Institutionen för medicin, Solna

A Study of Biomarker Analysis in Association with Type 1 Diabetes and Their Shared Features in Rheumatoid Arthritis

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av

Chengjun Sun

Huvudhandledare:
Docent Carani B. Sanjeevi
Karolinska Institutet
Institutionen för medicin, Solna

Bihandledare:
Professor Lars Klareskog
Karolinska Institutet
Institutionen för medicin, Solna
Enheten för reumatologi

Professor Åke Lernmark
Lunds Universitet
Institutionen för kliniska vetenskaper, Malmö
Enheten för diabetes och celiaki

Fakultetsopponent:
Professor Anna-lena Spetz
Stockholms universitet
Institutionen för Molekylär biovetenskap, Wenner-Grens institut

Betygsnämnd:
Docent Nandakumar Kutty Selva
Karolinska Institutet
Institutionen för medicinsk biokemi och biofysik
Enheten för medicinsk Inflammationsforskning

Professor Peter Bang
Linköpings universitet
Institutionen för klinisk och experimentell medicin

Docent Md.Shahidul Islam
Karolinska Institutet
Institutionen för klinisk forskning och utbildning, Södersjukhuset

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From the Department of Medicine, Solna (MedS)
Karolinska Institutet, Stockholm, Sweden

A STUDY OF BIOMARKER ANALYSIS IN ASSOCIATION WITH TYPE 1 DIABETES AND THEIR SHARED FEATURES IN RHEUMATOID ARTHRITIS

Chengjun Sun

Karolinska Institutet

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To my mother and father

ABSTRACT

Type 1 diabetes (T1D) is mediated by abnormal immune system (autoimmunity) that targeting specifically to self insulin-producing cells (β-cells). People with T1D require treatments based on life-long insulin substitution. In addition to the damages in health caused by T1D complications, the complexity of insulin treatment and the fear of glucose dysregulation often place extra burden to the affected family. There is a current need for better understanding of the disease etiology therefore guide the construction of successful prediction and prevention strategies for the disease.

There are many immune-related genes playing important roles in T1D etiology. In addition, there is a trend of autoimmune diseases segregating within individuals and families where those genes are critically involved. Exploration of these genes can provide knowledge related to the disease pathogenesis. In my studies, we select two genes functioning in the immune system and explored their potential roles in T1D. In addition, we analyzed the association between *HLA* alleles and T1D autoimmune markers (autoantibodies) in rheumatoid arthritis patients.

Killer cell immunoglobulin like receptors (KIRs) is a group of receptors expressed on the surface of natural killer cells and subgroups of T cells. KIRs could accelerate autoimmune diabetes in rodent models. However their roles in human T1D are not clear. In **Study I**, we studied the T1D association of *KIR* genes and their combination with *HLA*-C ligand genes in Chinese Han population. Our results indicated that *KIR* modifies the T1D association of *HLA*-C ligand genes. Recent studies indicated that KIRs exert their function in a collective fashion and their effects can initiate as early as life in uterus by maternal-fetal interaction. Therefore in **Study II**, we studied the T1D association with the collection of maternal *KIR* genes and their combination with fetal *HLA*-C ligand genes in the Chinese Han population. Results from study II indicated that the accumulation of maternal activating *KIR*s along with fetal *HLA*-C2 genes predispose T1D in the fetus.

Alpha-B crystallin (encoded by *CRYAB*) is a major autoimmune target in multiple sclerosis (an autoimmune disease occurred in central nervous system). In **Study III**, we tested the association between *CRYAB* gene and islet autoantibodies in T1D using two well-established Swedish cohorts. Our results suggested that genetic variant in the promoter region of *CRYAB* is associated with increased T1D risk and islet autoantibodies in T1D patients.

In **Study IV**, we aimed to identify genetic factors that cause the aggregation of the two autoimmune disorders, T1D and rheumatoid arthritis (RA). We measured islet autoantibodies among RA patients and analyzed the association between *HLA* and islet autoantibodies in RA patients and in subgroups of RA positive for anti-citrullinated protein antibodies. We identified that *HLA* DR4 alleles were associated with increased islet autoantibodies in RA patients, however *HLA* DR3 alleles were the major genetic contributors toward elevated islet autoantibodies among RA patients positive for both anti-CCP and anti-CEP-1.

In conclusion, our studies indicated that *KIR*, *CRYAB* are among the genetic factors predisposing T1D. In addition, *HLA* alleles are the major contributors to the presence of islet autoantibodies among RA patients.

A FRIENDLY PROLOGUE

After my medical education, I started to work as a pediatrician in endocrinology from 2008 in China. I saw children with diabetes and their families. Most of the time the parents were happy at first when their children recovered from the very sick state but sorrowing soon after being told that a life-long treatment to their children is required. Thank to the insulin which makes these children no longer die shortly after the disease onset. But the family's afraid of uncontrolled blood sugar and diabetes related illness will never go away for the rest of those children's life. At that time, I also saw within many families where the unawareness of the disease led to serious diabetes related illness occurring very early to their children. In fact, we doctors are the ones who are responsible for delivering knowledge to the patients' families. However I feel sheepish at that moment that I was not able to aid much to help the patients due to my own lack of knowledge on the disease. Therefore when I was offered the opportunity to study the disease in a country with much advanced health care, Sweden, I hardly hesitated.

During my years in Sweden, aside from catching up in English skills, there was much leant from my supervisors and colleagues. I feel I am able to explain more to my patients after my return. The diabetes that I studied is type 1 diabetes. It often occurs in children and in the disease, the destruction of the insulin producing cells makes the affected person have to rely on artificial insulin replacement for survival. The destruction of insulin producing cells mediated by our own immune system is influenced by many factors. The study on these factors is constructing the base of a pyramid on top of which stands the cure of the disease. While reminded by the patients' families' wish that their children can live a life as healthy children do, from a doctor's view I felt that the pyramid is still awaiting its completion. In fact, there are many encouraging new treatments under development and providing happier lives to those T1D families.

In this book, I arranged the knowledge that I gained during my last five years regarding type 1 diabetes along with my studies. With a feeling of more to study in the future, I wish my work in these past years aided our understanding of the disease.

LIST OF SCIENTIFIC PAPERS

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II. **Sun C**, Luo F, Zhi D, Sanjeevi CB. Interactions between maternal killer cell immunoglobulin receptor genes and fetal HLA ligand genes contribute to Type 1 diabetes susceptibility.
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III. **Sun C**, Sedimbi SK, Ashok AK, Sanjeevi CB; Swedish Childhood Diabetes and the Diabetes Incidence in Sweden Study Groups. CRYAB-650 C>G (rs2234702) affects susceptibility to Type 1 diabetes and IAA-positivity in Swedish population.
Human immunology. 2012 Jul;73(7):759-66.

IV. **Sun C**, Ramelius A, Israelsson L, Lernmark Å, Klareskog L, Sanjeevi CB. Autoantibodies against Type 1 Diabetes autoantigens in Rheumatoid Arthritis.
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* Contributed equally

OTHER PUBLICATIONS NOT INCLUDED IN THESIS

1. **Sun C**, Zhi D, Shen S, Luo F, Carani B.Sanjeevi. SNPs in the exons of Toll-Like Receptors are associated with susceptibility to Type 1 diabetes in Chinese population.
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   Diabetic medicine. 2014 Feb;31(2):219-26.

3. Zhao Z*, **Sun C***, Wang C, Li P, Wang W, Ye J, Gu X, Wang X, Sheng S, Zhi D, Lu Z, Ye R, Cheng R, Xi L, Li X, Zheng Z, Zhang M, Luo F. Rapidly rising incidence of childhood type 1 diabetes in Chinese population: epidemiology in Shanghai during 1997-2011.
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   * Contributed equally

CONTENTS

LIST OF ABBREVIATIONS

ACPA	Anti–citrullinated protein antibody
APC	Antigen presenting cells
CCP	Cyclic citrullinated peptides
CNV	Copy number variant
DC	Dendritic cell
DIAMOND	Multinational Project for Childhood Diabetes
ENCODE	Encyclopedia of DNA elements (project)
GADA	Glutamatic acid decarboxylase autoantibodies
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
IA-2A	Insulinoma associated antigen-2 autoantibodies
IAA	Insulin autoantibodies
KIR	Killer-cell immunoglobulin-like receptor
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NK	Natural killer (cell)
NOD mice	Non-obese diabetes mice
OMIM	Online Mendelian inheritance in man
PCR	Polymerase chain reaction
RA	Rheumatoid arthritis
RIA	Radioimmunoassay
SE	Shared epitope
SNP	Single nucleotide polymorphism
T1D	Type 1 diabetes
VNTR	Variable number tandem repeat
WHO	World Health Organization
ZnT8A	Zinc transporter 8 autoantibodies

1 INTRODUCTION

1.1 Type 1 diabetes

People affected by diabetes are not able to maintain their blood glucose at a normal (low) level. The improperly controlled diabetes can create metabolic disturbance which in turn leads to acute life-threatening conditions (hyperosmolarity or acidosis) and, chronic hyperglycemia (the elevated blood glucose). Chronic hyperglycemia is able to produce a wide variety of complications, including nephropathy (damages in the kidney), neuropathy (damage in the nervous system), cardiomyopathy/angiopathy (damages in the cardiovascular system) and, retinopathy (damage in the retina).

Type 1 diabetes (T1D) is a subgroup of diabetes and accounts for around 10% of total diabetes [1]. T1D is described as diabetes with a sudden, severe presentation at diagnosis–i.e., diabetic ketoacidosis, a lack of endogenous insulin production therefore absolute requirement of exogenous insulin and, the major form of diabetes that occur in childhood/early adulthood while obesity is not commonly found among affected patients.

Owing to the above characteristics, T1D was termed childhood diabetes/juvenile diabetes, thin diabetes and insulin dependent diabetes in the literature. It was in the late 19th century when physicians noticed diabetes occurring in childhood (T1D) could be divided from those occurring later in adulthood by lower body mass and an acute form of natural course [2, 3]. After the discovery of insulin, the insulin-dependent (T1D) and insulin-independent characteristics were further used to divide the two disease groups [4]. However, these characteristics alone could not clearly separate T1D from other forms of diabetes. Intended to describe T1D closer to its etiology, the concept of "type 1 diabetes" was introduced in 1940 [5] and reinforced in 1976 [6] owing to the identification of autoimmune origin in the disease [7]. However not until late 90s was it widely accepted along with the paradigm shift on our understanding of the disease pathogenesis (Figure 1.1).

***Diabetes*: excessive discharge of urine**
***Mellitus*: honey-sweet**

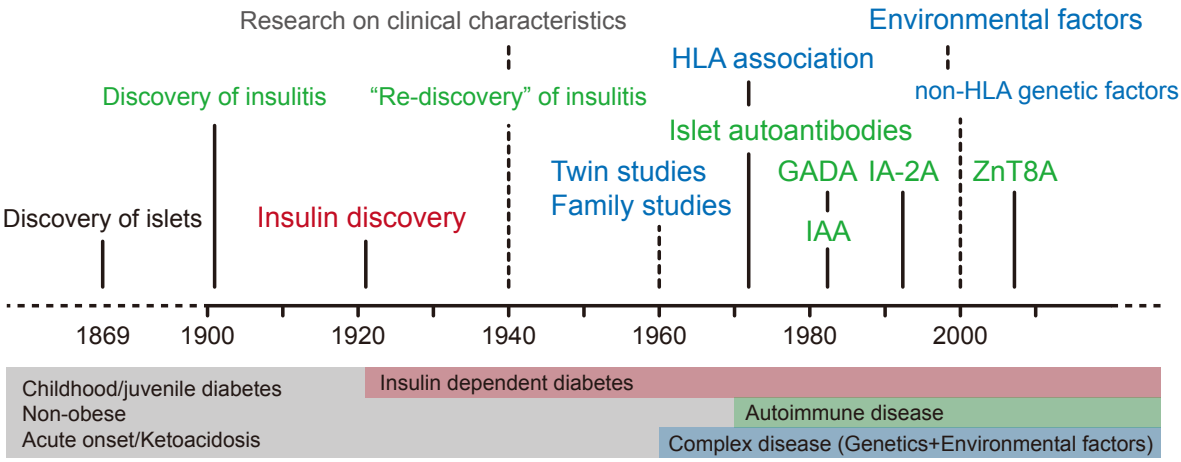

Figure 1.1 – The history of type 1 diabetes

Once a person was diagnosed with T1D, he/she will need exogenous insulin to compensate the loss of function in his/her own insulin secretion. The prognosis of T1D has miraculously improved after the discovery of insulin. Nevertheless, the current technology is

not able to supply a perfect replacement of our own insulin production. The current treatment for T1D depends on a collection of management consisting of life style control and insulin supply echoing the monitoring of glucose metabolism. In the last century, the quality of life among people with T1D has been improved tremendously by advances in insulin analogues, regimes, its administration electronics as well as glucose monitors [8-10].

The life-long requirement of treatment and monitoring equipment, combining with the fear of diabetic complications and "adverse effect" (hypoglycemia) along with insulin treatment, are still placing heavy financial and emotional burden on the family with T1D. In the current mountaineering towards a better healthcare in T1D patients, there are many ongoing promising approaches, including prediction/prevention strategies [11], inhaled insulin [12], islet transplantation [13], immune modulation therapies [14, 15] that are in close relation to bedside and, supported by breakthrough from "benchside" such as the generation of autologous insulin producing cells [16, 17], remarkable changes will be made to improve the management of T1D in the coming years.

1.2 Incidence and prevalence

T1D can strike at any age however it starts primarily among children under the age of 15 years and it is the most common form of diabetes in childhood (age < 15 y) [1]. In 1990, the world health organization (WHO) initiated the Multinational Project for Childhood Diabetes (DIAMOND) to collected T1D incidence around the world [18]. The DIAMOND investigations revealed a large variation in the incidence of T1D between populations during 1990-2000 [19]. In that period, the highest T1D incidence was located in southern Europe (Sardinia, incidence 36.8/100,000 person-years), followed by Nordic countries Finland (36.5/100,000 person-years) and Sweden (27.5/100,000 person-years), whereas the lowest was identified in east Asia (China and Japan, incidence < 5/100,000 person-years, Figure 1.2A) [19].

The incidence of T1D changed over time. Early studies showed that the incidence remained at low levels before 1950, however thereafter a rising T1D incidence was observed in many populations (Figure 1.2B and reviewed in [20]). The global annual increase of T1D incidence is 3% [1] and this worldwide T1D epidemic was not distributed evenly among populations/regions. Faster annual increase is observed in some regions (east and central European countries: 6%; Japan: 6%; east China: 14%) than that of others (north European countries: 3%, Sardinia: 2.1%) [21-24].

T1D incidence increases with age up to puberty (age 10–14 years) and thereafter drops drastically [25-29]. The two genders had similar T1D incidence before puberty, however a clear male dominance was often recorded after puberty [28-31]. In addition, T1D incidence had a latitude variation (polar-equatorial decreasing gradient) in the 1980s [32]. However the rapidly changing incidence among populations had weakened this latitude variation by the end of 1990s [19]. Moreover, a seasonal variation in the incidence of T1D has been noticed since 1926 [33]. Recent studies confirmed that regions further from the equator were more likely to exhibit significant seasonality where the T1D incidence is higher in cold seasons [34]

It is estimated that there are currently 497,100 children (aged < 15 years) worldwide living with T1D and additional 79,100 develops T1D annually [1].

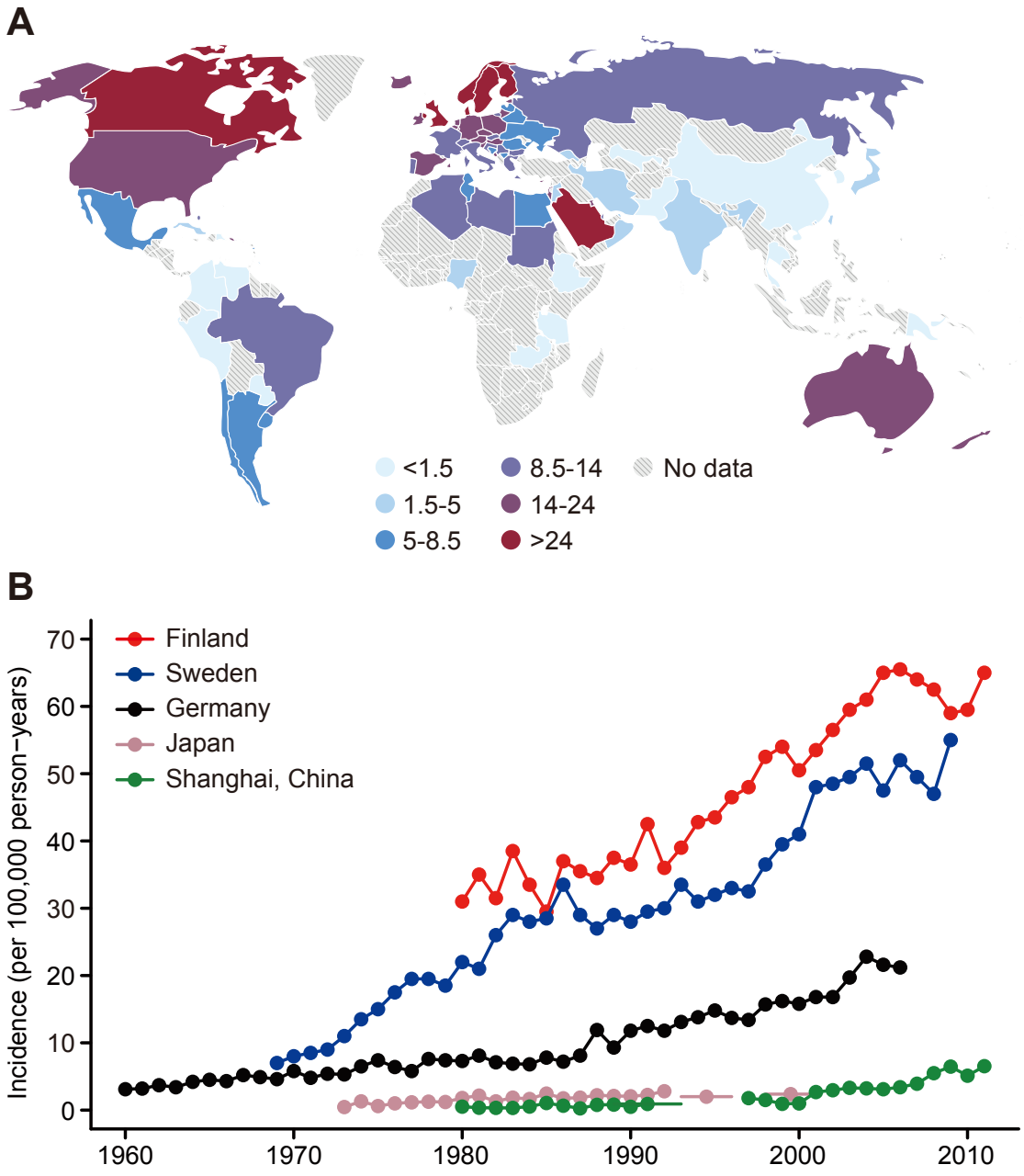

Figure 1.2 – Incidence of type 1 diabetes

(A) Estimated global incidence of T1D by region in 2013 (Page 43 in the 6th edition of IDF Diabetes Atlas-English Version, 2013 [1], reproduction with permission). **(B)** T1D incidence over time in children age 0-14 years in areas with high to low incidence rates [24, 35-41].

1.3 Pathogenesis

People with T1D have decreased capability of insulin production. Insulin is produced primarily by cells (namely β-cells) in the islets of Langerhans (also termed "islets", the small group of endocrine cells resides in the pancreas). It is widely considered nowadays that the infiltration of immune cells in the islets (insulitis) and subsequent autoimmune destruction of β-cells is the cause of T1D.

The anatomic structure of islets were discovered in 1869 and named after their discoverer Langerhans [42]. The infiltration of lymphocytes in the islets was also noticed as early as in 1902 [43] and later termed "insulitis" in 1940 [44]. Although the link between

diabetes and injured islets/pancreas was firmly established by the discovery of insulin in 1921, probably owning to the difficulties in biopsy and histology at that time, the limited data on pathological changes of islets in people with T1D only supported insulitis as a rare occasion among extreme cases [45, 46]. It was not until the middle 1960s when researchers started to discover insulitis as a common event in T1D with advanced immunohistochemistry techniques [47]. Confirmed by other human histology studies soon after [48, 49] and supported by the success of induced insulitis in animals [50, 51], insulitis was then considered the fundamental pathological change in T1D. Based on later observations that antibody against islet self-antigens could occur long before overt T1D [52], a widely accepted pathogenic model of T1D was proposed in 1986 [53] that hypothesized the process of insulitis can be triggered in early stage of life and proceed insidiously over years towards the destruction of β-cells, subsequently lead to overt diabetes once insulin production fails to meet the bodies' requirement. The identification of circulating antibody against β-cell antigens (autoantibodies) (see below in section 1.5) and self-reactive T cells both in peripheral blood [54, 55] and in draining lymph nodes of pancreas [56] led to the concept that insulitis in T1D is the consequence of autoimmune events targeting β-cells mediated by our adaptive immune system. Studies on the etiology of T1D have provided substantial novel insights on potential T1D-triggering factors (Figure 1.3) [57].

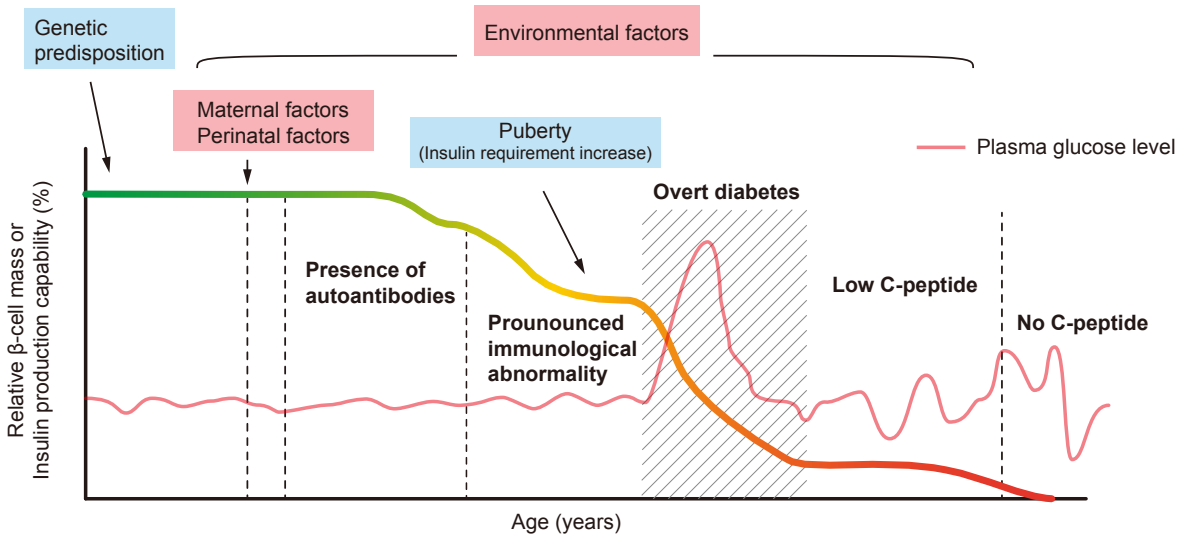

Figure 1.3 –Pathogenesis model of type 1 diabetes

1.3.1 Insulitis

Much knowledge on insulitis was derived from studies using animal models. One commonly used animal model for studying human T1D is the non-obese diabetic (NOD) mouse. NOD mouse develops spontaneous insulitis subsequent overt diabetes typically at 12 to 14 weeks of age [58]. The insulitis in NOD mice starts after approximately 4 weeks of age and most of the mice have severe insulitis by 10 weeks of age [58]. The initiation of insulitis in NOD mice showed low level of lymphocytes infiltration in 10% of islets [59]. Followed by increasing number of infiltrated immune cells in islets surroundings and increased number of affected islets (50%-60% at week 8 of age) [59, 60], insulitis in NOD mice proceeds to β-cell cytotoxicity and leads to a progressively declining in the number of islets [61]. However the NOD model and human T1D vary in many aspects (reviewed in [62]) such as immunological defects in innate immune system(impaired function of macrophage and natural killer (NK)

cells [63-65]) and in adaptive immune system (impaired function of T regulatory cells (Tregs) [66]). Along with its susceptibility to other autoimmune diseases [67-69], there are concerns raised to NOD mice that they might only resemble the pathogenesis of human T1D in a fraction of patients [62].

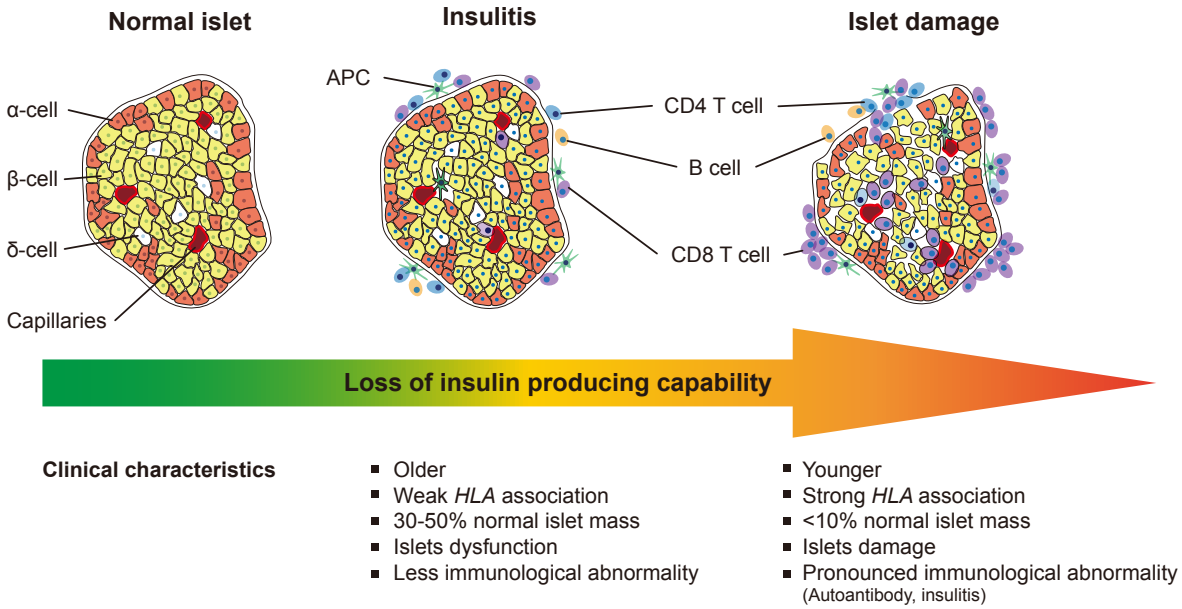

Figure 1.4 – Insulitis. APC: Antigen presenting cells.

In humans, the number of islets declines naturally with age [70]. It was generally believed that at the time of T1D diagnosis, the patients are likely to have only 10-15% of total β-cell mass. However a recent re-analysis of previous autopsy data showed that this concept only applies to T1D diagnosed before 5 years of age, whereas people with T1D diagnosed around puberty can still have 30% of β-cell mass left [70]. In addition, a meta-analysis using the available data on human insulitis among T1D patients confirmed that insulitis and β-cell damage were more common among younger patients than older patients [71]. Furthermore, major islet autoantibodies were found more frequent among younger patients (see below in section 1.5). Adding the fact that the genetic associations between immune-related genes and T1D were stronger in childhood than that in adulthood (see below in section 1.6.2), these evidence together suggested that T1D is a heterogeneous group of diseases with insulitis/aggressively declining β-cell mass among the young to one extend and non-insulitis/β-cell dysfunction among the old towards the other (Figure 1.4).

The lesion in islets prior to overt T1D in humans is not clear. The limited data from investigations on organ donors showed that only low levels of insulitis can be detected in 3% of those non-diabetic adults with T1D autoantibodies [72-74]. While consider the above evidence that adults are less likely to have conspicuous insulitis, it might only be possible to identify overt lesion in islets prior to disease among younger subjects. However owing to the very limited access to samples, insulitis before T1D in childhood is currently unknown.

1.4 Pathogenic role of cells in adaptive immune system

It is currently unclear whether insulitis is the primary lesion of T1D or secondary to events occurring even earlier. The current concept that autoimmunity specifically targeting β-

cells is the cause of T1D roots from its key pathology (i.e. insulin deficiency). Despite there are at least additional three cell types in human islets, the ability to produce insulin centers β-cells in the glucose homeostasis and diabetes. In people with T1D, aside from the identification of multiple immunological abnormalities (reviewed in [75]) and autoantibodies targeting a broad range of islets antigens (reviewed in [76]), antibody against glucagon producing cells (α-cells) was also clearly identified [77]. Nevertheless in T1D, the autoimmunity and subsequent damage of β-cells mediated by adaptive immune system is strongly supported by evidence.

1.4.1 T cells

In accordance with the dogma of immunology which centers $CD4^+$ T cells in specificity of adaptive immune response and, the strong association between major histocompatibility complex (MHC) class II molecules and T1D (see below in section 1.6.2) highlighted the importance of antigen presentation to $CD4^+$ T cells in T1D etiology. In T1D patients, $CD4^+$ T cells were observed in insulitis [78] and, autoreactive $CD4^+$ T cells against insulin were present in peripheral blood [54, 55] and pancreatic draining lymph nodes [56]. In addition, the occurrence of T1D in individual with B-cell deficiency highlighted the necessities of T cells in the development of T1D [79].

The importance of $CD4^+$ T cells in T1D were further strengthened by observations in NOD mice model. Similar to that in humans, $CD4^+$ T cells were clearly identified in islets throughout the development of insulitis in NOD mice [59, 60]. In addition, T cell clones from diabetic NOD mice were able to accelerate insulitis/diabetes in other recipient NOD mice [80-85]. Treatments with antibody against $CD4^+$ T cells ($CD4^+$ T cell depletion) were able to prevent insulitis and improve hyperglycemia [86, 87].

In contrast to the incapability of direct cytotoxicity by $CD4^+$ T cells, diabetogenic $CD8^+$ T cells were able to directly destroy β-cells [88] and induce diabetes in NOD mice without the aid from $CD4^+$ T cells [89]. In humans biopsied and autopsied pancreases, $CD8^+$ T cell were also constantly observed in islets with insulitis [78, 90, 91]. Further supported by the overexpression of MHC class I molecule in islets during insulitis [92], it is likely that $CD8^+$ T cells are direct players in β-cell cytotoxicity and islet damage of human T1D.

The above evidence suggested that both $CD4^+$ and $CD8^+$ T cells participate in the pathogenesis of T1D. The important roles of T cells in T1D is further supported by the successful induction of diabetes with co-transferred $CD4^+$ and $CD8^+$ T cells in NOD-scid mice (NOD mice with deficient adaptive immunity) [93-95] and other non-diabetic-prone mouse strains [96, 97]. In addition, treatment with anti-CD3 monoclonal antibody (T cell depletion) creates both diabetes remission in NOD mice [98] and clinical improvements in human T1D [14]. There is substantial evidence showing that autoreactive T cells were able to recognize islet antigens in both NOD mice and human T1D patients (reviewed in [99]). More recent study suggested that these islet specificities are essential to the entrance of T cells into islets [100].

Regulatory T cells (Tregs) is crucial in maintaining immune tolerance. In NOD mice, the depletion of Tregs resulted in accelerated diabetes [101] while transfusion of islet-specific Tregs can delay or prevent diabetes [102, 103]. The ability of re-introducing immune tolerance potentiates the therapeutic usage of Tregs. However, the compromised function of Tregs in NOD mice [66] might make it improper to translate results from this rodent model directly to humans. In fact in humans, Tregs infiltration was rarely found neither in normal

human islets nor islets with insulitis [78]. The number of Tregs in peripheral blood of T1D patients varied between studies (reviewed in [104]) which indicates the possibility that true effector Tregs exert their function inside the lymph nodes [105] therefore difficult to trace within other tissues. Indeed, a recent study discovered that T1D patients had less Tregs in the pancreatic draining lymph nodes [106] and the remaining Tregs had impaired ability to suppress the proliferation of other T cells [106]. Together these findings highlighted the potential of Tregs in human T1D and their roles in the lost of immune tolerance.

1.4.2 B cells

Aside from its ability to produce autoantibodies (see below in section 1.5), the role of B cells in human T1D is largely unclear. Despite that T1D could develop in people without B cells [79] and, T cells from NOD mice alone is sufficient to induce diabetes in recipient NOD mice without B cells [93-95], the possibility of these findings being based on cases of diverse pathogenesis and the potential role for B cells in pathogenesis of general T1D cannot be ruled out. In fact, the deficient monocyte proliferation in NOD mice made them heavily depend on B cells for antigen presentation [63, 64]. B cells are actually critical antigen presentation cells to initiate diabetes in NOD mice [107, 108]. Cytokine produced by B cells were capable of transforming islets naïve $CD8^+$ T cells into cytotoxic effector T cells [109]. While taking the evidence that B cells is observed in the insulitis of both human T1D and NOD mice [60, 78], and treatment with anti-CD20 monoclonal antibody (B cell depletion) were effective in both mice and humans [15, 110], it seems that B cells have similar pathogenic roles between mice and humans.

1.4.3 Immune tolerance

Although the importance of autoreactive T cells in the pathogenesis of T1D was clearly identified, how these cells gain their auto-reactivity is currently unclear. Regardless of the fact that thymic deletion of autoreactive T cells is particularly efficient [111], recent studies revealed that self-reactive T cells can actually be found abundant in normal T cell repertoire (reviewed in [112]). Nevertheless, the escape of autoreactive T cells from both regulatory checkpoints (thymic deletion and peripheral regulation) seems to be inevitable, as can be seen in extreme cases such as Omenn syndrome (OMIM 603554) in which deficient rearrangement of T cell receptor (*TCR*) gene leads to an excess of monoclonal autoreactive T cells. However under normal TCR rearrangements, by what mechanism can MHC class II (e.g. DQ molecules in T1D, see below in section 1.6.2) contribute to the gaining of auto-reactivity is currently unclear. It was suggested that the peptide presenting preference might influence the formation of T cell repertoire therefore facilitate the formation of self-reacting T cells. However the dual function of MHC class II molecule in both the selection of naïve T cells and immune response of effector T cells creates a dilemma that reflects the importance of MHC class II molecule in tuning between autoimmunity and effective immune response.

In addition, autoreactivity can be trigger by pathogens possessing similar molecule structure with human self-antigens (a process called “molecular mimicry”). A recent study clearly confirmed that TCRs bear loose specificities in binding to MHC-peptide complex [113]. This finding indicated that the “molecular mimicry” is able to be triggered by a broad range of pathogens.

Inspired the “hygiene hypothesis” (reviewed in [114]), early studies observed increased diabetes incidence in NOD mice raised in germ-free condition [115, 116], however

these observations were not obvious in latest studies where NOD mice raised in germ-free environment had similar incidence of diabetes to that not raised germ-freely [117, 118].

The innate immune system is crucial in the differentiation between self and non-self by recognizing common components of pathogens through pattern recognition receptors. The "danger model" proposed that innate immune system can trigger autoimmunity when it was improperly activated during the renew/clearance of self damaged cells by sensing the self "danger signals" (reviewed in [119]). However, other evidence showed that innate immune system is actually crucial to maintain immune tolerance. In humans, monocyte deficiency (OMIM 614172, in which the key defects in dendritic cells) is associated with deficient Tregs which explained the autoimmune phenotype of the deficiency [120, 121]. These evidence highlighted the role of innate immune in autoimmunity (see below in section 1.8).

Nevertheless, autoreactive T cells were commonly found among people with T1D. Studies in NOD mice showed that thymic chimera with non-diabetogenic MHC could enhance positive selection of non-autoreactive T cells [122]. Meanwhile, MHC mismatched bone marrow chimerism in NOD mice had MHC-II dependent (mediated by thymic dendritic cell) deletion of autoreactive T cells [123]. These facts indicated that the diabetogenic MHC in NOD mice could enhance the production of autoreactive T cell during both positive selection and negative selection. The diabetogenic mice MHC had stronger binding affinity to islet-epitopes [124] which would ideally facilitate the deletion of autoreactive T cells in negative selection. The mechanisms linking diabetogenic MHC and autoreactive T cells are likely to be found outside the thymic negative selection.

1.5 Autoantibodies

The first observation of circulating antibody against islets in newly diagnosed T1D was reported in 1974 [52, 125]. The detection assay (using fixed blood group type O human pancreas section and indirect immunofluorescence) with improvements later succeeded to identify islet cell antibodies (ICAs) in 38% of T1D patients compared with none in non-diabetic controls [126]. Taking together with later discovered antibodies against specific self-antigens (autoantibodies), there is no doubt that the development of autoantibodies is one of the pathological changes in T1D. Nevertheless, the ICA assays were found to be inconvenient in terms of access to reagents and assay efficiency [127, 128]. It was nowadays replaced by assays detecting major T1D autoantibodies (GADA, IAA, IA-2A and ZnT8A, see below).

1.5.1 **GADA** (Glutamic Acid Decarboxylase Autoantibody)

In patient with T1D, autoantibodies against a 64 kD (64 kilo-Dalton, relative molecular mass) protein were first identified in 1982 [129]. In 1990, the 64 kD protein was identified to be the glutamic acid decarboxylase (GAD) [130]. The two isoforms of GAD was distinguished that human islets express the 65 kD isoform in the islets whereas the other isoform is expressed only in the brain [131, 132]. The frequencies of GADA among T1D are around 70% in Caucasian populations [133, 134]. Among Asian newly onset T1D, the GADA is less prevalent at around 40%-55% [135-140]. GADA was found to be persistent after T1D diagnosis in both Asian and Caucasians [139, 141, 142]. The prevalence of GADA do not differ with onset age in general T1D population [143], however interestingly, its prevalence decreases slightly with T1D onset age among those with high-risk human leukocyte antigen (*HLA*) genes whereas the reverse is found among subjects without HLA risk [143].

1.5.2 **IAA** (Insulin Autoantibody)

Autoantibodies against insulin (IAA) were detected as early as in 1959 in sera of patients treated with bovine insulin [144]. In 1983, IAA were described in diabetic people before insulin treatment [145]. In clinics, the assessment of IAA is made prior to the development of insulin antibodies a few weeks after insulin treatments. IAA can be found among 85% of T1D patients less than 10 years old whereas only in about 55% T1D patients older than 10 years old [133, 146]. IAA is the first autoantibody developed in T1D which can occur as early as in 3-6 months of age among genetically predisposed infants identified from general population [147].

1.5.3 **IA-2A** (Insulinoma associated antigen-2 Autoantibody)

In T1D patients, the autoantibodies against a 40 kD protein in islets were identified in 1990 [148] and the antigen was soon resolved to be tyrosine phosphatase-like protein (also known as insulinoma associated antigen-2, IA-2) [149, 150]. The frequencies of IA-2A among T1D varies among Caucasian populations from 55% to 75% in newly diagnosed T1D (reviewed in [151]). Similar to that of IAA, the prevalence of IA-2A also decreased with age of T1D onset [143, 152]. Among Asian population, the prevalence of IA-2A is 47% among Koreans [135], 60% among Japanese [139] and 36% among Chinese [137].

1.5.4 **ZnT8A** (Zinc Transporter 8 Autoantibody)

The identification of autoantibodies against zinc transporter 8 (ZnT8) rooted from analysis on expression arrays of human and mice islet autoantigens [153]. ZnT8A was found to be present among 63% of newly diagnosed patients [154]. The ZnT8A frequency increased with age, peaks in 10 years old and drops thereafter [154]. Similarly, in Asian populations ZnT8A was present in 58% of childhood T1D and dropped to 34% in adults [155]. ZnT8A was found to have specificity to three ZnT8 isoforms owing to a common nonsynonymous genetic variation in the *SLC30A8* gene which results in amino acid variants (Trp, Arg and Gln) at 325th position of ZnT8 protein [156].

1.5.5 **Other autoantibodies**

Despite the above major autoantibodies, autoantibodies against other islet antigens were also identified in T1D (reviewed in [76]). There are around 5-10% of childhood T1D patients diagnosed without detectable autoantibodies [157, 158] and more among T1D diagnosed in adulthood [159]. A recent investigation demonstrated that ICAs could be detected among 5% of newly diagnosed childhood T1D subjects negative for all major autoantibodies [160]. These results suggested that T1D is a disease group with heterogeneous autoimmune specificities. Further discovery of novel autoimmune target in T1D can aid both understanding of the disease and disease diagnosis/prediction.

1.6 Genetic factors

Owing to the lack of diabetes classification and the well-established law of Mendelian inheritance, early studies dissecting genetic factors of T1D often generated inconsistent results [161]. Later observation that juvenile diabetes (later classified as T1D) trended to segregate within families suggested that this form of diabetes can be inherited [162]. The low genetic penetrance in T1D identified by later studies and relatively lower T1D incidence in siblings of probands (compared with Mendelian diseases) suggested that T1D had a pattern of multi-loci inheritance [163]. Subsequent studies investigating monozygotic twins showed the concordance of T1D between the twins can be as high as 65% after 40 years follow-up [164].

After the clearer classification, the aggregation of T1D within families was clearly observed (Table 1). These results indicated that genetic factors are crucial in the etiology of T1D.

Table 1 – Diabetes prevalence in relatives of people with T1D

Population	Frequency of occurrence	Reference
Monozygotic twin of T1D patient	13–65%	[164-167]
Dizygotic twin of T1D patient	3–4%	[165, 166]
Siblings of T1D patient	3–4%	[168, 169]
Parents of T1D patient	3–6%	[169-171]
Children of mothers with T1D	2–4%	[171, 172]
Children of fathers with T1D	6–7%	[171]
General Caucasian childhood population	0.3%	[173]

The current genetics of T1D is a complex involving many genes (reviewed in [174] and [175]). *HLA* constitute the highest proportion of T1D genetic etiology, followed by other genes detected in recent genome wide association studies (GWAS) [176-178]. In the Caucasian population, there are more than 40 genetic locus identified to have association with T1D (Figure 1.5, data derived from *www.t1dbase.org* as of 2014 August).

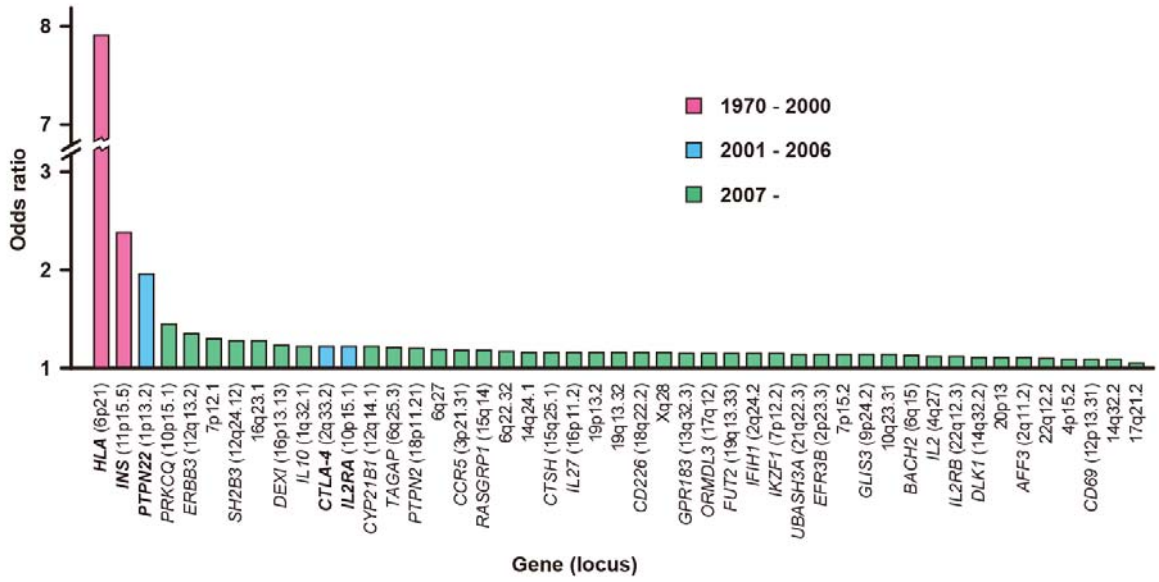

Figure 1.5 – Genetics of T1D

1.6.1 Major Histocompatibility Complex (MHC)

The MHC region is located on chromosome 6 (6p21.3) in humans. The region contains genes encoding molecules that are critical in the immune system. Genes in the MHC region are divided into three groups: MHC class I, II and III. MHC class I genes encode α-chain of human leukocyte antigen (*HLA*) class I molecules and they are named *HLA-A*, *-B* and *-C*. These α-chains, along with β_2-microglobulin, forms HLA class I molecules that present peptide epitopes to $CD8^+$ T cells. MHC class II genes are grouped into three pairs, namely *HLA-DR*, *-DP* and *-DQ*, encoding α- and β-chains of HLA class II molecules that present peptide epitopes to $CD4^+$ T cells. These *HLA* genes are highly polymorphic therefore creates a remarkable diversity of their protein products within human population [179]. MHC class III genes encode other proteins with immune-response related functions (aside from direct antigen presentation), such as complement.

1.6.2 *HLA* and T1D

The association between *HLA* and T1D was first reported in 1973-74 with association to *HLA* class I genes [180, 181]. Followed by studies using improved typing methods in the early 1980s, stronger associations were located to *HLA*-DR3 alleles (odds ratio, OR ~ 5), -DR4 alleles (OR ~ 8) and, DR3/DR4 heterozygosity had the highest risk (OR > 10) [182-184]. These results indicated that earlier observed *HLA*-class I associations were likely a reflection of linkage disequilibrium (LD) with MHC region [182]. Indeed, after *HLA* typing was replaced by genetic methods later, it was resolved that the highest genetic risk was conferred primarily by *DQB1**0302 whereas part of the *DR4* association identified previously was due to the strong linkage between *DR4* and *DQB1**0302. *DQB1**0302 is present in more than 90% of *DR4* positive T1D [185-187], 35% of all T1D patients [186, 188] and 10% of general Caucasian population [188, 189].

The heterozygous of DR3/4 has been observed to confer the highest T1D risk in Caucasian populations [182-184, 190, 191]. One of recent large study with finely mapped *HLA* confirmed previous finding that DR3-DQ2 (*DRB1**03-*DQA1**0501-*DQB1**0201) and DR4-DQ8 (*DRB1**0405-*DQA1**0301-*DQB1**0302) in a heterozygous combination conferred the highest T1D susceptibility [192]. Owing to the strong *DR-DQ* linkage identified in Caucasians (*DRB1**03 with *DQA1**0501-*DQB1**0201, *DRB1**04 with *DQA1**0301-*DQB1** 0302), it was hypothesized that the formation of *trans* heterodimers (i.e. heterodimers formed by products of *DQA1**0501-*DQB1**0302 and *DQA1**0301-*DQB1**0201) could explain the genetic associations [174]. In addition, compared with that in adulthood, the association between *HLA* susceptibility and T1D were stronger in childhood where more immunological abnormalities are found [193, 194]. The above finding from *HLA* association studies highlighted the potential of a key residue, position 57 of the class II DQ-β chain (β57), in autoimmunity [195]. Amino acid Asp at β57 is associated with protection against diabetes whereas Ala, Val or Ser correlates to T1D susceptibility [195]. Similar associations can also be found in mice strains (Asp confers diabetes resistance whereas Ser, in NOD MHC I-A^{g7}, confers diabetes susceptibility) [195].

The functional importance of MHC molecules was further demonstrated by studies using the NOD mice. Transgenic expression of diabetes-resistant murine MHC (I-A^{d}) in NOD mice prevented diabetes [196]. Replacement by transgenesis of Asp β57 into I-A^{g7} reduces the incidence of diabetes [197]. Study on the crystal structure of I-A^{g7} showed that this murine diabetogenic MHC (I-A^{g7}) had normal stability however a different peptide binding preference with remarkably increased ability to present epitopes of GAD65 [124]. In addition, a recent crystal structure analysis on *trans* MHC molecule formed by DQA1*0301 and DQB1*0201 showed it had higher binding affinity to peptide derived from gluten [198]. These results indicated that diabetogenic MHC is likely to function by increasing the presentation of self-epitopes to autoreactive T cells. Nevertheless, the binding preference of these MHC molecules might also promote negative selection in thymus mediated by thymic dendritic cells. In addition, attributes from other genetic factors as well as their interactions with environmental insults also alters the actual consequence of same MHC allele [199].

The large variation in T1D incidence among populations has been noticed for long, the difference in genetic background of populations is crucial in the determination of T1D incidence. It seems that the T1D incidence correlates the frequency of risk *HLA-DQ* (Figure 1.6). In addition, the common *DR-DQ* haplotypes identified in Caucasian population are rare

among Asians (Figure 1.6) and led to variations in the Asian replication of the above HLA associations in Caucasians.

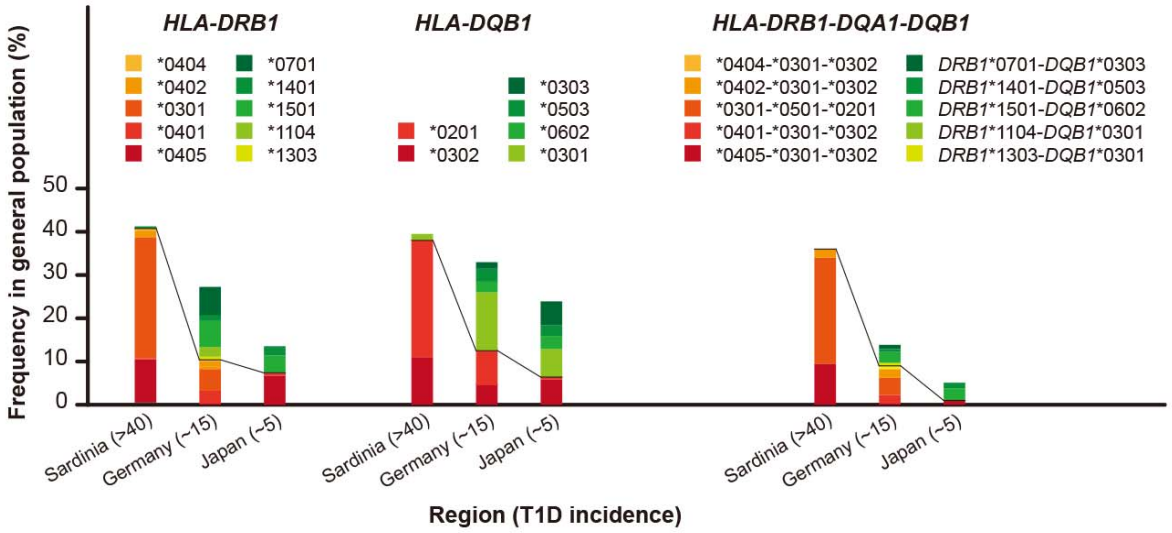

Figure 1.6 – HLA allele distribution among population. Incidence is per 100,000 person-years; data derived from *www.allelefrequencies.net* [189].

In addition to their association with T1D, *HLA* had also influence on the prevalence of T1D autoantibodies. GADA was associated with *HLA DR3-DQ2* (OR ~ 6) [143, 200, 201]. The frequency of IA-2A was higher among T1D with *DR4-DQ8* [143, 200-202] however lower among those with *DR3-DQ2* [143, 201, 203]. Similarly, IAA frequency was slightly higher in *DR4* positive T1D patients [143, 203, 204]. Nevertheless, DR3/4 heterozygous had the strongest association with GADA and IA-2A (OR > 20) [200, 203]. Interestingly, the latest identified T1D autoantibody, ZnT8A, was found more prevalent in T1D without *DR3* or *DR4* [154].

1.6.3 *INS*-VNTR

INS-VNTR, discovered in early 1980s, is a copy number variant (CNV) located in the 5' flanking region of *INS* (insulin gene, on chromosome 11p15.5) [205, 206]. *INS*-VNTR contains a variable number of tandem repetitions (VNTR) with a 14- to 15-bp nucleotide sequence (ACAGG GGTGT GGGG in consensus). The alleles of *INS*-VNTR were divided into three groups: the class I alleles with 30-60 repeats (short) were present in around 70% of the Caucasian individuals; the class III alleles with 120-170 repeats (long) found in ~30% of Caucasians and; the class II alleles with repeats between class I and class III (intermediate) were rarely found [143, 207-210]. Shortly after its discovery, the association between *INS*-VNTR and T1D was identified by many studies [208-212]. In the Caucasian population, the class I alleles were associated with an elevated risk (OR > 2) of T1D whereas class III alleles were protective [213]. Among Asians, the rarity of class III alleles made it difficult to assess the T1D association of *INS*-VNTR [214, 215]. Due to the pattern of linkage disequilibrium (LD) within region around *INS*-VNTR, genetic studies were not able to distinguish VNTR as the primary genetic association site [213], however functional studies demonstrated that VNTR was able to regulate *INS* expression, particularly in the thymus [216-219]. The protective class III VNTR alleles induced more than 2-fold higher expression of thymic insulin than the diabetogenic class I alleles [218, 219]. Regulated by aire (abbreviation for Auto-Immune Regulator), the ectopic insulin expression in thymus increases the probability of removing insulin autoreactive T cells during negative selection [220]. The importance of

thymic insulin expression is further support by studies using mice models where mice engineered to have deficient thymic insulin expression had increased T cell reactivity against insulin [221, 222] and therefore predisposition to diabetes [223, 224].

1.6.4 *PTPN22*

The *PTPN22* gene on chromosome 1p13.3-13.1, encodes cytoplasmic lymphoid tyrosine phosphatase (LYP) [225]. The importance of *PTPN22* in T1D was first discovered in 2004 [226]. Thereafter, the association between *PTPN22* gene and T1D was intensively agreed in studies among western populations [227, 228] as well as in GWAS using Caucasian-based populations [177]. The minor allele (T allele) of single nucleotide polymorphism (SNP) 1858C>T (rs2476601), present in 10% of Caucasian population, conferred genetic susceptibility to T1D. This T allele of 1858C>T results in an amino acid substitution (R620W) in the highly conserved N-terminal, proline-rich motif of LYP. The 620W substitution could impede the interaction between LYP and C-src tyrosine kinase (CSK), leading to reduced phosphorylation of LYP and subsequent enhanced inhibition on T cell activation [229, 230]. It was hypothesized that the inhibition of T cell activation caused by 620W substitution leads to weaker TCR signaling subsequently decreases either the deletion of autoreactive T cells during thymic selection or activity of regulatory T cells in peripheral [229]. However, owing to the extreme rarity of this allele among Asian individuals, this association was hardly observed in Asian populations [231-233]. A recent meta-analysis on genetic association studies clearly showed that the T1D association of R620W was highly dependent on ethnicity [234-237]. Among Asian populations, the T1D association in *PTPN22* region was located in the promoter region of the gene [232, 238].

1.6.5 *CTLA4*

The *CTLA4* gene on chromosome 2q33, encodes Cytotoxic T lymphocyte associated antigen 4 (CTLA-4). CTLA-4 is expressed on the surface of T cells and functions as a negative regulator of immune response mediated by T cells [239]. CTLA-4 is able to induce trans-endocytosis and subsequent degradation of its ligands, namely CD80 and CD86, on antigen presenting cells [240]. CD80 and CD86 are also ligands for costimulatory receptor CD28, therefore the degradation of CD80 and CD86 induced by CTLA-4 compete the ligation of costimulatory signals subsequently results in T cell inhibition, T cell anergy or Treg proliferation [239]. The importance of CTLA-4 in maintaining immunologic homeostasis is supported by disease caused by CTLA-4 deficiency which leads to lymphoproliferation, multiogran lymphocytic infiltration and tissue destructions in both mice and human (Chediak-Higashi syndrome) [241, 242]. In T1D, the most studied *CTLA4* polymorphism, SNP +49A>G (rs231775) in its first exon, was first identified as a T1D locus in Belgian population [243] and confirmed thereafter in other populations that the G allele of this SNP confers predisposition to T1D [244-246]. The G allele in +49A>G results in a threonine to alanine replacement in the 17th amino acid of CTLA-4 molecule and leads to aberrantly processed CTLA-4 in the endoplasmic reticulum thus a reduced cell surface expression [247].

1.6.6 *IL2RA*

The IL-2Rα and IL-2Rβ encoded by *IL2RA* and *IL2RB* are subunits of the high-affinity IL-2 receptor (IL-2R) which vitalizes T cell-dependent immune response [248]. IL-2 and IL-R interaction also stimulates Tregs and enhances immune suppression [249]. The association between *IL2RA* and T1D was first reported in 2005 [250]. It was later shown that

T1D susceptible allele in SNP located in the 5' flanking region of *IL2RA* (T allele in rs11594656) collated to reduced serum levels of sIL-2RA (soluble IL-2RA, cleaved from membrane-bound IL-2RA after the induction of T cell proliferation) [251]. The dual function of IL-2 in immune system was further clarified by a recent study investigating association between *IL2RA* promoter and *IL2RA* expression in T cells [252] showed that the T allele of rs12251836 residing in this region is associated with a allele-specific binding of transcription factor YY1 during the activation of $CD4^+$ T cells however not in Tregs, subsequently led to enhanced *IL2RA* expression and activity of T helper cells.

1.6.7 Genetic association discovered by GWAS

Classic genetic linkage studies on candidate genes have demonstrated that T1D is a multifactorial disease which cannot be explained by a limited number of genetic variants with large effects (Figure 1.7). Based on the hypothesis of "common disease, common variants", the GWAS using tagSNPs (SNP within LD regions which extends over typically 10- to 100-kilobases across human genome) succeeded in the confirmation of previous identified T1D locus and the discovery of novel common variants in human populations that are associated with T1D [176-178, 250]. There are currently more than 40 T1D association loci discovered (Figure 1.5).

1.6.8 Post-GWAS

GWAS proved its powerfulness and generated great insights into disease with complex genetics, however several limitations of these type of study were noticed [253, 254]. Although GWAS are conducted using very large sample size with finely designed statistical protocols, false positives are still unsolved problems that require future better techniques [255-257]. In addition, the basis on tagSNPs in GWAS reveals only the association of LD regions that might contains actual causative variants with larger effects (Figure 1.7). Furthermore, SNP genotyping arrays are limited from the detection of other genetic polymorphisms in human genome, including large indels (insertions or deletions) and structural variants.

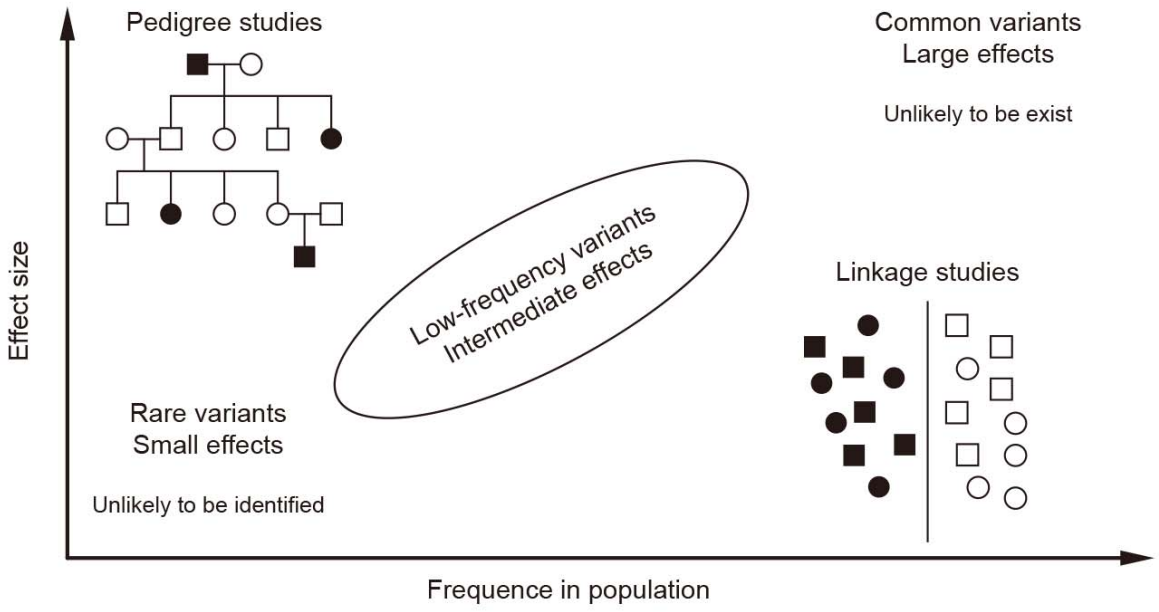

Figure 1.7 – Mapping genetic factors in complex diseases. Figure modified from [175].

Nevertheless, with recent developed techniques, GWAS on copy number variants (CNVs) started to provide additional information in T1D genetic association [258]. In addition, the rapid development of next-generation sequencing makes the analysis of rare variants possible. These techniques are promising to further provide insights to T1D genetic etiology. The random monoallelic expression pattern of human genes discovered recently [259] suggested additional difficulties in the determination of genetic causality in complex diseases. Nevertheless, the ENCODE project with RNA-seq and ChIP-seq is currently rapidly annotating cell-line/tissue specific functional non-coding sequences to the genome, which will facilitate determining causality of T1D association variants in noncoding genome regions [260].

1.7 Environmental factors

In monozygotic twins with one affected by T1D, about 35% of the other will not develop T1D even after a 60-year follow-up [164]. It was estimated that in general newly diagnosed T1D, more than 90% do not have a relative with the disease [175]. Meanwhile, more than 80% of individual with high-risk *HLA* alleles did not develop T1D before 15-years of age [261]. In addition, the rapidly rising incidence in many countries was not able to be explained by genetic factors alone [23]. These observations indicated that genetic factors alone are not able to determine the disease.

1.7.1 Viruses and bacteria

Links between T1D and viral infections stemmed from the finding of seasonality in T1D epidemiology (see above in section 1.2). Investigations on viral triggers of T1D have provided substantial evidence to support that viral infections can enhance autoimmunity in T1D (reviewed in [262]). Probably owing to the current classification of T1D which groups diseases with heterogeneous pathogenesis, there is no known viral strains can determine T1D. It is likely that viral infections are causative in subgroups of T1D patients therefore an enhancing factor in general.

The Finnish T1D studies demonstrated that the seasonality in the initiation of islet autoimmunity among genetic susceptible individuals correlated to population enteroviral infection rates [263]. This observation was supported by the finding that newly onset T1D patients had neutralizing IgM antibodies against many viruses among which antibodies against coxsackievirus were the most prevalent [264]. Despite there are inconsistent results produced by other studies where T1D do not have more frequent antibodies against coxsackievirus than general population [265, 266], the causative role of coxsackieviral infection in T1D was supported by direct evidence. Some studies isolated coxsackievirus from the pancreas of T1D patient [267] and others observed enhanced T cell immune response against coxsackievirus in T1D patients [268, 269]. The findings of abundant HLA class I and IFN-α levels in islets of T1D patients [270] further supported the view that viral infection in islets can trigger islet damage by activating $CD8^+$ T cells. However a recent study revealed that elevated enteroviral protein can be found in islets of both T1D patients and non-T1D diabetic patients [271]. These results showed that viral infections can be affected by other factors to determine the disease. Among those factors, Natural killer (NK) cells were recently identified as mediators between the islet viral infection and insulitis [272].

Many studies have reported associations between T1D and other viral infections, such as cytomegalovirus [273] and parvovirus [274] (reviewed in [275]). Among these

associations, congenital rubella viral infection was considered T1D causative and classified as a specific T1D subtype in the latest ADA (American Diabetes Association) guideline [276]. However, the diabetes caused by congenital rubella viral infection is considered as one clinical feature of the congenital rubella syndrome (reviewed in [277]). The actual role of congenital rubella viral infection in autoimmunity was not clearly resolved.

The molecular mimicry hypothesis (sequence similarities between pathogen and self-epitopes can induce cross-activation of autoreactive T/B cells by pathogen-derived epitopes) inspires the investigation on shared epitopes between viral antigens and β-cell self-antigens. Indeed early studies showed that aside from the above mentioned coxsackievirus which exhibits similarity between its C protein with GAD [278], rotaviral antigens share similarities with GAD and IA-2 [279]. However at the same time, the viral molecular mimicry was questioned by the direct findings that the diabetes induced by coxsackievirus in mice was restricted to MHC class I molecules [280] and coxsackieviral protein lacks humoral cross-reactivity with human self antigens [281]. Owing to the weak TCR-pMHC affinity which subsequently generate a board cross reactivity (see above in section 1.4.3), future studies will probably need to investigate larger amount of peptide epitopes.

Recent studies using NOD mice model highlighted the role of intestinal bacterial composition in T1D. NOD mice lacking TLR response had a modified intestinal bacteria spectrum which subsequently leads to the protection of diabetes [118]. In addition, intestinal bacteria spectrum was found to alter sex hormone levels in male mice and protect them from diabetes [117].

1.7.2 Dietary factors

The cross-reactivity identified between albumin from cow's milk and β-cell surface proteins suggested cow's milk might be a T1D trigger [282]. However, inconsistent results from later studies challenged the causality of cow's milk [283]. Although there were studies showing early introduction of cow's milk predispose T1D when compared with breastfeeding [284, 285], the lower T1D incidence owing to the potential protection effects of neutralizing antibodies from breast milk cannot be excluded.

Celiac disease (CD), caused by the irritant gluten (a protein abundant in wheat), shares HLA association with T1D [286]. While the co-occurrence of T1D with CD is commonly observed, it is logical that wheat proteins can also be a potential source of dietary triggers in T1D. However, elimination of gluten from diet did not ameliorate islet autoimmunity in people positive for T1D autoantibody [287]. Whether gluten can contribute to events earlier that initiate islet autoimmunity is yet to be studied.

Inspired by early finding of the latitude and seasonal variations in T1D epidemiology, it was suggested that lack of sun exposure can be a T1D predisposing factor. The finding that vitamin D levels were lower in newly diagnosed T1D gave an explanation for those epidemiology findings [286]. Observational studies also confirmed that vitamin D supplementation reduced risk of T1D [288]. In addition, haplotype of vitamin D receptor gene (*VDR*) contributes to T1D genetics [289]. However trials investigating the protective effect of vitamin D on residue β-cell function in newly onset T1D generated inconsistent results, with one showing benefits [290] while the other two did not [291, 292]. Nevertheless, the effect of vitamin D supplementation before overt T1D is unclear. A randomized open label, pilot trial is currently under way (NCT00141986) to test whether increased dose of

vitamin D (2,000 IU/day instead of the current practice of 400 IU/day) will prevent T1D among children genetically at risk.

There were other dietary factor association with T1D observed in retrospective studies and pilot trials, however not many succeeded in later prevention trials. For instance hydrolyzed infant formula was shown to have prevention effect against T1D in population with high HLA risk [293] but not confirmed by later study in larger population [294]. Similarly, it has been shown early that synthesis of IL-1β, IL-1α, and tumor necrosis factor can be suppressed by dietary supplementation with long-chain n-3 fatty acids [295]. An ongoing trial (NCT00333554) investigating the effect of long-chain n-3 fatty acids in the prevention of T1D will be able to demonstrate its effect.

1.7.3 Maternal and perinatal factors

Father with T1D had a increased risk for offspring to develop T1D than T1D mothers (see above in Table 1).The protection from mother can be partly explained by the T1D autoantibodies transmitted from mother which protect the offspring from islet autoimmunity, especially among those offspring who do not carry T1D risk HLA alleles [296]. Nevertheless, there are many other maternal factors associated with increased risk of T1D in the offspring, such as older maternal age [297], caesarean section [298]. Interestingly, maternal smoking has been identified as a protective factor against T1D ([299] and reviewed in [300]. In addition, higher birth weight [301], neonatal jaundice [302] had a small risk for those children to develop T1D later in life.

1.7.4 Other factors

The global rising incidence (see above in section 1.2) clearly indicated the importance of environmental factors in the etiology of T1D [23, 303]. The rising T1D incidence reflects the collective effects of environmental changes along with time. However the complexity of this effect collection made the dissection of each factors alone difficult and often result in inconsistent results between populations/studies.

Puberty: It is currently not clear why T1D incidence increases with age, peaks in puberty and declines soon after (see above in section 1.2). Puberty is a process during which remarkable changes occurs in endocrine system. As a prerequisite for growth in puberty, the activation of GH-IGF-1 (growth hormone – insulin-like growth factor-1) leads to increasing level of circulating IGF-1 which peaks in age 12-15 years [304]. This activation during normal puberty subsequently results in increasing peripheral insulin resistance which correlating circulating IGF-1 levels [305-308]. While taking findings from histological studies where overt diabetes occurs when the residue β-cell function fails the body's requirement of insulin (see above in section1.3.1), puberty can be one important factor which promote the penetration from asymptomatic islets destruction to overt disease. However interestingly in NOD mice, diabetes occurs only after puberty [117, 118] which reflects a substantial difference between human T1D and diabetes in NOD mice model.

Gender: A slight male dominance was found in T1D epidemiology, particularly among T1D occurs in adults (see above in section 1.2). While grouped as a member of autoimmune diseases, T1D might be the only one in the group that exhibits male dominance [309]. There are studies showing that women trended to develop more vigorous Th1 response after encountering infection which subsequently enhance autoimmunity [310]. Meanwhile, girls are found to have higher insulin resistance than boys in puberty [307]. However, these observations paved the route towards an increased incidence of autoimmunity in women that

contradicts the T1D epidemiology. A recent study using NOD mice model discovered a hormone-dependent regulation of autoimmunity with link to gut bacteria composition [117]. However, in NOD mice, despite that diabetes occurs only after puberty [117, 118], in contrast to human T1D, those mice showed an obvious pattern of female dominance. These disparities indicates that the inbred NOD mice model might not reflect the pathogenesis of disease among the randomly breed humans.

In the above two sections, the discussed factors that affect T1D predisposition, discrepancy from studies might indicate different patterns of factors' interaction between populations/studies. At the same time, it is more and more clear that the current classification of T1D, which is based on a collection of clinical symptoms, is a group of disease with extreme heterogeneity.

1.8 Role of innate immunity in T1D

The innate immune system is the frontline immune system that responds immediately upon encounter with infectious agents and activates the removal of pathogens. The innate immune system is a collection of factors that forms physical, chemical, biological and cellular barriers against pathogens to enter the body. Once pathogens breaches defense of the innate immunity, the adaptive immune system will be activated subsequently to enhance the clearance of these pathogens. The interaction between innate and adaptive immune systems is mediated by antigen presenting cells (APCs) such as monocytes (dendritic cells, macrophages) and B cells which present antigen to $CD4^+$ T cells through the MHC class II-peptide-TCR complex. The autoimmune process and immune response against pathogen often share mechanisms and pathways. Many autoimmune diseases can be grouped with etiology contributed by both environmental factors and genetic factors centering MHC class II genes. Currently there is an increasing body of evidence that the dysfunction of innate immunity, whose function connects environmental factors and adaptive immune system, can be an early triggering event in autoimmunity.

The analysis of islets from T1D subjects showed that macrophages remained constant during insulitis [78, 311]. Along with infiltrated dendritic cells, these monocytes were the major sources of local TNF-α and IL-1β (proinflammatory cytokines) and associated with a higher T cell infiltration into islets [312].

Similarly in NOD mice, APCs were among the cells during the earliest islet infiltration [59]. Followed by more lymphocytes co-localization, those APCs were capable of producing TNF-α and IL-1β therefore they are considered to initiate insulitis in NOD mice [313]. This observation was further supported by the finding that NOD mice with deficient IL-1 receptor have slower progression diabetes [314].

However on the other hand, NOD mice were known to have deficient hematogenesis and subsequent reduced level of monocyte proliferation [63] and they relied heavily on B cells to present antigens [64]. In fact in NOD mice, monocytes are crucial in the maintenance of peripheral tolerance. The transgenically modified dendritic cells with antidiabetogenic MHC could mediate the transformation of autoreactive T cells into Tregs in mice [315]. In addition, MHC-mismatched bone marrow chimera experiment showed that bone marrow from non-diabetic mice is able to induce anergy in the residual NOD autoreactive T cells [316].

1.8.1 *KIR* and NK cells

Killer-cell immunoglobulin-like receptors (KIR) are a group of membrane receptors expressed on the surface of NK cells and $CD8^+$ T cells [317]. In humans, the *KIR* genes are located on chromosome 19q13.4 [317]. Sixteen different KIR genes have been identified (eight encoding inhibitory KIRs, six are activating KIRs and two are pseudogenes) [317]. KIRs bear signaling motifs which are triggered upon binding to their human leukocyte antigen (HLA) ligands of HLA-C, -B and -G molecules [318]. Based on the binding specificity of the ligands, the HLA-C ligands are divided into two groups: HLA-C1 group carrying asparagines and -C2 group carrying lysine at position 80 within the epitope for KIR binding [319]; the HLA-B ligands is divided into two groups: -Bw4 group with isoleucine or threonine at position 80 while -Bw6 carrying Asparagine [320]. KIRs 2DL2, 2DL3, 2DS2 and 2DS3 bind HLA-C1 whereas 2DL1 and 2DS1 bind HLA-C2 ligands, and KIRs 3DL1 binds HLA-Bw4 ligands [318]. In addition, a distinct haplotype has been determined for the KIR genes, namely "A haplotype" which is defined by the presence of only one activating KIR gene, KIR2DS4, and often considered as "inhibitory" haplotype [317].

NK cells are not common among infiltration cells in human insulitis [78]. In contrast to that in humans, there is ample evidence that NK cells were present in murine insulitis and they were able to damage β-cells directly [321, 322]. However, the deficiency in NK cell function (dysfunction in NKG2D receptors) identified in NOD mice indicated that activated NK cells could actually be crucial in the maintenance of immune tolerance [64, 65]. It was later resolved that NKG2D contributed to β-cell damage in mice through $CD8^+$ cytotoxic T cells [323]. Furthermore, neither the depletion nor transfer of NK cells in NOD mice resulted in a significant protection against the development of diabetes [324].These results create a need for the study in subgroups of NK cells in mice. Whereas in humans, the role of NK cells in T1D remains unclear. A recent study showed that NK cells are present in human insulitis and damage β-cells which were associated with coxasckie B4 viral infection [272]. Together these observations indicated that the role of NK cells is probably limited in the autoimmune process and might be important either in a fraction of T1D patients or in events occurred much earlier during the autoimmunity of T1D.

Nevertheless, data from genetic association study suggested a genetic imbalance towards more activating *KIR*s conferred susceptibility to human T1D [325-327]. In addition, *KIR*-2DS2 was found to be more frequent among T1D diagnosed before 5 years of age [327]. Although large variation has been identified between populations on the association between individual KIR gene and T1D [325, 327-334], these data might reflect the complex interaction between environmental factors (viral infections) and *KIR* genes in the disease pathogenesis. Together the above facts indicated that *KIR*s are involved in diabetogenic pathways.

Among patients with T1D, the occurrence of disease predictive islet autoantibodies before 6 months of age indicated that initiation of the disease can be triggered as early as life in uterus [335]. Maternal NK cells actively interacted with HLA ligands on fetal extravillous trophoblasts [336-338]. Previous studies demonstrated that maternal immune cells directly enter fetal circulation and probably influenced the fetal immune system later in life [338]. Indeed, compared with people without T1D, T1D patients seemed to have more circulating maternal immune cells [339]. The disease susceptibility caused by improper interaction between maternal *KIR*-child *HLA* ligand genes can be found during the development of preeclampsia where polymorphisms of *KIR* and *HLA-C* interactions between mothers and

fetuses was able to influence maternal NK cell function where the activation of maternal NK cells is critical in the maintenance of immune tolerance during pregnancy [336, 337]. Nevertheless, whether maternal-KIR/child-HLA interaction could contribute to the initiation of T1D in the children remained to be studied.

1.8.2 *CRYAB*

The *CRYAB* gene is located on chromosome 11q22.3-q23.1, and encodes αB-crystallin, a member of small heat shock protein (sHSP) [340]. The αB-crystallin was originally identified as a member of crystallin which concentrates in the eye lens. Crystallins were first termed and described in 1830 by Berzelius and fractionated in 1894 (C. T. Mörner). Nearly after a century in the early 1980s, a remarkable similarity was identified between homology region of mammalian αB-crystallin and Drosophila sHSP [341]. It was soon found that αB-crystallin is expressed universally in the body rather than tissue (lens) specifically [342-345] and the role of αB-crystallin as sHSP has been recognized thereafter [346].

The importance of αB-crystallin in autoimmunity was first discovered in multiple sclerosis (MS, an autoimmune disease in the central nervous system). In MS, αB-crystallin was a strong self-targets for both T cells [347, 348] and B cells [349]. This importance of αB-crystallin was further supported by its elevated expression in MS brain lesions [350]. In addition, independent genome-wide screen studies showed that microsatellite markers of *CRYAB* region: D11S2000 [351] and D11S898 [352] were associated with MS. It was recently resolved that three frequent polymorphisms in the promoter region: *CRYAB*-652 A>G (rs762550), -650 C>G (rs2234702) and -249 C>G (rs14133) could be responsible for the disease and disease phenotypes. In people with MS, *CRYAB*-652 A and -249 C was associated with the disease whereas -650 C is associated with a rapidly progressive form of MS [353, 354]. It was hypothesized that these polymorphisms in the promoter region might have an influence on transcription rates of the *CRYAB* gene and subsequently results in precipitation to autoimmune disease.

1.9 Prediction and prevention

1.9.1 Prediction

There are many ongoing international collaborative efforts to determine the genetic and environmental factors for T1D. These studies will identify individuals with elevated risk for T1D who would benefit from emerging therapies for the prevention of β-cell destruction.

TEDDY: The environmental determinants of diabetes in the young (TEDDY, URL: teddy.epi.usf.edu) study, comprising six clinical centers located in USA and Europe, was a prospective investigation to analyze the role of HLA-DR-DQ haplotype and environmental triggers on signs of islet autoimmunity (presence of autoantibodies) [355]. By the end of 2011, the study had screened more than 421,000 children from general population and more than 6000 first-degree relatives of T1D patients [356]. ***BABY-DIAB***: BABY-DIAB is a prospective German multicenter study followed from birth of children born to mothers with insulin dependent diabetes or gestational diabetes and fathers with insulin dependent diabetes to investigate the temporal sequence of ICAs, IAA, GADA, and IA-2A [357]. ***ABIS***: All Babies in Southeast Sweden (ABIS) is a population-based (population of 1.1 million in the investigated area), prospective study follows 78.6% of children (17,055 out of 21,700) born in the southeast of Sweden between 1997 and 1999 [358] with an aim to investigate the possibility to screen and to determine factors affecting the development of T1D in general

population [359]. ***DIASY***: Initiated in 1993 in US to determine environmental triggers for T1D, the Diabetes Auto Immunity Study in the Young (DIASY, URL: http://0316829.netsolhost.com/DAISY/DAISY_home.htm) is a population based, single center, prospective study that followed babies in general population with high T1D genetic risk and children with T1D in first degree relatives [360].

However currently there is no known large scale prospective study among Asian populations. Owing to the low T1D incidence in those populations, greater efforts will have to be made to launch such studies scaled similar to those among Caucasian populations. Nevertheless the efficiency of screening strategy in general population is heavily dependent on healthcare system and degree of social development [361, 362]. Such investigations would be likely to initiate in Asian populations in the future.

1.9.2 Prevention

TrailNet: Evolved from the Diabetes Prevention Trial-Type 1 (DPT-1), TrialNet is an international collaborative network on clinical trials to prevent, delay and reverse the progression of T1D (URL: www.diabetestrialnet.org) [363]. ***TRIGR***: Trial to reduce IDDM in the genetically at risk (TRIGR, URL: trigr.epi.usf.edu) study is an European based collaborative randomized trial which aims at testing the hypothesis that weaning to an extensively hydrolyzed infant formula will decrease the incidence of T1D in children who carry high-risk HLA or have a first-degree relative with T1D [364]. ***DIPP***: Diabetes Prediction and Prevention Project (DIPP) is also a population-based, prospective study launched in Finland [365]. In the study, general population newborns are screened for increased T1D genetic risk in the University Hospitals of Turku, Tampere, and Oulu. ***BABYDIET***: BABYDIET study is a prospectively designed, primary prevention trial in Germany to investigate whether modifications in dietary factors can prevent the development of islet autoimmunity in newborns with a first-degree relative with T1D [366].

In addition to the above investigations, there are many other promising attempts to prevent of β-cell function (reviewed in [11]), such as the administration of DiaPep277 (24 residues analog to 437–460 amino acid of human islet autoantigen hsp60 [367]), anti-CD3 monoclonal antibodies [368, 369], anti-CD20 monoclonal antibody [370], CTLA4-Ig [371], anti-TNF-α monoclonal antibodies [372]; autologous hematopoietic stem cell transplantation [373] and; stem cell education therapy [374]. Nevertheless, most trials are in early stages of clinical development whereas the expansion of successful results to general population is yet to happen in the near future.

1.10 Co-occurrence of type 1 diabetes and rheumatoid arthritis

During 1970s, the finding of co-occurrence between T1D and other autoimmune disease (autoimmune thyroid disease) aided the concept that T1D could be of autoimmune origin [125]. Rheumatoid arthritis (RA) was among one of the diseases that co-occurred with T1D [375-378]. RA causes joint damage and can strike at all age, however its incidence rise with age and results in prevalent cases mostly among elders (older than 60 years of age) [379, 380]. Owing to the different disease epidemiology, the co-occurrence of RA and T1D was considered as a rare event [375-377]. Nevertheless, a recent systematic review of literature revealed that around 1.7% RA patients developed T1D whereas relatives of RA patients had increased T1D risk (OR ~ 4) compared with controls from general population [378].

Similar to that of T1D, RA patients also develop humoral autoimmunity against citrullinated self-proteins (i.e. anti-citrullinated protein antibodies, ACPAs) before clinical symptoms occur [381]. ACPAs can be found in 50-70% of RA patients however are rare in healthy individuals [382]. The subsequently developed ELISA assay using a mixture of antigens demonstrated that the captured anti-cyclic citrullinated peptide antibodies (anti-CCPs) had substantial overlap with ACPAs [383]. Further dissection of anti-CCPs using specific citrullinated antigen showed that citrullinated-fibrinogen (Cit-fib), citrullinated peptide-1 of α-enolase (Cit-CEP-1), citrullinated type-II collagen (Cit-C1) and citrullinated vimentin (Cit-vim) are major targets of anti-CCPs [384, 385].

The etiology of RA is contributed by both genetic factors and environmental factors [379]. Among the genetic factors, *HLA-DRB1* "shared epitope" (SE) alleles (alleles of *HLA-DRB1**01, -*DRB1**04 and -*DRB1**10, encodes a conserved amino acid sequence (QKRAA, QRRAA, or RRRAA) at positions 70–74 of their products) confer predisposition to RA [386] whereas *HLA-DRB1**03, *07, *13 and *15 have protective effect [387]. Among the *HLA-DRB1* SE, *DRB1**04 had strongest association with ACPA positive RA (OR ~ 4.3), followed by *DRB1**01 and *DRB1**10 (OR ~ 1.5) [387]. Further investigation on relation between *HLA-DRB1* SE and anti-CCPs also showed that the *HLA-DRB1* SE were strongly associated with Cit-CEP-1 positive RA (OR ~ 5) and Cit-vim positive RA (OR ~ 6) [385].

Clinical trials showed that agents suppress specific immune components (such as anti-CD20 and anti-TNF-α therapies) benefit clinical manifestation of both RA and T1D [370, 372, 388]. Interestingly, among RA patients, co-morbid T1D cases were restricted to anti-CCP positive patients [389]. By exhibiting similar immune-pathological abnormalities (autoantibodies) and genetic susceptible factors (*HLA-DRB1**04), it is likely that RA and T1D share mechanisms in disease pathogenesis.

2 AIMS AND HYPOTHESES

1. To test whether *KIR* along with their *HLA-C* ligand genes modifies suseptibility of T1D (activatory *KIR* and *HLA-C* increase susceptibility to type 1 diabetes (T1D) where as inhibitory *KIR* /*HLA-C* decreases T1D risk).
2. To test whether the combination of maternal *KIR* along with their *HLA-C* ligand genes in fetus modifies suseptibility of fetal T1D.
3. To test the hypothesis that SNPs in the promoter region of *CRYAB* gene is associated with an increased susceptibility of T1D and T1D autoimmunity.
4. To test whether T1D autoantibodies are increased among patients with rheumatoid arthritis (RA) and, whether HLA-DR alleles are associated with T1D autoantibodies in RA patients.

3 SUBJECTS AND METHODS

3.1 Subjects

3.1.1 Chinese T1D cohort

The Children's Hospital of Fudan University provided a cohort which consisted of 259 children with T1D and 262 hospital-based non-diabetic children as control. DNA from peripheral blood was obtained for all subjects. Data on status of GADA, IA-2 autoantibodies were assayed using ELISA and were available only in 128 and 88 subjects respectively. These subjects were studied in paper I.

3.1.2 Chinese T1D children-mother cohort

The Children's Hospital of Fudan University provided a cohort (independent from the above) that consisted of 59 children with T1D and their mothers, together with 158 hospital-based non-diabetic children and their mothers as control. DNA from peripheral blood was obtained for these subjects and studied in paper II.

3.1.3 Swedish Childhood Diabetes Study (SCDS) and Diabetes Incidence in Sweden (DIS) Study

The Swedish Childhood Diabetes study (SCDS) is a collaborative study involving 44 pediatric clinics in 24 countries of Sweden. SCDS invited T1D patients who were registered between September 1, 1986 and December 31, 1987 in the Swedish Childhood Diabetes Registry [390] to participate in the study. Out of the 515 patients agreed to participate, 497 people who donated blood samples, along with 423 control subjects (12% hospital-based and 88% selected from general population using civic registration number matched by birth date, gender and region) formed the SCDS cohort [391].

The Diabetes Incidence in Sweden (DIS) registry is a collection on incident T1D between the age of 15 and 34 years. Blood samples were collected from 633 T1D of the DIS registry were obtained during 1987 and 1988 along with 282 age, gender and region-matched healthy controls [392].

Among the cohort above, DNA from 444 T1D patients and 350 healthy controls were available for genotyping. Those subjects were studied in paper III.

3.1.4 Epidemiological Investigation of Reumatoid Arthritis (EIRA)

Initiated in 1995, the Epidemiological Investigation of Rheumatoid Arthritis (EIRA) (URL: www.eirasweden.se) is an ongoing, Swedish population-based case-control study in which cases were incident rheumatoid arthritis individuals aged 18-70 years identified according to the 1987 revised criteria of the American College of Rheumatology [393, 394]. Controls were age, gender and residential area matched individuals randomly selected from the Swedish national population registry. A total of 1000 incident RA cases and 500 healthy controls were selected from the EIRA cohort and analyzed in paper IV.

3.2 Methods

3.2.1 *KIR, HLA-C* and *-B* genotyping

KIR, *HLA-C* and *-B* genotypes were determined using PCR-sequence-specific primers. In paper I, the 14 *KIR* genes (*KIR-2DL1*, *-2DL2*, *-2DL3*, *-2DL4*, *-2DL5*, *-2DS1*, *-2DS2*, *-2DS3*, *-2DS4*, *-2DS5*, *-3DL1*, *-2DS1*, *-2DP1*, *-3DP1*) were genotyped using sequence specific primer-PCR method [395]. Two internal control primers to amplify the third intron of *DRB1* gene were included in each reaction to distinguish the difference between true

negative and false negatives. In paper II, in addition to the above mentioned KIR genes, KIR-*3DL2* and -*3DL3* were genotyped using duplex sequence specific primer-PCR which utilized framework *KIR* genes in each reaction as internal controls [396]. In both papers the genotyping of *HLA-C* and -*B* ligand genes were also performed using sequence specific primer-PCR with internal control primers [395, 397]. The genotyping were performed in a reaction volume of 15µl with a final content of 50-100ng genomic DNA, 0.5µM of each primer (Eurofins MWG Operon, Ebersberg, Germany), 200µM of each dNTP (Fermantas, Thermo Fisher Scientific), 3mM $MgCl_2$ and 0.5U Taq DNA polymerase (Promega, Madison, USA). The PCR was conducted using an MJ research PTC200 thermal cycler with the following conditions: 95ºC for 3 min, then eight cycles of 20s at 94ºC, 20s at annealing temperature plus 5ºC, and 90s at 72ºC; 35 cycles of 20s at 94ºC, 20s at annealing temperature, and 90s at 72ºC, and a final extension at 72ºC for 5 min. The 15µl of PCR product was visualized by electrophoresis on ethidium bromide stained 2% agarose gels. The presence or absence of *KIR*, *HLA-C* and -*B* genes were determined based on the presence of corresponding PCR amplicons.

3.2.2 *CRYAB* genotyping

To genotype SNPs in *CRYAB* gene (in paper III), we used sequence specific primer-PCR for allelic differentiation. Three SNPs in the promoter region (*CRYAB*-652A>G [rs762550], -249C>G [rs14133] and -650C>G [rs2234702]) were genotyped using primers designed and described previously [353]. A pair of primers that amplifies HLA-DRB gene is used as internal control. The content of PCR reaction, cycling condition and PCR products visualization were conducted similar to that mentioned above and described in details in paper III.

3.2.3 Measurement of T1D autoantibodies

GAD65 and IA-2 autoantibodies: Autoantibodies against GAD65A and IA-2A were analyzed using radioimmunoassay (RIA) as described previously [398, 399]. In this RIA, the recombinant GAD65 and IA-2 were labeled with [^{35}S]-methionine (GE Healthcare Life Sciences, Amersham, U.K.) with *in vitro*-coupled transcription and translation in the TNT SP6 coupled reticulocyte lysate system (Promega, Southampton, U.K.) using full-length cDNA coding for human GAD65 and IA-2 in the pTNT vector (Promega) (pThGAD65) or the intracellular domain (aa 603-980) of IA-2 in the pSP64 Poly(A) vector (Promega) (IA-2ic) [398, 399]. The ^{35}S-labeled GAD65 or IA-2 were then incubated (in separate sets of experiment) with 5µL plasma (in duplicates) overnight at 4°C and then precipitated with protein A-Sepharose (PAS; Amersham Biosciences, Uppsala, Sweden). After the washing of unbind antigen, the radioactivity of antibody bound ^{35}S-labeled GAD65 or IA-2 was counted in a Wallac Microbeta Trilux (PerkinElmer) β counter. The levels of GAD65A and IA-2A were expressed as units per milliliter derived from the World Health Organization standard 97/550 [400]. ***ZnT8 autoantibodies***: Autoantibodies against ZnT8 were also analyzed by RIA [401]. Similar to that described above, the *in vitro* transcription translation was performed using vector mixture containing the three variants of the autoantigen at amino acid position 325 representing ZnT8-R (Arginine), ZnT8-W (Tryptophan) and ZnT8-Q (Glutamin). After the incubation of antigen tracer with serum sample and washing off unbound antigens, the radio activity was counted and results were expressed in arbitrary units derived from in-house positive and negative standard samples. These assays were performed on plasma samples included in paper IV.

3.2.4 ACPA and anti-CCP assay

The anti-CCP antibody status was determined in serum from both RA patients and control subjects using the CCP2 assay (Immunoscan CCPlus, Euro-Diagnostica, Malmö, Sweden) according to the manufacturer's instructions [402]. Results were quantified and the cut-off for positivity was 25 AU/ml. Detection of IgG antibodies against citrullinated peptides from α-enolase (CEP-1), fibrinogen (Cit-fib), collagen type II (Cit-C1) and vimentin (Cit-vim) were assayed in RA patients using ELISA.[402]. ACPAs were measured with ELISA using the following citrullinated antigens: citrullinated α-enolase peptide 1 (CEP-1) [403], fibrinogen (full-length protein), and triple-helical peptides of the C1 epitope of type II collagen ($C1^{III}$), amino acids (aa) 359–369 [404, 405], citrullinated vimentin (aa 60-75 or aa 2-17) [402]. A standard curve constructed with a serum sample positive for specific antigen was included on each ELISA plate and was further used to translate OD values to unit values. The cutoff value for each of the citrullinated antigens was set at 99% specificity and was determined according to the reactivity of control sera. The cut-off for positivity was 10 AU/ml for all ACPA fine specificities. All ACPA measurements were performed in duplicates. These assays were performed on plasma samples included in paper IV.

3.2.5 *HLA* genotyping

HLA-DRB1 subtyping was performed on DNA samples by sequence-specific primer PCR assay (DR low-resolution kit (2-digit); *DRB1*04* subtyping kit (4-digit), Olerup SSP). The PCR products were run on 2% agarose gels for electrophoresis and analyzed using an interpretation table according to the manufacturer's instructions [406]. Among the *HLA-DRB1* genes, *DRB1*01* and *DRB1*10* were defined as SE genes. We determined a frequency of *DRB1*0101* of 89% and a frequency of *DRB1*0401, *0404,*0405,*0408* alleles of 98%, and for practical reasons, we decided to restrict the *DRB1*01* genotyping to only low-resolution analysis [394]. The following alleles were classified as SE of *DRB1*: *DRB1*01* (except *DRB1*0103*), *DRB1*04* (with the exception of some *DRB1*0402* and *DRB1*0403* alleles from limited subtyping experiments) and *DRB1*10*.[406]

3.2.6 Statistics

Paper I: The frequencies of KIR genes and HLA-C ligand genes between T1D patients and non-diabetic controls were analyzed using Fisher's Exact test using R (www.r-project.org,v2.10.0). Results were expressed with odds ratio (OR) and 95% confidence interval (95% CI). Stratification analyses were done using Svejgaard and Ryder method to detect the strongest disease association [407]. Bonferroni correction for multiple testing was applied to comparisons of gene frequencies among T1D and healthy controls. The corrected p values (Pc) less than 0.05 were considered statistic significant.

Paper II: The Fisher's exact test was used to compare the frequencies of genes and maternal-*KIR*/fetal *HLA* ligand gene combinations between patient group and controls. Data analyses were conducted in R (v3.0.2). Probability (P) values were considered significant if they were less than 0.05. With the investigated sample, the Fisher's exact tests have 80% power to detect an OR of 1.8 in at 50% of gene frequencies in controls. We define the AA genotype as those carrying *KIR-2DS4* as the only activating KIR gene. In addition to analysis on individual *KIR* gene, we performed analysis to compare whether the amount of activating *KIR* genes or *KIR* genotype (and in combination with fetal *HLA* genes) in the mothers differs between T1D and controls.

Paper III: The Hardy-Weinberg equilibrium (HWE) was first tested separately for all SNPs in controls. No significant deviation from HWE has been shown in control groups for any of the SNP. The Allele and genotype frequency of each SNP is compared using fisher's exact test with R (v2.10.0). Bonferroni correction was applied in correction for multiple comparisons. The comparisons of genotype frequencies were first made between T1D patients and controls in the following groups: (1) all subjects; (2) high, neutral and low-HLA-risk categories. Those T1D patients were then grouped according to their autoantibody (GADA, IAA, IA-2A and ICA) status, age, and gender. The comparisons were then made according to HLA-risk categories in subgroups also. We then calculated frequencies of haplotype estimates using program *Haploview* with P values assessed by permutation tests [408]. In the analysis of haplotypes, the association between haplotypes and T1D, IAA and IA-2A positivity were analyzed. Results were expressed with OR and 95% CI calculated using fisher's exact tests. P value less than 0.05 after bonferroni correction was considered statistical significant.

Paper IV: In this study, we first determined the cut-off value for GADA, IA-2A and ZnT8A with the quantile-quantile (QQ) plots on autoantibody levels of healthy controls (98.5^{th} quartile of autoantibody level in controls). We then used logistic regression analyses (adjusted by age, gender) compared the frequencies of T1D autoantibodies positivity between RA patients and controls. To determine the association between *HLA-DR* alleles and T1D autoantibody among RA patients, we divided RA patients according to their *HLA-DR* allele carrier status, anti-CCP/ACPA status and in their combinations. The association between T1D autoantibodies and anti-CCP/ACPA or *HLA-DR* alleles among subgroups (according to anti-CCP/ACPA status) were also conducted using logistic regression (adjusted for age and gender). P value less than 0.05 is considered as statistical significant. Statistics was performed using R (v3.0.2).

4 RESULTS AND DISCUSSION

4.1 Paper I

4.1.1 *HLA*-C1 is positively associated with T1D however no association of individual *KIR* genes with T1D in Chinese patients cohort

The frequencies of *KIR* genes are similar between T1D patients and controls, whereas HLA-C1 genes are more frequent among patients (64%) than in controls (46.5%, OR 2.05, 95% CI 1.44–2.90). *HLA*-C2 gene tended to be more frequent among patients (49% vs 37.4% in controls), however this difference is not statistical significant after correction.

The genetic association between *KIR* genes and T1D has been investigated in several populations/ethnic groups which generated inconsistent results [325, 327-334]. *KIR*-2DL2, 2DL5, 2DS1, 2DS2, 2DS3 were more frequent among T1D patients in Latvian population [328, 329]. *KIR*-2DL5 and -2DS5 were less frequent among Korean T1D patients [330] and -2DS5 was also less frequent among Finnish T1D patients [333]. However, the association between individual *KIR* and T1D was not observed in Basque population [334], Dutch population [325] or Brazilian population [331]. Meanwhile, the association between *HLA*-C1/-C2 genes were identified in Latvian population [328, 329]. The inconsistent results can be attributed to ethnicity [330], age of T1D onset [327, 334] or relatively small sample size which can not exclude the possiblity of identifying true association.

4.1.2 *KIR* modified the association between *HLA*-C1 and T1D in Chinese cohort

In the analysis of T1D association with *HLA-C*/*KIR* combination, we demonstrated that the combinations *KIR*-2DL2−/*HLA*-C1+; -2DL3+/C1+; -2DS2−/C1+ were positively associated with T1D whereas the combinations *KIR*-2DL1+/*HLA*-C2−; -2DL2−/-C1−; -2DL3+/-C1−; -2DS2−/-C1− were negatively associated with T1D. In further stratification analysis, we confirmed that *HLA*-C1 was positively associated with T1D among those carrying *HLA*-2DL3 (OR 2.02) and those without -2DL2 or -2DS2 (OR 2.42 and 2.30 respectively). Meanwhile, *HLA*-C2 was positively associated with T1D among those carrying *KIR*-2DL1 and -2DL3 (OR 1.73). Nevertheless, the strongest T1D association was found with *HLA*-C1/-C2 double positivity (OR 3.04).

Our results showed that the genetic interaction between individual *KIR* and *HLA*-C conferred additional susceptibility to T1D. The presence of *KIR*-2DL3, absence of -2DL2 and -2DS2 contributes *HLA*-C1 association whereas the association of *HLA*-C2 is enhanced by the presence of *KIR*-2DL1 and absence of -2DL3. These results indicated that the interaction of *KIR*-2DL1/*HLA*-C2 and *KIR*-2DL3/*HLA*-C1 were important in T1D pathogenesis whereas *KIR*-2DL2/*HLA*-C1, *KIR*-2DS2/*HLA*-C1 contributed to the protection against T1D. These results were similar to those found among Latvians except the reversed effect that *KIR*-2DL2+/*HLA*-C1+ were associated with T1D in Latvians [329]. In addition, a different *KIR*/*HLA*-C interaction were identified in central Chinese populatioin where absence of *KIR*-2DL1/*HLA*-C2 or -2DS1/-C2 were negatively associated with T1D [409]. Meanwhile no association of *KIR*/*HLA* conbination were observed in other studies. These inconsistent findings from different populations reflected the ethinicity difference between populations and the biological consequence of KIRs as a collection of both activating and inhibitory effects.

KIR is a group of receptors that have both activating and inhibitory effect and the effect (immune activating or inhibition) is a result of collective KIR-HLA interaction. The need of analysing *KIR* gene collections can be one approach to resolve these discrepancies. The hypothesis that collection of *KIR* genes modifies T1D susceptibility appeared in the first report in this field and van der Slik et.al. showed that individuals with two or more activating *KIR* genes had increased T1D risk [325]. However this observation was not confirmed by later studies [333] and, actually the inhibitory haplotype of KIR were found to be dominant among Korean T1D patients [330]. Owing to the ethinicity difference among these studies, the exact biological role of *KIR*s in T1D will be resolved by future investigations involving other genetic and environmental factors. In addition, the statistical adjustments within each report is unlikely to prevent publishing false-positive results. Future studies need to involve standardized study protocols, centralized samples to exclude possible confounding factors.

4.2 Paper II

4.2.1 Maternal *KIR*-2DS1–/fetal *HLA*-C2+ and maternal AA genotype+/fetal -C2+ were associated with T1D

In our cohort, among children with *HLA*-C2 ligand gene, absence of *KIR*-2DS1 gene in mother was significantly less frequent in T1D than in controls (6.8% vs 18.7% respectively, OR=0.32, P<0.05). However, among children who did not carry HLA-C2 ligand gene, there is no apparent difference in frequencies of mothers with or without *KIR*s between patients and controls. In addition, we observed that mothers carrying AA genotype were significantly less frequent in patients than in controls among children carrying *HLA*-C2 ligand gene (3.4% vs 12.7% respectively, OR=0.24, P<0.05, Table 1), whereas mothers carry activating KIR genotypes (non-AA genotype) trended to be more frequent in patients compared with controls (22% vs 18% respectively). Among children who did not carry HLA-C ligand gene, there is no difference in frequencies of mothers with presence or absence of KIR AA genotype between patients and controls.

Our study is the first analysis using maternal *KIR*/fetal *HLA* ligand gene combination. These results indicate the importance of maternal KIR/fetal HLA ligand interaction towards an activated immunological reaction in the development of T1D pathogenesis in-utero.

4.2.2 The major inhibitory genotype was less frequent among T1D patients however more frequent among T1D mothers

The major inhibitory genotype was more frequent among T1D children when compared with controls (OR 1.87, P<0.05). This genotype segregated more among children without HLA-C2 ligand gene (OR 2.86, P<0.005). However in mothers, maternal inhibitory genotype interacting with HLA-C2 was more frequent among controls (OR 0.13, P<0.05).

The results among T1D patients (*KIR* inhibitory genotype is associated with increased T1D risk) was similar to that observed in Koreans [330] and Latvians [328, 329]. However, these results contrast with those identified in Caucasian populations where inhibitory *KIR* genes tended to segerate in control subjects [325, 327, 333]. The cause for these disparities can be argued by the variation in the activation of immune system driven by the collective effect of KIRs among different populations. While the decreased *KIR* inhibitory genotypes in T1D mothers indicated the possiblity of maternal KIRs involved in the autoimmune process among offsprings. Meanwhile, the re-analysis of data from our first cohort (larger than the

second) did not show statistically significant difference in *KIR* genotype distribution between T1D and controls. The current cohort is smaller therefore has a higher risk to present false positive findings. In addition, after statistical correction for multiple comparisons, the statistical significance no longer persist. We are currently planning a larger cohort of mother-child pairs for replication.

4.2.3 Mothers to those T1D children with *HLA*-C2 have more *KIR* activating genes

We analyzed the association between the number of activating *KIR* genes and susceptibility of T1D. We found that mothers possessed two or less activating *KIR* genes were more frequent in controls than in patients (60% vs 42% respectively, P=0.033). This difference in frequencies of mother with activating KIR was observed only among children with HLA-C2 ligand gene however not in children without HLA-C2 ligand gene.

These results indicated a trend that maternal activatiing KIRs can predispose T1D in their children. While taking results from studies on human reproduction where maternal inihibitoy KIR genotype increased the risk for reproductive failure among fetus with *HLA*-C2 [336, 337]. It seems that the consequence of maternal *KIR*/fetal *HLA*-C2 interaction falls on the balancing point to maintain the species between reproduction success and fatal diseases. Nevertheless, the results of our study need confirmation from future studies in a larger cohort.

4.3 Paper III

4.3.1 *CRYAB*-650*C and *CRYAB* haplotype was associated with T1D, IAA and IA-2A positivity in T1D

In our study, we were able to show that *CRYAB*-650*C allele was significantly more frequent in patients (84.2%) than in controls (78.2%), with an OR of 1.48 (95% CI = 1.12–1.97, Pc = 0.03). In addition, *CRYAB*-650*C allele was more frequent in IAA-positive patients (92.0%) than in IAA-negative patients (58.2%), with an OR of 8.17 (95% CI = 4.99–13.4, Pc < 0.0001). *CRYAB*-650*C allele was also dominantly present in IA-2A positive T1D patients (OR = 2.14, 95% CI = 1.41–2.33, Pc = 0.005).The haplotype GGC (in the order of *CRYAB*-652/-650/-249) is significantly more among control subjects (OR 0.60, P = 0.02).

4.3.2 The *CRYAB* association with IAA and IA-2A was stronger among T1D with high-risk *HLA* alleles

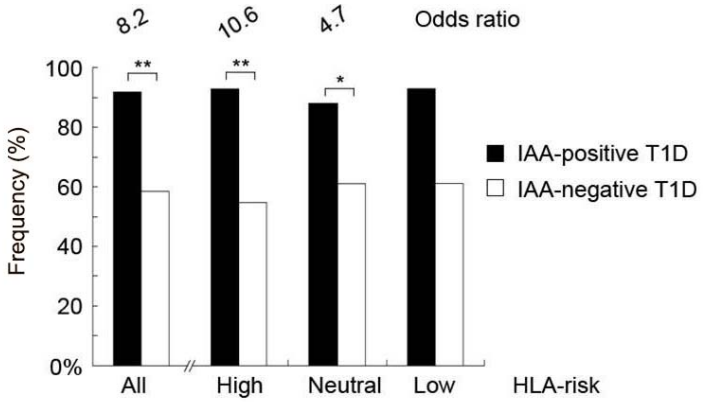

Figure 4.1 – Association between *CRYAB*-650*C and IAA (stratified by HLA) among T1D patients.
* P<0.05; ** P<0.01

CRYAB-650*C allele frequency was higher in IAA-positive and high HLA-risk positive T1D patients, the OR of this association was 10.6 (95% CI = 5.52–20.7, Pc < 0.0001, Figure 4.1). In neutral HLA-risk category, an association was also found between *CRYAB*-650*C allele and IAA-positivity, but with a lower OR (OR = 4.73, 95% CI = 2.00–11.3, Pc =

0.01, Figure 4.1). The association between *CRYAB* haplotypes and IAA positivity only remained in high HLA-risk group.There was no difference in frequencies of CRYAB-650 allele/genotype between IA-2A positive and IA-2A negative T1D patients among each HLA-risk groups. The difference in frequencies of CRYAB haplotypes between IA-2A positive and IA-2A negative T1D patients remains only among high HLA-risk subgroup.

CRYAB-650*C allele had an association with a primary progressive course but later onset subtype of MS [353], however this association between *CRYAB*-650 and MS was not confirmed in later study [354]. While consider the finding that healthy individuals and MS patients both have a strong immune response to alpha B crystallin suggested a more complex role played by alpha B crystallin in MS and autoimmunity [410, 411]. In fact, alpha B-crystallin also plays a role in immune modulation where it suppresses NF-κB in astrocytes during demyelination and results in less inflammation [349]. Our haplotype analysis suggested that the association between haplotypes and T1D (or the presence of antibodies in T1D) is mainly contributed by SNP *CRYAB*-650. Nevertheless the association between *CRYAB*-650 and T1D autoimmunity (IAA production) was not observed in previous studie in MS despite that alpha B-crystallin is a major autoimmune target in MS. This disparity can be owing to the MS autoimmune markers which are yet to be clearly defined. Furthermore, our results showed that the *CRYAB*-650*C frequencies in IAA positive and negative groups fall on each side of its control frequencies, especially among high HLA-risk group. While taking the biological function of alpha B-crystallin in MS, this could be due to the balance between the autoantigenicity and immune suppression of alpha B-crystallin. Increased overall alpha B-crystallin expression might lead to a non-specific autoimmunity while the decrease of alpha B-crystallin expression results in less inflammation and enhanced autoimmune specificity. Previous studies which showed that the promoter region between -836 and -622 of *CRYAB* had a negative regulatory function on the gene expression [412]. Taking together these evidence indicated a possiblity that *CRYAB*-650*C allele acts by inhibiting alpha B-crystallin expression and subsequent an weak inhibition of NF-κB in immune cells which inturn results in a shift toward less inflammation but enhanced autoimmune specificity.

4.4 Paper VI

4.4.1 Islet autoantibodies were increased among RA patients with DR4 alleles

GADA were present in 4.2% RA patients, meanwhile IA-2A and ZnT8A were found in 10.8% and 4.8% of RA patients. GADA was more prevelant among RA patients with DR4 (5.8%) and DR3/4 heterozygosity (14.7%). These frequencies were significantly higher than GADA frequencies among RA patients without DR4 (2.0%, OR 2.98) or DR3/4 heterozygosity (3.3%, OR 5.03). In addition, we identified significant increase in ZnT8A among RA patients with DR4 and DR3/4 heterozygosity (6.5% and 10.3% respectively). We did not observe significantly higher islet autoantibody in DR3 positive RA compared with DR3 negative RA.

4.4.2 GADA and ZnT8A were increased among RA patients positive for anti-CEP-1

GADA and ZnT8A were more commonly found among RA patients positive for anti-CEP-1 compared with RA patients negative for anti-CEP-1 (OR 2.20 and 2.02 respectively). There was no statistic difference in frequencies of islet autoantibodies between RA patients subgrouped by anti-CCP, anti-Cit-fib), anti-Cit-C1 or anti Cit-vim.

4.4.3 DR3 alleles mediated the increase of islet autoantibodies in ACPA positive RA

We divided our RA patients acording to their anti-CCP/anti-CEP-1 status and compared the frequencies of islet autoantibodies among those subgroups. When compared with our RA patient population in general, GADA was prevelant among RA patients positive for both anti-CCP and anti-CEP-1. In this subgroup of RA patients, GADA were significantly higher among those positive for DR3/4, followed by patients positive for DR3 and DR4 (Figure 4.2). IA-2A was only found to be higher among DR3 carriers in RA patient positive for both anti-CCP and anti-CEP-1. ZnT8 was slightly higher among RA patients positive for both anti-CCP and anti-CEP-1 whereas it is significantly more frequent in DR3/4 heterozygous group in regardless to anti-CCP or anti-CEP-1 status (Figure 4.2).

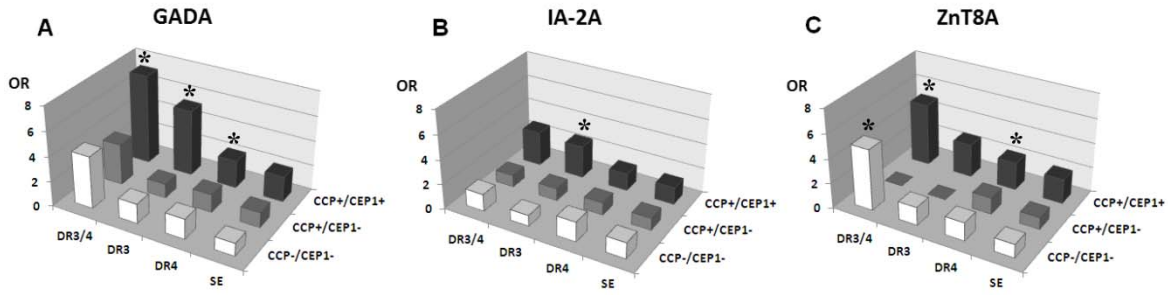

Figure 4.2 – Association between HLA and islet autoantibodies among RA patients stratified by anti-CCP/anti-CEP-1 status. Association were made in comparison to general RA patient population and are presented in **(A)** GADA, **(B)** IA-2A, **(C)** ZnT8A. OR: odds ratio; CCP: anti-CCP status; CEP1: anti-CEP-1 status.
***** P<0.05.

Our results demonstrated that RA patients have significantly elevated islet autoantibodies. *HLA-DRB1**04 (DR4) alleles confer susceptibility to both RA and T1D. DR4 alleles has the highest OR (around 8) among *HLA* alleles that confers T1D susceptibility [182-184]. It is likely that the elevated islet autoantibodies were due to the diabetogenic effect of DR4 alleles. Our results showed that RA patients carrying DR4 alleles had higher frequencies of islet autoantibodies (GADA and ZnT8A) compared with those do not carry DR4.

The subgroup of RA patients positive for ACPAs differs from those RA patients negative for ACPA in several aspects. ACPA positive RA patients is associated with increased joint damage [413, 414]. Studies using rodent models also supported the pathogenic role of ACPAs in the induction of arthritis [415, 416]. In humans, DR4 alleles are the major component of SE and they had the strongest association with ACPA positive RA [387].

Based on the above findings, it would be resonable to identify increased islet autoantibodies among ACPA positive RA patients (which is enriched for DR4 alleles). However, we did not observe significantly increased islet antibodies among ACPA (measured by anti-CCP) positive RA patients. Nevertheless, we observed significantly higher islet autoantibodies (GADA and ZnT8A) in anti-CEP positive RA patients. Interestingly, our results showed that DR3 alleles had stronger association with islet autoantibodies among anti CCP+/anti-CEP+ RA patients than DR4 alleles.

It was shown that the ACPA epitopes (measured by anti-CCP) overlaps primarliy with anti-Cit-fib, anti-CitC1 and, with anti-CEP-1 to a lesser extent [384]. These results indicated that anti-CEP+ RA patients might stem from distinct etiology than general RA

patients. In fact, the dominant environmental factor, smoking, exerts its etiological effect only among RA patients positivity for both anti-CCP and anti-CEP-1 [406, 417, 418]. In addition, the effect of *PTPN22* gene and its interaction with smoking was also identified only among anti-CCP and anti-CEP-1 double positive RA patients [406]. Noting that the EIRA cohort consists of incident RA cases and matched controls with older age (mean age 50.5 years in the analysed cohort), interpretation of our results will depend heavily on T1D etiology among the elders. In T1D, the gene-disease association in DR4 decreases with age, whereas the association of DR3 remains [201]. In addition, IA-2A and ZnT8A are less frequent among older patients however the frequency of GADA remains (see above in section 1.5). Moreover, the association between DR4 with IA-2A becomes trivial among older T1D patients whereas association between DR3 and GADA remains [143]. Together these evidence indicated that GADA is a stable marker of T1D and GADA remains to have strong association with DR3 alleles among older individuals. Indeed, our results demonstrated that this strong association between GADA and DR3 alleles was prominent among RA patients positive for both anti-CCP and anti-CEP-1, whereas the *HLA* association of IA-2A and ZnT8A was obscure.

In accordance to previous finding in our cohort that T1D is restricted to RA patients positive for ACPA [389], we also identified that RA patients positive for ACPAs had higher frequencies of islet autoantibodies. It is worth to note that DR3 is negatively associated with ACPA positivity in RA [387, 419]. The tuning of GADA association with DR4 in general RA towards an association with DR3 in anti-CCP and anti-CEP-1 double positive RA could be influenced by non-HLA genetic and enviromental factors that enhanced the GADA specific autoimmunity linked to DR3 alleles. Although it was showned that the effect of most non-*HLA* genetic factors (including *PTPN22*) among older T1D patients are weaker than those in younger patients [201], the fact that *PTPN22* contributes to increased T1D among ACPA positive RA patients in our studied population [389] indicated that the contribution of *PTPN22* towards T1D among ACPA positive RA was probably not the same as those observed in T1D. There is a need for future investigations to identify the difference in T1D etiology among RA patients positive for ACPAs (anti-CCP and anti-CEP-1). Nevertheless, the weak T1D genetic association among older patients in general raised the question whether individuals with T1D are the same as those individuals who developed islet autoantibodies. In addition, the strong association between DR3/4 and ZnT8A was not identified in younger T1D patients. While in our cohort with older subjects, this association was identified in regardless to ACPA status require further studies to reveal the underlying mechanism.

5 CONCLUSIONS

The following conclusion can be drawn based on the results obtained in papers I to IV.

***KIR* and T1D:** The collective number of inhibitory and activating *KIR* genes could contribute to susceptibility to T1D in ethnic specific manner. Maternal activating *KIR* predisposed T1D among fetus carrying *HLA*-C2 ligand genes. These results will require larger studies for confirmation and functional studies for validation.

***CRYAB* and T1D:** The promoter region of *CRYAB* gene modifies risk for islet autoimmunity (measured by the presence of IAA and IA-2A) and T1D. The effect of *CRYAB* gene is stronger among patients carrying high-risk *HLA* alleles

Islet autoantibodies in RA: Islet autoantibodies are increased in RA patients compared with the healthy individuals. DR4 alleles are the link towards this increase in RA patients. However among anti-CCP and anti-CEP-1 double positive RA patients, DR3 alleles were the major contributor to the increase of islet autoantibodies. This difference indicated distinct etiology of autoimmunity in the subgroup of RA patients to be defined by future studies.

6 PROSPECTS

The Chinese population has very low incidence of T1D. The major genetic risk factors for T1D are less common among Chinese populations. Consider the different *DR-DQ* linkage among Chinese population, the fine mapping of T1D susceptibility in this population will probably produce additional insights to T1D genetic etiology. There were sporadic studies in the Chinese population investigating association between *HLA* and T1D. However the less standardized study protocols, different methods and relative small sample size made the genetic risk from *HLA* still obscure in T1D of this population. There is a need to overcome the above mentioned weakness in future studies.

Aside from *HLA*, many studies in Chinese (and East Asians) indicated that there is substantial difference in the composition of genetic factors compared with Caucasian populations. Some of common T1D association alleles (such as *PTPN22* 1858T) are very rare among Chinese. Take these results and the success of GWAS in the Caucasian population, the sequencing of the whole T1D genome will be necessary to map T1D genetics in Chinese populations. However, to perform such studies among Chinese with comparable scale to those among Caucasians might be less likely to happen (due the low incidence and lower social status). A possible alternative approach can initiate with sequencing using small sample size and replicate in and independent larger samples using lower-throughput genotyping methods.

In addition, analysis combining data of islet autoantibodies assays might provide further interesting findings in Chinese population. Standardized assay using reliable method is crucial to the quality of such studies. Nevertheless, these prospective will rely heavily on the adequate sample size which will only be resolved by collaborations of centers across China.

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