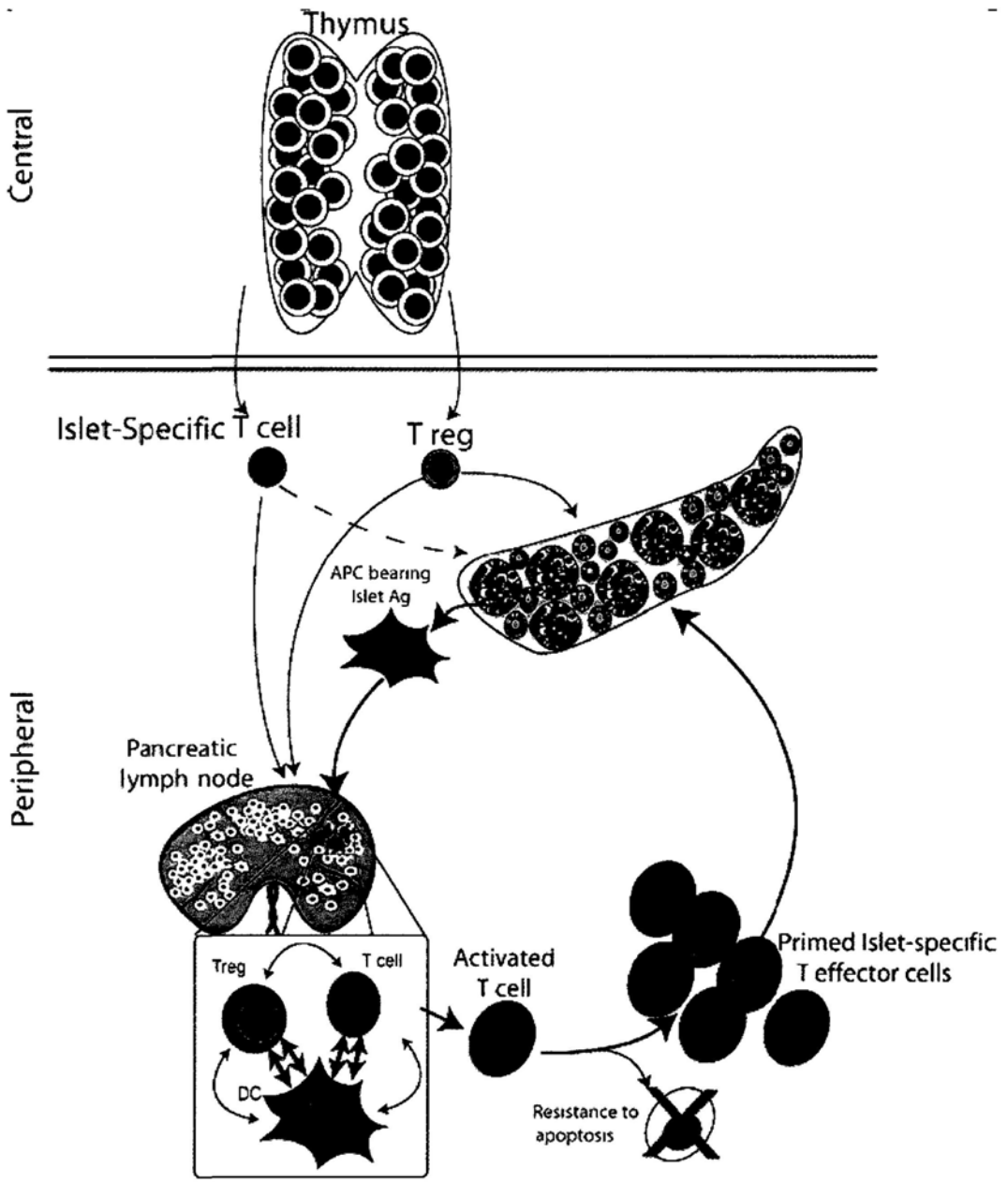

Figure 36. Immune response in NOD mice [204].

β cell antigens are taken up by antigen presenting cells (APC) such as dendritic cells (DCs) in the islets, then transported to the draining pancreatic lymph nodes (PLNs), where the antigens are processed with activation of islet-specific Teff cells to cause the inflammatory insulitis infiltrates and eventually β cell destruction. As a counter mechanism, the DC cells can also activate Treg cells which can modulate these autoimmune responses.

4.4 Amylin stimulates induction of CD4+CD25+ Treg cells

The second finding of my study is the possible causal association between human amylin and Treg cells. I observed a higher percentage of Treg cells in hIAPP transgenic mice which was further confirmed in the *ex vivo* experiment where treatment of splenocytes with human amylin stimulated expansion of Treg cells purified from nontransgenic controls. This finding was further supported by the effects of human amylin on expansion of Treg cells using human PBMC in blood donors.

In the field of type 1 diabetes, there is explosive evidence on the existence and physiological significance of Treg cell-mediated immune suppression as a key mechanism of self-tolerance and immune regulation. The β cell destruction in Type 1 diabetes results from breakdown of self-tolerance which is a consequence of a disequilibrium between Treg and Teff cells [205]. Deficiency or dysfunction of Treg cells can cause autoimmune disease such as type 1 diabetes [139], and innate immune disease such as type 2 diabetes [206]. Indeed, every adaptive immune response recruits and activates not only effecter T and B cells, but also the Treg cells. The balance between these two cell populations is critical in controlling the quality

and magnitude of immune response and maintaining a balance between tolerance to self or non-self antigens [207]. Dysregulation of Treg cells is now considered a primary cause of autoimmune and inflammatory diseases in humans and animal models [129].

Using NOD.Cd28-/- nonobese diabetic mice deficient in Treg cells as an example, these mice develop diabetes at an accelerated rate compared with NOD mice [166]. Injection of NOD Treg cells into NOD.Cd28-/- mice delays, and in some cases, prevents the development of diabetes [166]. Islet antigen–specific Treg cells isolated and expanded from BDC2.5 T cell receptor (TCR)–transgenic mice routinely prevent and even reverse spontaneous autoimmune diabetes in NOD mice [208, 209].

The importance of Treg cells in maintaining immune tolerance in NOD mice [210] is evidenced by their controlling effect on progression from peri-insulitis to destructive insulitis in murine autoimmune diabetes [211]. Defective generation and low numbers of Treg cells predispose NOD mice to autoimmune diabetes [212]. Similarly, protection from autoimmune diabetes by natural killer T cells also requires the activity of Treg cells [213]. Recently, Treg cells have been shown to inhibit the islet innate immune response and promote islet engraftment [214] while islet-specific

Treg cells restore normoglycemia in diabetic NOD mice [215]. Despite these observations, the *in vivo* mechanism of Treg cell in controlling autoimmune diseases remains to be elucidated. Apart from limiting migration, proliferation, and accumulation of Teff cells within the PLNs, Treg cells may also modulate antigen presentation of DC and the consequent Teff cell responses [216-218] [219].

Against this background, I have demonstrated the higher percentage of Treg cells in hIAPP transgenic mice and the induction of Treg cells by amylin *ex vivo.* These findings strongly suggest that these immune changes might play a causal role in suppressing PLN formation, thereby attenuating the priming and activation of destructive CD4+ T cells and preventing the onset of insulitis and autoimmune destruction of pancreatic β cells.

4.5 Association of hIAPP with toll-like receptor

The third finding of this study is that the expression level of Toll-like receptor 4 (TLR-4) was lower in transgenic mice than non-transgenic littermates. The family of TLRs is a major class of receptors expressed in many different types of immune cells such as DCs, T cells, neutrophils, eosinophils, mast cells, macrophages, monocytes and epithelial cells [220, 221]. These receptors recognize a limited but highly

conserved set of molecular structures (pathogen-associated molecular patterns or PAMPs), which are produced by microorganisms including bacteria, viruses, fungi and protozoa. Apart from endogenous and exogenous ligands, TLRs can also recognize and bind to degradation products of endogenous macromolecules such as heparin sulphate and polysaccharide fragments of hyaluronan. The latter are markers of tissue injury, infection and tissue remodeling [141, 222]. There is emerging evidence showing that toll-like receptors (TLRs) play dual role in innate and adaptive immune responses [223, 224]. In this context, patients with type 1 diabetes exhibit evidence of dysregulated innate immune functions which may contribute to the onset of autoimmune diabetes [225].

By activating signaling pathways that lead to production of proinflammatory cytokines and up-regulation of co-stimulatory molecules, TLR may modulate both innate and adaptive immune responses [223]. Here, TLR signaling may directly or indirectly regulate the immunosuppressive function of Treg cells in autoimmune diseases, infectious diseases, graft rejection and cancers [141, 143, 226-228]. By shifting the balance between CD4+ T-helper cells and Treg cells, TLR signaling may influence the outcome of the immune response [141]. Patients with insulin resistance and type 2 diabetes show increased expression and signaling of TLR-4 in skeletal

muscle together with activated inflammatory pathways, including inhibitor of κB (IκB)/nuclear factor κ (NFκB) [229]. Pasare and co-workers have shown that engagement of TLR-4 on freshly isolated mouse splenic DCs could abrogate the suppressive function of Treg cells through expression of IL-6. The latter can render Teff cells resistant to Treg cells-mediated suppression [143].

The effects of TLR stimulation on Treg and Teff cells appear to be highly complex. In one experiment, pretreatment of the murine Treg cells with LPS, one of the TLR-4 stimulator, enhanced the survival and suppressive capacity of Treg cells. By contrast, Treg cells isolated from TLR-4 deficient mice did not respond to the same stimuli [230]. However, these findings were not replicated in another study which used a highly purified LPS (free of contaminating TLR-2 ligands) to activate murine Treg cells [231].

In my experiments, I found decreased expression level of TLR-4 and increased percentage of Treg cells in hIAPP transgenic mice compared to nontransgenic mice, raising the possibility that overexpression of human amylin might prevent activation of the TLR signaling pathway which can contribute to the autoimmune destruction of β cell. Indeed, given the many types of TLRs and immune cells which can be

activated by different external/internal stressors to promote or protect against β cell destruction through innate and autoimmune mechanisms, the situation can be highly complex.

4.6 Effect of hIAPP on immune cytokines

The fourth finding of this study is that hIAPP transgenic mice had higher levels of the multifaceted cytokines, TGF-β and TNF-α, but lower levels of Interleukin-6 (IL-6) than control mice. TGF-β is involved in immuneregulatory role of Treg [232]. Treg cells maintain self-tolerance and suppress a wide array of targets by acting through a TGF-β-dependent pathway [233]. Progression to overt type 1 diabetes is related with escape by pathogenic T cells from TGF-β-dependent Treg immune regulation.[234]. Furthermore, a transient pulse of the cytokine TGF-β, when delivered to the islets, was able to regulate *in vivo* expansion of Foxp3-expressing Treg cells responsible for protection against diabetes [191]. Administration of TGF-β eliminates autoimmune diabetes in NOD mice and protects against effector lymphocytes [149]. Moreover, DCs stimulated by TGF-β1 induce differentiation of islet-protecting Foxp3+ Treg cells [235]. Other studies have shown that the number of Treg cells co-expressing Foxp3 and TGF-β1 decreased during autoimmune diabetes [236]. TGF-β1 gene therapy blocks islet destructive autoimmunity and

facilitates regeneration of beta-cell function in NOD mice with overt diabetes [237]. On the other hand, induction of suppressive signal of Treg cells requires the pathogenic T cells to be permissive to accept the suppressive signal. Studies have shown that pathogenic T cells with a dominant negative TGF-β type II receptor cannot be suppressed due to the lack of TGF-β signal. These findings provide further support that the suppressive effects of Treg are mediated in a TGF-β dependent manner [150, 189, 238]. Recently, Filippi *et al* showed that Tregs modulated by viral infection *in vivo* are capable of diminishing the incidence of Type 1 diabetes through TGF-β production [239].

These data indicate that TGF-β signaling is required for the function of both Treg T cells and pathogenic T cells in regulating the host immune status depending on the prevailing environment [190]. In my experiments, overexpression of hIAPP increased percentage of Tregs and expression of TGF-ß which are consistent with the TGF- ß dependent nature of the suppressive function of Treg cells.

In this study, I also found increased TNF-α expression in the hIAPP mice of 12 month old with increased Treg cells compared with non-transgenic littermates. Here, TNF-α is an anti-inflammatory cytokine with pleiotropic effects and plays an

important role in autoimmune type 1 diabetes. Treatment with TNF-α can delay onset of diabetes in the adult NOD mice [154, 158, 193]. This was in part mediated by increasing the number and function of Treg cells [240]. Local expression of transgene-encoded TNF-α in islets prevents autoimmune diabetes in NOD mice by inhibiting auto-reactive islet-specific T cells and by induction of Treg cells [193]. On the other hand, increased hIAPP and TNF-α expression has been associated with insulin resistance in human subjects [241], which theoretically can lead to type 2 diabetes especially in subjects with reduced beta cell mass/function for other reasons. Since both TNF-α and TGF-β are key mediators of innate immune responses, these findings highlight the complex interplay between adaptive and innate immune responses which can be triggered by different external/internal stressors. Thus, it is plausible that an efficient innate immune response characterized by increased cytokines (TGF-β and TNF-α), in part mediated by human amylin, may prevent autoimmune type 1 diabetes but increase risk of type 2 diabetes through increased insulin resistance.

4.7 IL-6 in autoimmune disease through Treg cells

IL-6 is a multi-functional cytokine implicated in a variety of autoimmune diseases, including antigen-induced arthritis, collagen-induced arthritis, myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE) and pristine-induced lupus [242-245]. IL-6-deficient mice are resistant to all these diseases [246-248] which may be due to the ability of IL-6 in rendering pathological T cells refractory to suppression by Treg cells. In support of this idea, postnatal deletion of IL-6 by administration of anti-IL-6 antibodies in NOD/Wehi mice inhibited the development of autoimmune diabetes [163]. On the other hand, during chronic infections, secretion of IL-6 can lead to the activation of autoreactive T cells. Over-expression of IL-6 in the pancreatic islet beta cells in IL-6 transgenic mice further induced B lymphocyte differentiation to cause autoimmune type 1 diabetes [249]. The important role of B cell in type 1 diabetes is evidenced by the improved outcome of autoimmune diabetes in B cell-deficient NOD mice [250]. Thus, apart from attenuating the ability of Treg cells to suppress autoimmune tenancy, IL6 can also activate self-reactive B and T cells, all of which will contribute to the initiation, perpetuation and/or acceleration of autoimmune process. Thus, in my experiment, the decreased IL-6 expression in transgenic hIAPP mice is expected

to suppress the autoactive T cells and increase the suppressive function of Treg cells with reduced number and size of PLN.

4.8 Human amylin or amyloid associated with immune modulation

It is well recognized that hIAPP forms islet amyloid in type 2 diabetes [1]. An important question arising from this study is whether it is hIAPP itself or hIAPP-derived amyloid that alters immunity. Consistent with other reports [251], in these transgenic mice fed with normal chow, I failed to detect hIAPP amyloid deposits in the islets of hIAPP mice up to 12 months of age. However, there was evidence of increased expression of RAMP and altered immune responses in these transgenic animals confirming the presence of amylin signaling. In a mice model of Alzheimer's disease with overexpression of amyloid β (Aβ), extensive brain amyloid deposits were found in animals over the age of 12 months [252]. In the mice model of Alzheimer's' disease kept in our laboratory, we did not find differences in the number and size of PLNs between the transgenic mice with brain amyloid deposits and nontransgenic amyloid-free littermate control mice. Furthermore, we only found increased expression of the amylin receptor (RAMPs) in the hIAPP transgenic mice but not the Aβ transgenic mice [unpublished data]. These data strongly suggest that

human amylin itself, rather than human amylin-derived amyloid, induced Treg cell expansion and associated PLN suppression in the hIAPP transgenic mice.

5

CHAPTER 5: Conclusions and Future Research

5.1 Conclusions

In this thesis, I set out to test the hypothesis that amylin oligomerization or amyloid formation might cause type 2 diabetes in a hIAPP transgenic mice model. During the metabolic profiling of the mice fed with a normal diet, I noted the marked reduction in PLN in the transgenic mice of 12 and 18 months of age. Detailed review of literatures suggests a possible immunomodulating role of human amylin although this has not been systematically studied. In a series of experiments, I have made the following observations:

1. The hIAPP transgenic mice showed improved insulin sensitivity with reduced insulin to amylin ratio from 3 months of age and onward.
2. On average, the levels of RAMP1, RAMP2, and RAMP3 were higher in the splenocytes of the transgenic mice than the control mice.
3. The hIAPP transgenic mice had reduced PLNs in terms of percentage number and size per islet area.
4. The hIAPP transgenic mice had decreased expression level of TLR-4 in the spleen tissue.
5. The hIAPP transgenic mice had augmented protein expression and secretion of the immune cytokines TGF-β, TNF-α but decreased level of IL-6 in the

supernatants of splenocytes

6. Expression levels of immune cytokines such as interleukin (IL) -1, IL-2, IL-4, IL-10, IL-17, interferon-γ and GM-CSF in the splenocytes were similar between the hIAPP transgenic and nontransgenic mice.
7. *Ex vivo* treatment with human amylin but not rat amylin increased the percentage of Treg cells in splenocytes from non-transgenic hIAPP littermates and PBMCs from healthy blood donors.
8. *Ex vivo* treatment of splenocytes from nontransgenic mice with hIAPP tended to increase secretion of TNF-α and TGF-β albeit short of significance.

Based on these findings, I conclude that human amylin may cause induction of human RAMPs to activate APC such as macrophages to secrete cytokines (TGF-β and TNF-α) which may be associated with the expansion of Treg cells to suppress formation of PLNs. Human amylin can also down-regulate the expression level of IL-6 through engagement of TLR-4 receptor in dendritic cells. These changes may enhance the ability of splenic Foxp3+CD4+CD25+ Tregs to suppress activation of autoreactive T cells. The complex interplay between these innate and adaptive immune responses may prevent the onset of insulitis characterized by infiltration of destructive lymphocytes and phagocytes and thus autoimmune type 1 diabetes.

5.2 Limitations

Despite these novel findings, the possible immunomodulating role of amylin in preventing autoimmune type 1 diabetes is largely hypothesis-generating. To test this hypothesis further, more experiments are needed to address the following issues:

1. Additional investigations are needed to determine whether human amylin-induced changes in the functions of Treg cells and other cell subsets such as natural killer cells, B lymphocytes and monocytes in the spleen of hIAPP transgenic mice.

2. The suppressive ability of FoxP3+ Treg cells on Teff cells in a dose-dependent manner should be measured.

3. The interrelationships between human amylin and cytokines such as TGF-β and TNF-α in causing the changes in Treg cells need to be clarified.

4. Lymph nodes in anatomical sites other than the PLN of the hIAPP transgenic mice should be examined to determine whether this was a regional or generalized phenomenon.

5. Finally, the effects of human amylin treatment on onset of autoimmune diabetes in NOD mice will need to be examined followed by clinical studies.

5.3 Amylin in diabetes

In my study, human amylin stimulated transformation of Treg cells from splenocytes which are known to have effect on both autoimmunity and innate immunity. Both human amylin and insulin are co-synthesized and co-secreted mainly from differentiated islet β-cells. Studies have shown that enhanced human amylin expression may inhibit proinsulin synthesis [66] which is considered to be a major autoantigen associated with autoimmune pancreatic β-cell destruction in human and NOD mice [253, 254]. Although autoantibodies to human amylin have been detected in type 1 and type 2 diabetes and insulinoma [255, 256], these findings are not always replicable [257]. Besides, given the shared homology between insulin and amylin and their precursors and possible cross-reactivity between these antibodies, the significance of these findings remains to be confirmed.

Against this background, I hypothesize that since food consumption is inevitably associated with proinsulin/insulin release which can serve as autoantigens as well as increased risk of exposure to external toxins and microbial agents, an efficient amylin system with induction of human RAMP receptor may stimulate the expansion of Tregs which can suppress both innate immunity (decreased expression of TLR-4 and IL-6) and autoimmune immunity (Figure 36).

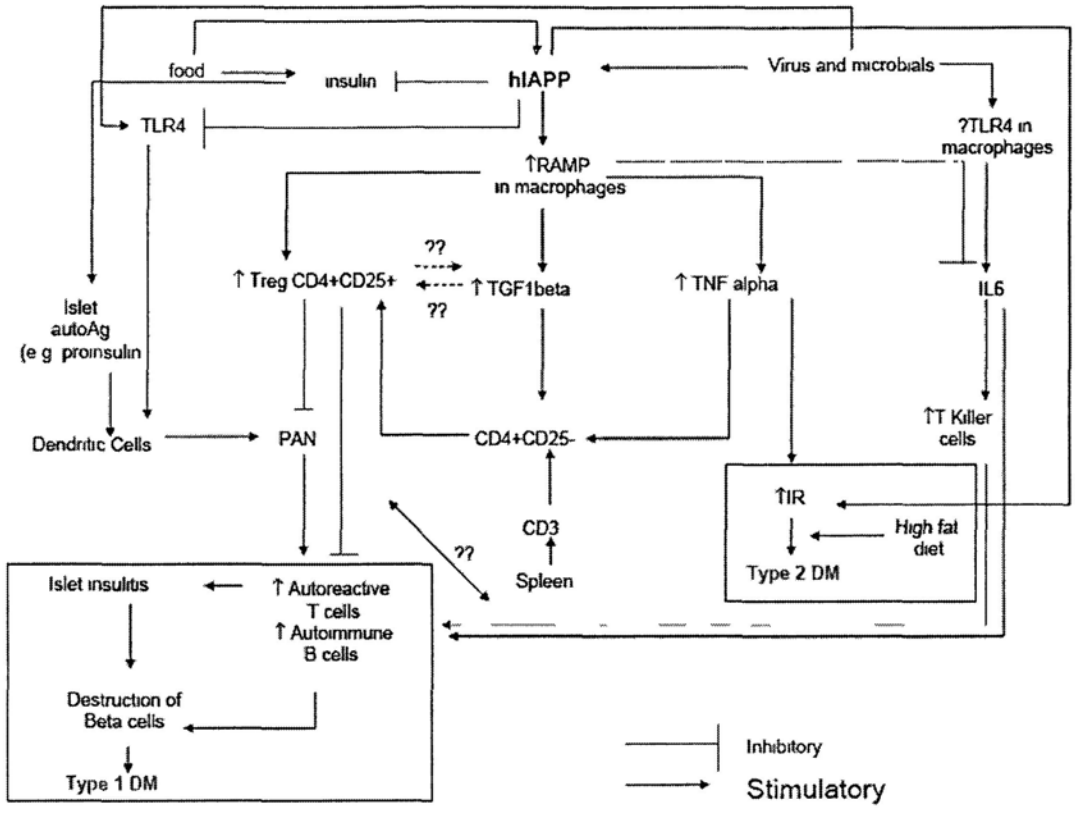

Figure 36: Possible dual role of amylin in the pathogenesis of type 1 and type 2 diabetes.

Amylin signaling through RAMP can modulate both innate and adaptive immune systems to increase CD4+CD25+Treg cells and suppress PLNs to reduce risk of autoimmunity and type 1 diabetes. From an evolutionary perspective, an efficient amylin system in a pathogen-rich environment may confer survival benefits. However, due to the pluripotent effects of amylin, in an obesogenic environment, an efficient amylin system may interact with obesity-associated adipokines to increase risk of insulin resistance and beta cell failure due to amylin toxicity resulting in increased risk of type 2 diabetes.

5.4 Implications and future research

Thus, evolutionally, in primitive and pathogen-rich communities, an efficient amylin system may be naturally selected to protect against exogenous microbials and

increase self tolerance to reduce risk of autoimmunity. While this efficient innate system may promote survival in a pathogen-rich environment and reduce risk of allergic and autoimmune disorders, in an obesogenic environment, a hyperamylinemic response may induce an insulin resistant state with secondary beta cell failure in part due to amylin toxicity. Clearly, more mechanistic studies are needed to confirm this double-edged effect of amylin in maintaining an optimal balance between innate and adaptive immune responses for survival. Confirmation of this hypothesis will have major implications on the prevention and amelioration of both type 1 diabetes and type 2 diabetes.

6

List of original articles and conference papers

6.1 Contribution to original articles published during my PhD study period

1. Sui Y, Zhao HL, Fan RR, Guan J, **He L**, Lee HM, Chan JC, Tong PC. Renin-Angiotensin System Activation in Renal Adipogenesis. Am J Physiol Renal Physiol. 2009 Nov 25. PMID: 19940035

2. Zhao HL, Guan J, Sui Y, **He L**, Lai FF. Islet amyloidosis and beta-cell neogenesis in chronic calcifying pancreatitis with non-insulin-dependent diabetes mellitus. Pancreas. 2009 Apr;38(3):342-4. PMID: 19307931

3. Guan J, Zhao HL, Baum L, Sui Y, **He L**, Wong H, Lai FM, Tong PC, Chan JC. Apolipoprotein E polymorphism and expression in type 2 diabetic patients with nephropathy: clinicopathological correlation. Nephrol Dial Transplant. 2009 Jun;24(6):1889-95. 2009 Feb 14. PMID: 19218599

4. Zhao HL, Sui Y, Guan J, **He L**, Gu XM, Wong HK, Baum L, Lai FM, Tong PC, Chan JC. Amyloid oligomers in diabetic and nondiabetic human pancreas. Transl Res. 2009 Jan;153(1):24-32. 2008 Nov 19. PMID: 19100955

5. Zhao HL, Sui Y, Guan J, **He L**, Lai FM, Zhong DR, Yang D, Baum L, Tong PC, Tomlinson B, Chan JC. Higher islet amyloid load in men than in women with type 2 diabetes mellitus. Pancreas. 2008 Oct;37(3):e68-73. PMID: 18815541

6. Zhao HL, Sui Y, Guan J, **He L**, Zhu X, Fan RR, Xu G, Kong AP, Ho CS, Lai FM, Rowlands DK, Chan JC, Tong PC. Fat redistribution and adipocyte transformation in uninephrectomized rats. Kidney Int. 2008 Aug;74(4):467-77. 2008 May 21. PMID: 18496513

7. Zhao HL, Sui Y, Guan J, Lai FM, Gu XM, **He L**, Zhu X, Rowlands DK, Xu G, Tong PC, Chan JC. Topographical associations between islet endocrine cells and duct epithelial cells in the adult human pancreas. Clin Endocrinol (Oxf). 2008 Sep;69(3):400-6. 2008 Jan 21. PMID: 18221396

6.2 Articles under preparation:

1. **Lan He***, Hai-Lu Zhao*, Heung Man Lee, Yi Sui, Jing Guan, Man Ting Siu, Pui Pui Tse, Fernand M Lai, Juliana C Chan. Diminished Pancreatic Lymph Nodes and Increased Splenic CD4+CD25+ Regulatory T cells in Transgenic Mice Overexpressing Human Amylin

2. **Lan He**, Hai-Lu Zhao, Xiao-ling Li, Yi Sui, Juliana C Chan. Green Tea Epigallocatechin-3-Gallate Prevents Oligomer Formation of human islet amyloid polypeptide

3. **Lan He***, Hai-Lu Zhao*, Yi Sui, Juliana C Chan. Oligomerization of human islet amyloid polypeptide and metabolic profile in transgenic mice

6.3 Conference abstracts

1. **Lan He**, Y Sui, X Zhu, LZ Liu, XM Gu, HL Zhao, Fernand MM Lai, Peter CY Tong, Juliana CN Chan. Oligomerization of Islet Amyloid Polypeptide Precedes the Development of Diabetes The American Diabetes Association 67th Scientific Sessions, 22-26 June 2007, Chicago, IL, USA. Diabetes 2007;56 (Suppl 1): A811 (2698-PO)

2. Xun Zhu, Yi Sui, Jing Guan, **Lan He**, LZ Liu, HL Zhao, LW Lam, T James, JC Chan, PC Tong. Pancreatic beta-cell function during oral glucose tolerance test in Zucker lean rats. The American Diabetes Association 67th Scientific Sessions, 22-26 June 2007, Chicago, IL, USA. Diabetes 2007;56 (Suppl 1): A675 (2666-PO).

3. **Lan He**, Hai-lu Zhao,Yi Sui,Ebenezer KC Kong, Fernand MM Lai, Peter CY Tong, Juliana CN Chan. Islet Oligomerization in Transgenic Mice Over-Expressing Human Islet Amyloid Polypeptide The American Diabetes Association 67th Scientific Sessions, The American Diabetes Association 68th Scientific Sessions, 6-10 June 2008, San Francisco, CA, USA. Diabetes 2008;57 (Suppl 1): A857 (1667-Poster).

4. Yi Sui, Hai-Lu Zhao, Rong-Rong Fan, **Lan He**, Jing Guan, Gang Xu, Juliana CN Chan, Peter CY Tong. Ectopic fat distribution associated with hyperlipidemia due to dysregulation of angiotensin ands HMG-CoA reductase in uninephrectomized rats. The American Diabetes Association 68th Scientific Sessions, 6-10 June 2008, San Francisco, CA, USA. Diabetes 2008;57 (Suppl 1): A707 (2594-PO).

5. Jing Guan, Hai-Lu Zhao, Yi Sui, **Lan He**, Harriet Wong, Peter CY Tong, Juliana CN Chan. Apolipoprotein E polymorphism and expression in type 2 diabetic patients with nephropathy: Clinicopathological correlation. The 6th Congress of the Asian Pacific Society of Atherosclerosis and Vascular Disease & 10th Hong Kong Diabetes and Cardiovascular Risk Factors East Meets West Symposium, 25 - 28 September 2008, Hong Kong Convention and Exhibition Center, Hong Kong. Programme & Abstract Book, p48 (Ab62).

6. **Lan He**, Hai-Lu Zhao, Xiao-Ling Li, Yi Sui, Peter C. Tong, Juliana C. Chan Green Tea Epigallocatechin-3-Gallate Prevents Oligomer Formation of Human Islet Amyloid Polypeptide The American Diabetes Association June 5 - 9, 2009, New Orleans, Louisiana, CA, USA.Diabetes 2009;(Suppl 1): (2404-PO)

7. **Lan He**, Hai-Lu Zhao, Yi Sui, Jing Guan, Heung Man Lee, Man Ting Siu , Juliana C Chan. Diminished Pancreatic Lymph Nodes and Increased Splenic CD4+CD25+ Regulatory T cells in Transgenic Mice Over-expressing Human Amylin. The American Diabetes Association 70th Scientific Sessions, June 25-29, 2010, Orlando, FL, CA. (1335-Poster) ,

7

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8

Appendix

8.1 Appendix 1

Specificity of Mercodia Rat Insulin ELISA (Mercodia, Sweden)	
Human insulin	167%
Human proinsulin	75%
Human C-peptide	< 0.05%
Insulin lispro (Humalog®)	167%
IGF-I	< 0.02%
IGF-II	< 0.02%
Rat C-peptide	<0.001%
Rat proinsulin	7%
Mouse insulin	75%
Porcine Insulin	476%
Sheep Insulin	179%
Bovine Insulin	78%

8.2 Appendix 2

Crossreactivity of Human Amylin ELISA kit (Linco Research, USA)	
Human Glucagon	<1%
Human GLP-1	<1%
Human Insulin	<1%
Human Pancreatic Polypeptide	<1%
Human Adrenomedullin	1%
Human Calcitomin	<1%
Calcitonin Gene Related Peptide	<1%

Note:
This kit is suitable for the measurement of amylin in rat and feline plasma; however, the precise percentage of crossreactivity has not been determined.

Sensitivity:
The lowest level of human amylin that can be detected by this assay is 1 pM (50 uL plasma sample size).