Tracking the Humoral Immune Response

In Type 1 Diabetes

by

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ABSTRACT

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by progressive autoimmune destruction of insulin-producing pancreatic β-cells. Genetic, immunological and environmental factors contribute to T1D development. The focus of this dissertation is to track the humoral immune response in T1D by profiling autoantibodies (AAbs) and anti-viral antibodies using an innovative protein array platform called Nucleic Acid Programmable Protein Array (NAPPA).

AAbs provide value in identifying individuals at risk, stratifying patients with different clinical courses, improving our understanding of autoimmune destructions, identifying antigens for cellular immune response and providing candidates for prevention trials in T1D. A two-stage serological AAb screening against 6,000 human proteins was performed. A dual specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2) was validated with 36% sensitivity at 98% specificity by an orthogonal immunoassay. This is the first systematic screening for novel AAbs against large number of human proteins by protein arrays in T1D. A more comprehensive search for novel AAbs was performed using a knowledge-based approach by ELISA and a screening-based approach against 10,000 human proteins by NAPPA. Six AAbs were identified and validated with sensitivities ranged from 16% to 27% at 95% specificity. These two studies enriched the T1D "autoantigenome" and provided insights into T1D pathophysiology in an unprecedented breadth and width.

The rapid rise of T1D incidence suggests the potential involvement of environmental factors including viral infections. Sero-reactivity to 646 viral antigens was

assessed in new-onset T1D patients. Antibody positive rate of EBV was significantly higher in cases than controls that suggested a potential role of EBV in T1D development. A high density-NAPPA platform was demonstrated with high reproducibility and sensitivity in profiling anti-viral antibodies.

This dissertation shows the power of a protein-array based immunoproteomics approach to characterize humoral immunoprofile against human and viral proteomes. The identification of novel T1D-specific AAbs and T1D-associated viruses will help to connect the nodes in T1D etiology and provide better understanding of T1D pathophysiology.

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CHAPTER 1

1 OBJECTIVES AND CONTRIBUTION

1.1 Significance and Objectives

Type 1 diabetes (T1D) is one of the most common chronic autoimmune diseases during childhood. It is characterized by immunologically mediated destruction of insulin-producing pancreatic islet β-cells (van Belle et al., 2011). The incidence rate of T1D is increasing at an annual rate of 3% particularly in those under the age of 10 years (Onkamo et al., 1999). Currently, there is no cure for T1D. T1D patients suffer from lifetime dependence of exogenous insulin injection accompanied by microvascular and macrovascular complications which are burdensome to both the individual and society (van Belle et al., 2011).

Genetic, immunological and environmental factors have been implicated in T1D development (Atkinson, 2012). Genetic factors include single nucleotide polymorphisms (SNPs) in human leukocyte antigen (HLA) and non-HLA genes. Autoreactive T-cells play a primary pathological role in T1D. Autoantibodies (AAbs) produced by B-cells targeting self-proteins is one significant hallmark during disease progression. Family history, HLA genotypes and AAbs can be combined to predict T1D risk. Discovering new AAbs may help improve risk prediction, stratify patients with different clinical courses, explain disease pathogenesis, identify cellular immune response antigens and provide candidates for prevention trials. There are some evidences showing a link between environmental factors and T1D pathogenesis. Viral infection is of particular

interest (Knip and Simell, 2012). Uncovering viruses that are associated with T1D development will aid in the understanding of T1D pathophysiology.

However, the challenge of discovering novel T1D-associated AAbs and viruses lies in the lack of high-throughput screening tools. The advert of the proteomic era gives us new opportunities to tackle these problems using protein mircoarrays. Hence, the goal of this doctoral dissertation project is to track the humoral immune response including AAbs and anti-viral antibodies in T1D by protein arrays in order to identify new T1D-associated AAbs and viruses. The specific aims of the projects are:

i) Perform a proteome-scale, two-stage serological AAb screening against 6,000 human proteins by Nucleic Acid Programmable Protein Array (NAPPA). The aim is to identify and validate novel AAbs in T1D. The candidate AAbs will be validated by an orthogonal immunoassay. The role of new AAbs in improving T1D diagnostic in combination with the major known T1D-associated AAbs will be evaluated.

ii) Conduct a comprehensive study for T1D-associated AAbs by a screening-based approach using NAPPA including 10,000 human proteins and a knowledge-based approach using individual immunoassay to test 126 pancreas enriched genes from literature and bioinformatics analysis. The aim is to enrich the T1D “autoantigenome” and provide new insights into T1D pathophysiology in an unprecedented breadth and width.

iii) Immunoproteomic profiling of anti-viral antibodies to 646 viral antigens in 42 new-onset T1D patients and 42 age-gender matched healthy controls. This will

allow us to compare the antibody positive rate of the studied viruses and individual viral antigens. The goal is to identify viral strains that are associated with T1D.

iv) Characterize high density (HD)-NAPPA platform including its sensitivity, reproducibility and application in profiling anti-viral antibodies in autoimmune diseases. The signal to background (S/B) ratio will be compared between the standard NAPPA and HD-NAPPA. The aim is to demonstrate HD-NAPPA as a platform to profile anti-viral antibodies in future applications.

1.2 Scientific Contributions

Based on the results of the above projects, all of the four objectives have been achieved successfully. The scientific contributions are as the following:

(i) A proteome-scale, two-stage serological AAb screening against 6,000 human proteins was performed and identified 26 novel AAbs including a known T1D-associated AAb (ZnT8A). An orthogonal immunoassay named Luciferase ImmunoPrecipotation System (LIPS) assay was developed and used to validate these candidate AAbs. A dual specificity typrosine-phosphorylation-regulated kinase 2 (DYRK2) was validated with 36% sensitivity at 98% specificity. The AUC for a combination of DYRK2 AAb (DYRK2A) and the classical IA-2 AAb (IA-2A) was 0.90 compared to 0.72 for DYRK2A and 0.64 for IA-2A alone. This is the first systematic screening for novel AAbs against large number of human proteins by protein arrays in T1D.

(ii) A comprehensive search for novel AAb biomarkers in T1D using a screening-based approach by NAPPA and a knowledge-based approach by enzyme-

linked immunosorbent assay (ELISA) was performed. Six AAbs were identified and validated with sensitivities ranged from 16% to 27% at 95% specificity. Their prevalence in T1D cases complements to the major known T1D-assocaited AAbs and varied in different age subgroups.

(iii) Seroreactivity to 646 viral antigens was assessed in 42 new-onset T1D patients and 42 age-gender matched healthy controls. Prevalence of anti-viral antibodies agreed well with the infection rates of the corresponding virus based on the previous epidemiological studies. Antibody positive rate of Epstein-Barr virus (EBV) was significantly higher in cases than controls (OR 6.6; 95% CI 2.0-25.7) while the other viruses did not differ between the two groups. The EBV and T1D association was significant in both genders and age subgroups (<=12 and >12). These results suggest a potential role of EBV in T1D development.

(iv) HD-NAPPA showed higher S/B ratio compared with standard NAPPA. HD-NAPPA also showed high reproducibility of protein display and serum profiling with average correlation coefficients within or between printing batches at 0.91 and 0.95, respectively. Common as well as unique antibody reactivity patterns were observed in T1D and juvenile arthritis.

With these results, two papers were published. One manuscript is in revision and one more manuscript in preparation for publication. All of them are listed below:

1. Miersch, S.*, **Bian, X.***, Wallstrom, G., Sibani, S., Logvinenko, T., Wasserfall, C. H., Schatz, D., Atkinson, M. A., Qiu, J., Labaer, J. (2013). Serological

autoantibody profiling of type 1 diabetes by protein arrays. J Proteomics. 94, 486-96. PMID: 24148850. (*Co-first authorship)

2. **Bian, X.**, Wallstrom, G., Wasserfall, C. H., Wang, J., Barker, K., Wang, H., Atkinson, M. A., Schatz, D., Qiu, J., Labaer, J. Identification of novel autoantibodies in type 1 diabetes by protein arrays. *In preparation.*

3. **Bian, X.**, Wallstrom, G., Davis, A., Wang, J., Park, J., Throop, A., Steel, J., Yu, X., Wasserfall, C. H., Atkinson, M. A., Schatz, D., Qiu, J., Labaer, J. Immunoproteomic profiling of Virus Antibodies in New-Onset Type 1 Diabetes on Protein Arrays. *In revision.*

4. **Bian, X.**, Wiktor, P., Kahn, P., Brunner, A., Khela, A., Karthikeyan, K., Barker, K., Wasserfall, C. H., Gibson, D., Rooney, M. D., Qiu, J., LaBaer, J. Anti-Viral Antibody Profiling by High Density Protein Arrays. Proteomics. 2015 Mar 11. doi: 10.1002/pmic.201400612. [Epub ahead of print]. PMID: 25758251.

CHAPTER 2

2 INTRODUCTION

2.1 Type 1 Diabetes

2.1.1 Overview

Type 1 diabetes (T1D), which is referred as insulin-dependent diabetes, is characterized by progressive autoimmune destruction of insulin-producing pancreatic β-cells (Atkinson, 2012). Type 2 diabetes (T2D), also named as noninsulin-dependent diabetes, is another major subtype of diabetes. T2D patients are resistant to the effects of insulin rather than the lack of insulin. Both of them suffer from a high blood glucose level. The boundary between these two subtypes is not as black-and-white nowadays (Odegaard and Chawla, 2012). Some autoantibody (AAb) positive T1D children are obese, which is a common characteristic of T2D patients. Alternatively, some latent adult onset diabetic (LADA) patients are positive for T1D-associated AAbs usually AAb to GAD65 (Falorni and Calcinaro, 2002). Although T1D only accounts for 5-10% of diabetes population (Atkinson, 2012), it affects individuals at their most productive age. So there is an emerging need to understand its natural history of disease development, improve current diagnostic tools and develop effective therapeutic strategies for T1D.

T1D is a growing and global health problem. According to the statistics from Juvenile Diabetes Research Foundation (JDRF) (http://jdrf.org/about-jdrf/fact-sheets/jdrf-and-diabetes-statistics/), as many as three million Americans were diagnosed with T1D. More than 15,000 children and 15,000 adults were diagnosed with T1D each year in

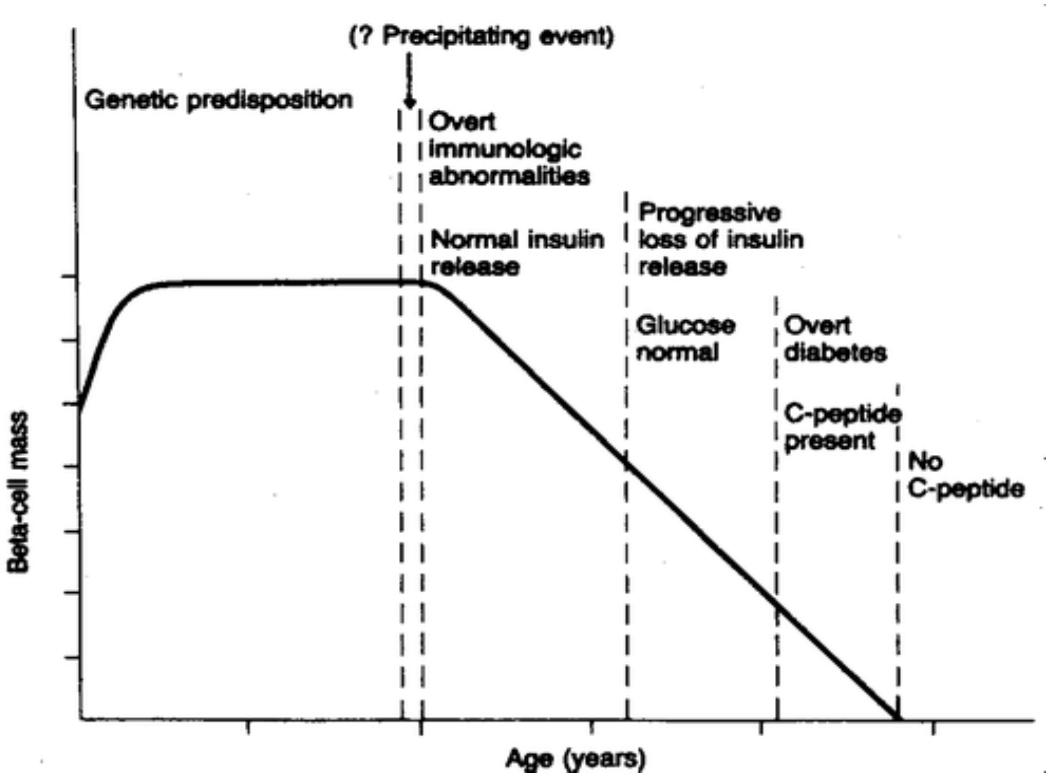
United States (US), respectively. Among people who are living with T1D, 85% are adults and 15% are children. Nearly 14.9 billion US dollars were spent on the healthcare of T1D.

The symptoms of T1D include extreme thirst, frequent urination, increased appetite, sudden weight loss and sugar in urine. A lot of children developed severe ketoacidosis before T1D diagnosis. T1D is often diagnosed by the symptoms, fasting blood glucose test, oral glucose tolerance test (OGTT) and hemoglobin A1c (HbA1c) test. T1D and T2D are distinguished based on the age of diagnosis and doctors' expertise. At the time of diagnosis, > 80-90% of pancreatic β-cells of T1D patients were destroyed (Atkinson, 2012). T1D patients need daily insulin injections and can live almost normal lives. However, it may still cause some effects at the microvascular and macrovascular level. It will lead to hyperglycemia or hypoglycemia if the blood glucose level is not carefully monitored (van Belle et al., 2011). There is a great impetus to have better understanding of this disease.

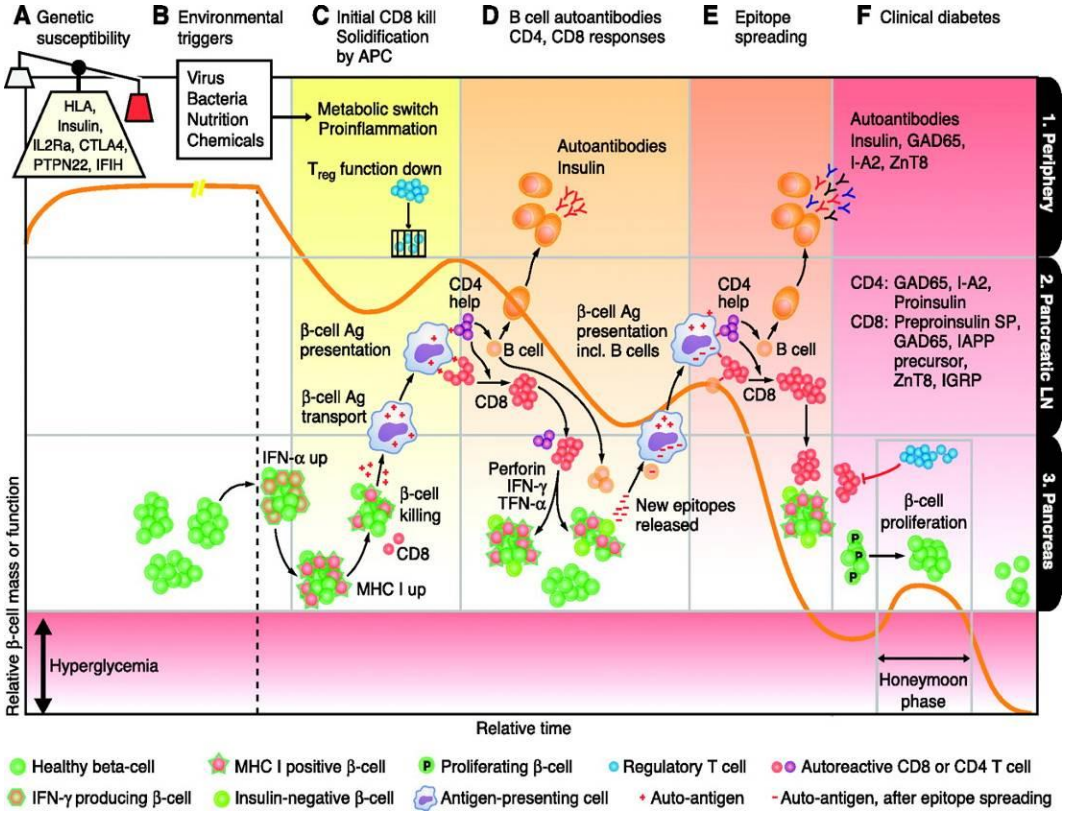
2.1.2 Natural History of T1D

The first model of the T1D natural history was proposed by George Eisenbarth in 1986 (Eisenbarth, 1986) (Figure 2-1). This model suggests individuals with genetic predisposition are exposed to putative environmental triggers which will finally lead to β-cell autoimmunity. During the process of autoimmune destruction, autoreactive T-cells are capable of destroying β-cells. AAbs are produced by autoreactive B-cells. The loss of pancreatic β-cells results in the reduction of insulin production which is reflected by the decreased C-peptide levels.

This model has been a benchmark and served the T1D community for years. The details in this model was enriched by ongoing research in the T1D field (van Belle et al., 2011) (Figure 2-2). During the initiation stage, cytokine expression is triggered by environmental factors. It further up-regulates the expression of major histocompatibility complex (MHC) I molecules on the antigen presenting cells (APCs). $CD4^+$ T-cells will activate B-cells to produce AAbs against specific antigens. $CD8^+$ T-cells are involved in direct killing of β-cells to release new epitopes. There is a short period of honeymoon phase after epitope spreading due to the stimulation of β-cell proliferation.



2 - 1 Stages in the Development of Diabetes Mellitus
Adapted from (Eisenbarth, 1986). Reproduced with permission from (scientific reference citation), Copyright Massachusetts Medical Society.



2 - 2 How T1D Arises
Adapted from (van Belle et al., 2011). Permission not required for reproduce in a dissertation.

2.1.3 Genetic Factors

As suggested by the classic model of T1D natural history, genetic predisposition is a primary T1D risk factor. A number of genomic wide associations studies (GWAS) revealed the linkage of human leukocyte antigen (HLA) regions on chromosome 6p21 with T1D. These include the HLA-DR3/4 and DQ2/8 class II antigens. Besides HLA genes, other genes contributing to T1D are involved in insulin production, metabolism, immunity, protection from β-cell apoptosis and some with unknown functions (Atkinson, 2012). INS encodes insulin which is a major known T1D-associated autoantigen for both

B-cells and T-cells. It is not surprising that single nucleotide polymorphisms (SNPs) in INS results a higher susceptibility of T1D. PTPN22 encodes tyrosine-protein phosphatase non-receptor type 22. It regulates T-cell receptor signaling by dephosphorylation of the Src family kinases LCK and FYN (Steck et al., 2009). CTLA4, which is short for cytotoxic T lymphocyte-associated protein 4, is a major negative regulator of T-cell responses (Pozzilli et al., 1996). The depth of genetic association studies was extended to epigenetic and transcriptome level.

2.1.4 Immunological Contributors

T1D is an autoimmune disease with various immunological contributors including $CD8^+$ T-cells, natural killer (NK) cells, $CD4^+$ T-cells, regulatory T-cells and B-cells.

Cytotoxic/$CD8^+$ T-cells that escaped from negative selection in the thymus are the primary killers of pancreatic β-cells. One way of their action is that they recognize β-cells as non-self and release cytotoxins including perforin, granzymes and granulysin. These cytotoxins are serine proteases and will trigger the caspase cascade in the apoptosis pathway in β-cells. Another way of their action is that they express FAS ligand on the cell membrane which will interact with FAS molecule on the β-cells to recruit the death-induced signaling complex (DISC) and further induce apoptosis. NK cells act similarly as cytotoxic T-cells by releasing cytotoxins to destroy β-cells. As shown in the natural history of T1D, $CD4^+$ T-cells activated by APCs will further activate B-cells to produce AAbs against specific antigens. The function of regulatory T-cells is to suppress autoreactive T-cells or B-cells. B-cells not only produce AAbs, as a feedback, they are also APCs and can further present antigenic epitopes to cytotoxic T-cells.

2.1.5 Environmental Triggers

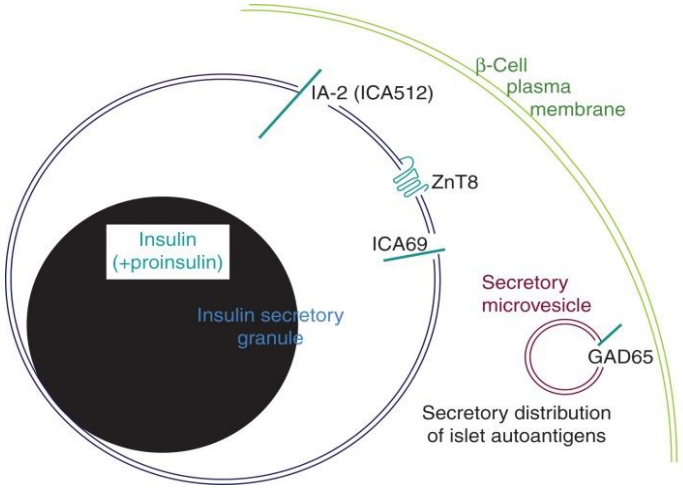
There are several evidences that suggest the involvement of environmental factors in T1D development: first, the incidence of T1D cases has been increasing at 3% a year which cannot be explained by genetic deposition alone (Onkamo et al., 1999); second, the concordance of monozygotic twins in developing T1D is around 66% (Redondo et al., 2008); third, in some countries, T1D shows seasonal and regional outbreaks which is similar to an infectious disease (Peng and Hagopian, 2006).

There are several possible environmental triggers: viruses, bacteria, dietary factors and psychosocial factors (Peng and Hagopian, 2006). Among these, viral infections have attracted of particular interest among. A variety of viruses have been implicated in the β-cell autoimmunity. Bacteria especially symbiotic microorganisms reside in the human gastrointestinal tract play a role in the metabolic process in T1D (Knip and Simell, 2012). Dietary factors include cow's milk consumption, breastfeeding, caloric and vitamin D intake. The impact of these factors in T1D is still controversial based on previous studies. They might be involved in initiation, acceleration or inhibition of T1D progression by some unknown mechanisms (Peng and Hagopian, 2006). Increased psychosocial stress has been connected with increased T1D risk. The stress could be direct to the mother of children who develop T1D or children themselves (Peng and Hagopian, 2006). The identification of environmental factors associated with T1D development will lead to a better understanding of disease pathogenesis.

2.2 T1D-associated Autoantigens

2.2.1 Known T1D AAbs

AAbs produced by B-cells are hallmarks of T1D development. Islet cell antibodies (ICAs) to pancreatic islet cells were found by immunofluorescence staining of human pancreatic tissue using sera from patients with multiendocrine deficiencies associated organ specific autoimmunity (Bottazzo et al., 1974). Insulin antibodies (IAA) were present in insulin-dependent diabetics before insulin treatment (Palmer et al., 1983). AAb to a 64 kDa protein from human pancreatic islet was detected in 23 of 28 ICA positive individuals, 4 of 5 ICA negative but IAA positive individuals, 26 of 31 newly diagnosed T1D patients (Atkinson MA, 1990). This 64 kDa protein was identified as the GABA-synthesizing enzyme named glutamic acid decarboxylase (GAD) (Baekkeskov et al., 1990). Protein tyrosine phosphatase-like IA-2 (islet cell antigen 512) was identified as the insulin-dependent diabetes-related 37/40K autoantigen in 1995 (Bonifacio et al., 1995a). ZnT8 was the most recent identified major T1D-associated autoantigen. It was

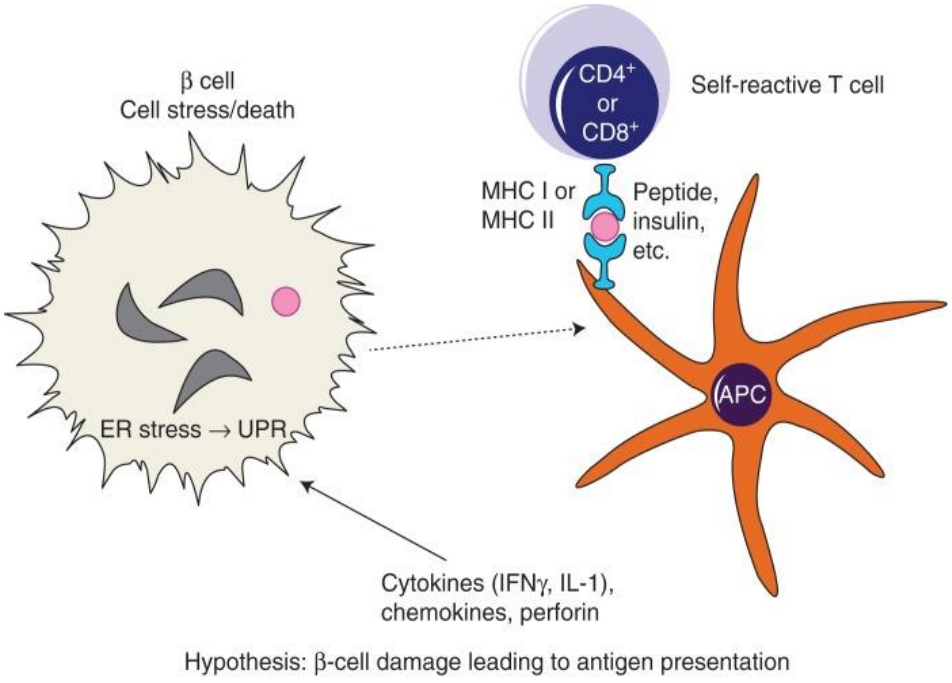


2- 3 Intracellular Distribution of Major T1D-associated Autoantigens
Adapted from (Arvan et al., 2012). Permission was requested from Cold Spring Harbor Laboratory Press.

discovered by analyzing genes highly expressed in pancreas using DNA microarray. AAbs to the candidate proteins were measured by radioimmunoprecipitation assay (RIA) in T1D patients and healthy controls (Wenzlau et al., 2007). The sensitivities of AAbs to these four major T1D-associated autoantigens are around at 60%-80% in new-onset T1D patients.

Insulin is the central regulator that maintains metabolic hemostasis (Figure 2-3). It is highly expressed in the pancreatic β-cells. After transcription and translation from mRNA, the (pro-) pre-insulin polypeptide is transported to the endoplasmic reticulum (ER) by the 24-residue signal peptide. (Pro-) pre-insulin is folded by forming three disulfide bonds and the signal peptide is cleaved. It is further cleaved by peptidase to generate mature insulin and release C-peptide. The mature insulin is stored in the secretory granules (Steiner and Oyer, 1967). Insulin expression is regulated by the promoter of insulin gene and responds to glucose stimuli. GAD65 is an enzyme that catalyzes the reaction to produce the neurotransmitter GABA. It is a soluble protein primary expressed in the cytosol of neuroendocrine cells (Baekkeskov et al., 1990). GAD67 is an isoform of GAD65. They are both expressed in human brains, but only GAD65 is expressed in human islets. IA-2 is a protein phosphotyrosine phosphatase that does not possess phosphatase activity. IA-2, together with IA-2β are transmembrane proteins localized at the membrane of insulin secretory granules (Arvan et al., 2012). ZnT8 belongs to a zinc transporter protein family with 10 members (ZnT1-10). It is a six-pass transmembrane protein that resides in the membrane of insulin secretory granules. ZnT8 is responsible for the processing and packing of insulin into these granules

(Cousins et al., 2006). One proposed hypothesis of T1D pathogenesis is the increased demand of β-cell proteins due to the increased metabolic stress leads to the generation of antigenic proteins from protein misfolding (Figure 2-4). Meanwhile, autoreactive T-cells recognizing these abnormal epitopes can kill β-cells to release more antigenic epitopes



2- 4 Model of β-cell Damage Leading to β-cell Autoimmunity
Adapted from (Arvan et al., 2012). Permission was requested from Cold Spring Harbor Laboratory Press.

and initiate the autoimmune process (Arvan et al., 2012).

The value of four major T1D-assocciated AAbs is well acknowledged. Their contribution in predicting T1D risk depends on factors such as genetic background, age, gender, family history and country. The 5-year risk is almost 80% when combining four major known AAbs in the Diabetes Prevention Trial of Type 1 Diabetes (DPT-1) study (Winter and Schatz, 2011). The addition of ZnT8A with GADA, IA-2A, or IAA results a

5-fold increase of the risk prediction from 7.3% to 36.8% among the Diabetes and Autoimmunity Study in the Young (DAISY) participants (Winter and Schatz, 2011). These four autoantigens were also explored as potential candidates for T1D therapeutics. DPT-1 trial investigated the preventive effects of oral insulin intake in the relatives of patients with diabetes (Group., 2002). Gene therapy using plasmid-encoded (Pro-) insulin successfully reduced (Pro-)insulin-specific $CD8^+$ T cells in T1D patients (Roep et al., 2013). Another two clinical studies looked at the therapeutic role of GAD protein in preserving C-peptide level among recently diagnosed T1D patients (Ludvigsson et al., 2008b; Ludvigsson et al., 2012).

Besides these major T1D-associated AAbs, many minor ones were reported in T1D. AAb to insulin receptor was reported in one T1D case (Elias et al., 1987). Among two GAD isoforms, AAb response in T1D is primary to the GAD65 isoform, AAb to GAD67 added little to IDDM detection (Hagopian WA, 1993). IA-2β (phogrin) is a homologue of IA-2. They are both expressed in neuroendocrine cells. AAb to IA-2β had lower sensitivity than IA-2 antibody (IA-2A) (Lu J, 1996). AAb to a 58 kDa ICA was detected in non-obese diabetic (NOD) mice but not in the other mice strains. This antigen was found to be expressed in neuroblastoma cells and identified as peripherin (Boitard et al., 1992). AAb to ICA69 was identified by immunoprecipitation of human islet expression library using sera of prediabetic patients (Pietropaolo et al., 1993). Sensitivities of AAb to ICA69 varied from 4.17% to 21% depending on different studies (Kerokoski et al., 1999; Lampasona V, 1994; Martin et al., 1995). Another study reported AAbs from T1D patients recognized a 51 kDa antigen. By screening the expression

library derived from rat insulinoma cells, the 51 kDa protein was identified as aromatic-L-amino-acid decarboxylase (DCC) (Rorsman et al., 1995). However, there were no follow-up studies on this AAb. The GM2-1 ganglioside islet autoantigen was reported to be a target of AAbs in the ICA positive relatives of T1D patients (Dotta et al., 1997b). It is expressed in secretory granules but not β-cell specific (Dotta et al., 1998). Glima 38 is a glycosylated islet cell membrane protein. AAb to glima 38 was detected in 19% of newly diagnosed T1D patients (Aanstoot et al., 1996). Another study investigated the association of AAb to glima 38 with IA-2A and ICAs. It was found AAb to glima 38 was detected in 38% of T1D patients, 35% of prediabetic siblings and 0% in control subjects (Winnock et al., 2001). AAb to glucose transporter 2 (GLUT2) was found in new-onset T1D patients. It may be involved in the process of the blockage of glucose uptake by binding to GLUT2 (Inman et al., 1993). Serum from T1D patients was reactive with rat GLUT2 at the molecular weight of 60 kDa (Pehuet-Figoni et al., 2000). AAbs to heat shock proteins (Hsp) including Hsp60 and Hsp70 was described in multiple studies by enzyme-linked immunosorbent assay (ELISA) in T1D (de Graeff-Meeder et al., 1993; Gruden et al., 2009; Ozawa et al., 1996). The sex-determining region Y-related protein SOX13, also named as ICA12, was first described as a T1D autoantigen in 2000 (Kasimiotis et al., 2000). Several follow-up studies investigated its prevalence in Indian, Swedish and Korean diabetes patients (Fida et al., 2001; Park et al., 2003; Tandon et al., 2002; Torn et al., 2002). However, an opposite conclusion which claimed AAb to ICA12 was unlikely to be a useful marker for pre-clinical T1D patients challenged the validity of this AAb (Lampasona et al., 2001). Human DNA topoisomerase II (Top II) was

characterized as an autoantigen in IDDM patients in 1996 (Chang et al., 1996). It was reported by the same group that the positivity of anti-TopII was 50.2% and 55.2% in two studies, respectively (Chang et al., 2004; Shiau et al., 2000). AAbs to carbonic anhydrase II and lactoferrin was reported in three different studies and had variable sero-prevalence (di Cesare et al., 2004; Hardt et al., 2008; Taniguchi et al., 2001). Importin β was identified as a novel autoantigen in T1D autoimmunity by screening random peptide libraries by phage display (Ola et al., 2006). CD38 is named as ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 1. AAb to CD38 was first described in Caucasian patients with diabetes (Pupilli et al., 1999). Different approaches such as immunoblot analysis or fluid radioimmunoassay were used to measure AAbs to CD38 and resulted in discrepancies in its sensitivity (Antonelli et al., 2002; Mallone et al., 2001; Mallone et al., 2002; Pupilli et al., 2005; Sordi et al., 2005). In 2008, a systematic study investigated the presence of AAbs to 56 secretory vesicle-associated proteins and identified VAMP2 and NPY as novel minor T1D AAbs with 21% and 9% sensitivity, respectively (Hirai et al., 2008a). CCL3 was reported to be the target of AAb with 87.4% sensitivity by a fluid phase radioassay (Shehadeh et al., 2009). However, it was later proved not to be markers of T1D when measured by a commercial ELISA kit (Ziegler AG, 2011). Desin and filtrin are presented in both the kidney podocytes and pancreatic islet cells. It was found AAbs to desin and filtrin were prevalent in 33% and 11% of T1D patients (Rinta-Valkama et al., 2007). Only a few studies explored both the protein level of autoantigens and the AAbs to autoantigens. Reg1α was described in other autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (Fujishiro et al., 2012). Reg1α protein level

was found to be significant higher in newly diagnosed T1D patients and anti-Reg1α antibodies were prevalent in 47% of T1D patients (Astorri E, 2010). AAb to aminoacyl-tRNA synthetase (aaRS) was detected in 37.9% of T1D patients compared with 1.54% of the non-diabetic controls (Park et al., 2010). Luciferase immunoprecipitation systems (LIPS) assay was a recently developed method to profile AAbs. Pancreatic duodenal homeobox 1 (Pdx1) is a transcription factor that regulates β-cell development and function (McKinnon and Docherty, 2001). AAb to Pdx1 (PAA) was found in 21% of the patients who were triple positive of the major T1D-associated AAbs (Donelan et al., 2013). Serological Proteome Analysis (SERPA) is a method to profile T1D AAbs from human pancreatic β-cells by two-dimensional gel electrophoresis (2DE) followed by immunoblotting. The AAb to Rab GDP dissociation inhibitor beta (GDIβ) was further validated by radiobinding assay (RBA) (Massa et al., 2013). AAb like anti-serpin B13 was found to be protective to autoimmune destruction by reducing inflammation in pancreatic islets (Baldzizhar et al., 2013). The dual specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2) was identified by a non-biased screening using an innovative protein array platform followed by LIPS assay for validation (Miersch et al., 2013). A Korean group performed a large scale screening for novel AAbs. Anti-EEF1A1 and anti-UBE2L3 antibodies were validated by an independent sample set (Koo et al., 2014).

Among these reported AAbs, few were validated in an independent sample set in an independent study by orthogonal assays. Some are not T1D specific but also presented in other autoimmune diseases (Raju et al., 2005). Most of the follow-up studies were

done by the same group who made the discovery. Inconsistent conclusions resulted from differences in the samples used (prediabetic, new-onset, long lasting or latent autoimmune diabetes of adults) and the technology applied (RIA, ELISA, immunoblotting, LIPS assay, protein array or luminex assay). Nonetheless, the enrichment of T1D AAb repertoire will benefit T1D research in the long term.

2.2.2 Methods for Identifying Novel AAbs

Methods for identifying novel autoantigens for B or T-cells are largely different. The methods used for identifying novel AAbs can be divided into two categories: screening/non-targeted or knowledge/targeted based approaches.

The screening/non-targeted approaches include: immunohistochemistry (IHC) staining of pancreatic tissue section, immunoprecipitation of phage display library constructed from brain or pancreatic islet tissues of human or mice, immunoprecipitation of phage display library made of peptides covering all human proteome together with next generation sequencing (NGS) technology, SERPA and protein microarray. Islet cell antigens were detected using IHC staining by applying patient sera on human pancreatic islet tissue sections (Bottazzo et al., 1974). There are several disadvantages associated with this method. It is hard to obtain pancreatic tissue sections. The process to prepare the tissue sections is time consuming and labor intensive. In addition, it is difficult to quantify the signal of fluorescence staining. nPOD which is short for the network for pancreatic organ donors with diabetes, was established and funded by JDRF (http://www.jdrfnpod.org/). It provides pancreatic tissues to T1D researchers and has great impact worldwide (Campbell-Thompson et al., 2012). Juvenile Diabetes Foundation

(JDF) unit is a standard to quantify the level of ICAs (Bonifacio et al., 1990). To uncover the protein identities of ICAs, a screening technology was developed using serum from T1D patients to precipitate phage displayed proteins from islet cells of human or mice. Several AAbs were discovered in this way including IA-2 (ICA512), ICA69 and importin β (Bonifacio et al., 1995a; Ola et al., 2006; Pietropaolo et al., 1993). Phage display was combined with NGS to screen T1D-associated AAbs. Using this method, a phage display library displaying peptides covering the whole human proteome was constructed (Larman et al., 2013). No specific AAb was discovered. However, they revealed a prematurely polyautoreactive phenotype in T1D patients compared with their matched controls. SERPA was used as a proteomic tool to identify novel AAbs in T1D. Proteins from pancreatic tissues were separated by 2DE. T1D specific candidate AAbs were identified by applying T1D patient serum on the membranes transferred from 2DE gels. Candidate AAbs were cloned and validated by RBA (Massa et al., 2013). Protein microarray enables high-throughput screening of AAb to individual antigen proteins. Shane Miersch used a novel protein array platform and validated a minor T1D-associated AAb by LIPS assay (Miersch et al., 2013). A group from Korean identified two novel AAbs by high-density protein microarrays (Koo et al., 2014).

The knowledge/targeted approaches include: RBA using pure proteins, western blotting, immunoprecipitation, ELISA and luminex assay. Most AAbs identified in the early days were focused in the landscape of pancreatic islets. It was already known that T1D patients had ICAs against pancreatic islet tissue sections and insulin is one of the most abundant β-cell specific proteins. It was reasonable to check the presence of AAbs

to insulin in T1D patients. The suspicion was confirmed by measuring insulin antibodies using a radiolabeled soluble insulin binding assay before insulin treatment (Palmer et al., 1983). The rationale to measure it before treatment is because insulin treatment may elicit antibodies to exogenous insulin which may not be distinguishable from endogenous insulin. Since ICA stains the whole human pancreatic tissue, it is reasonable to search for more proteins in the pancreatic islets as the source of AAb targets. Western blotting was used to identify specific proteins that are recognized by patient serum. A 64 kDa islet antigen from human pancreatic tissue was identified and had high prevalence in T1D patients (Atkinson MA, 1990). This 64 kDa protein was later proved to be GAD65 by immunoprecipitation of GAD prepared from brain and islet with anti-64 kDa antibody from T1D patients (Baekkeskov et al., 1990). ELISA was used to profile AAbs to purified proteins. Several AAbs were identified using ELISA including HSP60, HSP70, carbonic anhydrase II, lactoferrin and Aminoacryl-tRNA synthetase (di Cesare et al., 2004; Gruden et al., 2009). Luminex assay is a method to measure AAbs in a multiplexed manner. It was applied to quantify AAb to serpin in the serum of NOD mice (Baldzizhar et al., 2013).

2.2.3 Methods for Measuring Known AAbs

The identification of novel AAbs by various approaches described above, allows for more efforts to develop and standardize assays used for quantifying these AAbs for clinical or research purposes. The most common methods to measure T1D AAbs include: RIA, LIPS assay and meso-scale discovery (MSD) platform.

RIA is a classical assay to measure major T1D-associated AAbs. In this assay, radiolabeled antigens are produced by *in vitro* transcription and translation using Rabbit Reticulocyte Lysate (RRL) expression system. Serum samples from T1D patients are incubated with antigen proteins and pulled down by protein A/G coated beads. After washing away non-specific bindings, AAb levels are quantified by measuring the radioactivity. Diabetes Autoantibody Standardization Program (DASP) is a program to improve the measurement of T1D-associated AAbs. The aim of this program is to improve laboratory methods, evaluate and compare assay performance in different labs. Experiment materials such as plasmids used for *in vitro* protein expression, sample processing procedures and experimental protocols were standardized (Bingley et al., 2003; Burbelo et al., 2010; Schlosser et al., 2010).

LIPS assay was developed by Peter D. Burbelo at National Institute of Health (NIH) (Burbelo et al., 2005b). In this assay, genes encoding antigens of interest are cloned into a mammalian expression vector appended with a C-terminal renilla luciferase tag. Antigen proteins are expressed in mammalian cell lines and harvested for incubation with patient serum samples. Antigen-antibody complexes are formed and captured by protein A/G beads. The substrate coelecterazine is added to produce luminescent signal. It was shown that LIPS assay had high sensitivity in measuring AAb to IA-2, GAD65 and high reproducibility between RIA and LIPS (Burbelo et al., 2008). The method was adapted and modified in which they use a T7 promoter-based plasmid to encode antigen proteins that allows for cell-free expression of antigen proteins. This modified version of LIPS assay had similar performance in measuring IA-2A (Miersch et al., 2013). One

advantage of LIPS over RIA is the avoidance of radioactive materials. Because of its similarity and consistency with the more commonly used RIA, researchers were trying to develop and standardize it as a high-throughput platform to profile AAbs in T1D (Marcus et al., 2011).

MSD provides an alternative and multiplexed immunoassay platform for quantifying AAbs in T1D. It was developed as a nonradioactive and bivalent assay to measure insulin antibodies. In this assay, 96-well plates with electrodes were coated with streptavidin. Biotinylated and sulfo-TAG labeled insulin was incubated with serum samples from T1D patients. Biotinylated insulin was captured by streptavidin on the plates and pulled down insulin antibodies together with Sulfo-TAG labeled insulin. Electrochemiluminescent signal was produced when the Sulfo-TAG was close to the bottom of the plates. This assay has successfully demonstrated in its ability to measure persistent insulin in individuals with differential risk (Yu et al., 2012). It was also used to measure AAbs to GAD65 to identify high risk individuals. The advantage of MSD platform is that it has been approved by the Food and Drug Administration (FDA) for preclinical studies.

Many other assay platforms were developed to improve the measurement of known T1D-associated AAbs. A time-resolved immunofluorometric dual-label assay was developed to detect AAbs to GAD65 and IA-2 simultaneously (Ankelo et al., 2007). Triple chimeric islet autoantigen IA2-ZnT8WR was used to measure AAbs to IA-2 and ZnT8 with two different polymorphisms (ZnT8W and ZnT8WR) (Yu et al., 2010).

2.2.4 Autoantigens Recognized by T-cells

For years, AAbs were thought to be the "smoke of fire" and not play a role in T1D pathogenesis. Whereas autoantigens recognized by T-cells including $CD4^+$ or $CD8^+$ T-cells are more important. $CD4^+$ T-cells are the heart of this disease. The breakage of immune tolerance to pancreatic β-cells of $CD4^+$ T-cells is the key during T1D progression. $CD8^+$ T-cells also play a pivotal role in the process of autoimmune destruction. The identification of autoreactive T-cells may provide targets for diagnostic and therapeutic purpose of T1D.

Most of the reported T-cell autoantigens were not specified to be reactive to $CD4^+$ or $CD8^+$ T-cells as determined by T-cell proliferation assay or cytokine production assay. One early study showed peripheral blood mononuclear cells (PBMCs) of T1D patients responded to fractionated human pancreatic islet cell proteins. T-cell proliferation was observed in T1D patients but not in controls (Brooks-Worrell et al., 1996). PBMCs had a proliferative response to GAD65 in insulin-dependent diabetes (Atkinson et al., 1992). Autoreactive T-cells specific for ICAs including GAD65 and ICA512 were present during the prediabetic period (Durinovic-Bello et al., 1996). T-cell response to IA-2 was characterized in T-cell lines. Some of the T-cell lines responded to an immunodominant region in IA-2 (Hawkes et al., 2000). ZnT8 is a major AAb target in T1D. It was demonstrated that PBMCs from T1D patients were stimulated to produce IFNγ in response to a peptide pool from ZnT8 by Enzyme-Linked ImmunoSpot (ELISPOT) assay (Dang et al., 2011). Besides the major T1D-associated autoantigens, one study reported a recombinant fragment of JUNB (amino acids 1-180) stimulated peripheral blood T-cell

proliferation in 71% of new-onset T1D patients (Honeyman et al., 1993). T-cell proliferation responding to HSP60, HSP70 and HSP90 was profiled in children newly diagnosed with T1D. Epitopes of HSP70 and HSP60 were recognized by T-cells in 85% of the 25 children tested in this study (Abulafia-Lapid et al., 2003). RegII is a protein predominantly expressed in β-cells. Proteins from Reg family were overexpressed in islets of a patient who died after sudden onset of T1D. Reg-specific T-cells can transfer diabetes in NOD mice (Gurr et al., 2007).

Some autoantigens were clearly described that can stimulate both $CD4^+$ and $CD8^+$ T-cells. Glucose-6-phosphatase (G6PC2) is one of them. Also named as IGRP, it is highly expressed specifically in pancreatic islets. The gene was first cloned in 1996 (Arden et al., 1999). In one study, NOD mice immunized with peptides from IGRP showed a dominant $CD4^+$ T-cell response and had a preventive role during disease development(Mukherjee et al., 2005). In another study, IGRP-reactive T-cells were detected in the islets and peripheral blood *ex vivo* by a tetramer assay (Lieberman et al., 2003).

Autoantigens recognized by $CD8^+$ T-cells were investigated more because their direct involvement in β-cell destruction. GAD-specific cytotoxic T-cells may play a critical role in the initiation events inT1D (Panina-Bordignon et al., 1995). One study reported the insulin peptide B_{10-18} was presented by HLA-A2 (*0201). Cytotoxic T-cells recognizing this epitope was detected and correlated with recurrent of autoimmunity after islet transplant (Pinkse et al., 2005). A more recent study found cytotoxic T-cells recognizing a 10-mer signaling peptide of pre- (pro-) insulin killed β- cells in a glucose

dependent manner (Skowera et al., 2008). T-cell clones from T1D patients responded to proteins from insulin secretory enriched fractions of a hamster insulinoma cell line (Chang et al., 1995). Lots of studies tried to identify antigenic epitopes that can stimulate cytotoxic T-cells. In one study, peptides from islet amyloid polypeptide (IAPP), IGRP and IA-2 were presented by HLA-A (*0201) in T1D patients. Stimulation of IFNγ production was measured by ELISA (Ouyang et al., 2006). A quantum dot coupled major histocompatibility complex (MHC) multimer assay was used to measure the presence of $CD8^+$ T-cells restricted to the insulin, pre- (pro-) insulin, IA-2, GAD65 and IGRP epitopes (Velthuis et al., 2010). Islet-specific $CD8^+$ T-cell IFNγ enzyme-linked immunospot (ISL8Spot) assay was developed to quantify $CD8^+$ T-cell response to ICAs including HLA-A2 restricted β-cell epitopes derived from pre- (pro-) insulin, GAD and IGRP (Mallone et al., 2007).

There are some autoantigens that showed reactivity to $CD4^+$ T-cells. Imogen 38, also named as 28S ribosomal protein S31, is expressed in the mitochondrial and was identified as a novel autoantigen recognized by diabetic T-cell clone (1C6) in one newly diagnosed T1D patient by T-cell proliferation assay (Arden et al., 1996). It was reported later by another group that alteration of peptide of imogen 38 could inhibit antigen specific reactivity of Th1 clone (Geluk et al., 1998). A natural processed peptide WE14 from chromogranin A was reported to be recognized by $CD4^+$ T-cells in NOD mice (Stadinski et al., 2010). Mouse islet protein fraction containing islet amyloid polypeptide (IAPP) can stimulate the diabetogenic $CD4^+$ T-cell clone BDC-5.2.9 to produce IFNγ (Debug et al., 2011).

In general, the identification of autoantigens for T-cells is much more challenge than for B-cells. T-cell assays need the interaction between T cell receptors (TCRs), peptides from autoantigens and MHC encoded by HLA genes. B-cells, $CD4^+$ and $CD8^+$ T-cells may recognize same autoantigens. It is feasible to screen the autoantigens for B-cells using an easier approach and test their validity as autoantigens for T-cells by a more complicated method. Many T-cell autoantigens were initially discovered in NOD mice not in human. It is worthy to test whether they are valid in human. A systematic survey of autoantigens for T-cells is needed with detailed epitope mapping, interaction with specific HLA genotypes and T-cell clones, their frequency and dynamics during T1D progression.

2.2.5 Methods for Identifying Autoantigens Recognized by T-cells

Methods for discovering and monitoring T-cell autoantigens are much more complicated than for B-cells. Four strategies could be used to identify T-cell autoantigens. First, pancreatic β-cells are one of the most specialized cells in human body. Many pancreas enriched genes are potential targets for T-cell autoimmunity; Second, autoantigens recognized by B-cells are another source for T-cell autoantigens as they may share some common epitopes; Third, β-cell autoantigens could be identified by analyzing mRNA expression using cDNA subtraction libraries or DNA microarrays. Forth, using an "inverse translation" approach, antigens found in NOD mouse can be tested in human (Haskins et al., 1989).

The T-cell proliferation assay is a method to measure the proliferation of activated T-cells. In this assay, PBMCs are stimulated with specific antigens and incubated with

^{3}H-Thymidine. The proliferation signal is measured by the incorporation of radioactive nucleotide in DNA by a CPM reader. The stimulation index (SI) is calculated by using the mean CPM of the experimental wells divided by the mean CPM of control wells (Kruisbeek et al., 2004).

The ELISPOT assay is a method to detect a single antigen responsive T-cell within a population of PBMCs. It provides information about the specific cytokines that are secreted by T-cells after antigen stimulation and the frequency of reactive T-cells within the test population. In this assay, a PVDF membrane microtiter plate was coated with a capture antibody which binds specifically to the cytokines that will be measured. PBMCs from either T1D patients or healthy controls are seeded into individual wells together with different antigens. Cytokines secreted by reactive T-cells are captured by the capture antibody in the wells. A biotin labeled detection antibody that binds to the specific cytokine is applied and is followed by a streptavidin conjugated enzyme that can catalyze substrate reaction to produce signals. Each spot in the well represents a cytokine-secreting cell (Czerkinsky et al., 1983).

The FluoroSpot assay is a modified form of the ELISPOT assay. Instead of measuring one cytokine, FluoSpot assay measures the secretion of two or more cytokines in one well by coating two or more capture antibodies. The secreted cytokines can be captured simultaneously in the same well and detected by different fluorescently labeled secondary antibodies (Gazagne et al., 2003).This method is more attractive than ELISPOT assay when there is limited cell amount and many cytokines need to be measured.

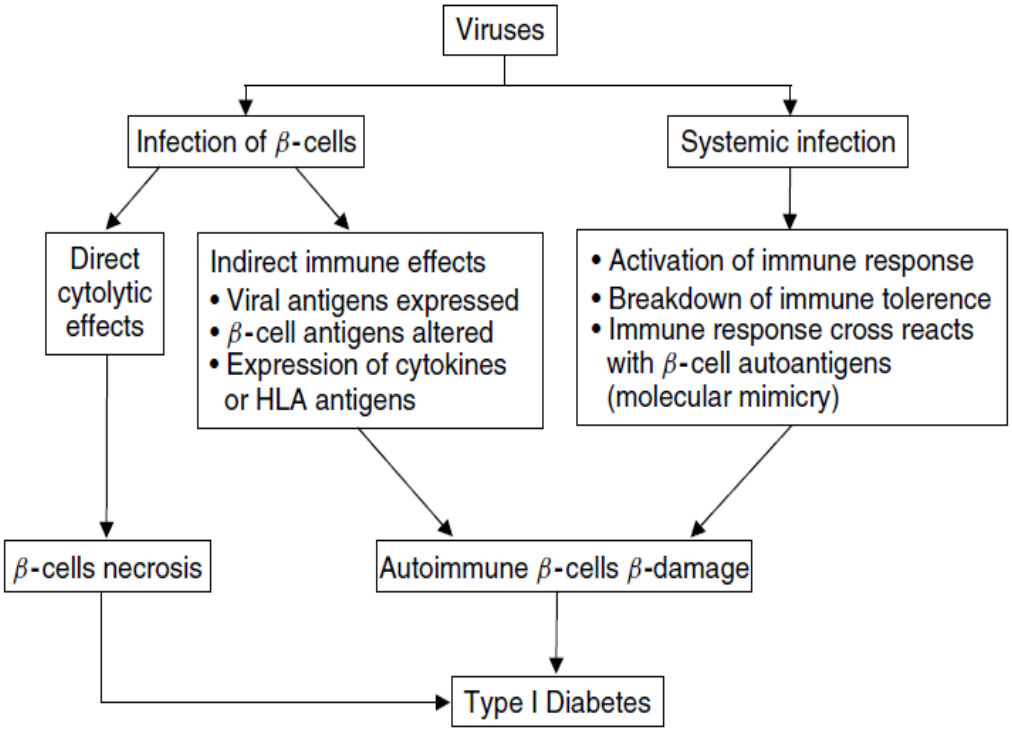
The MHC tetramer assay is a method to detect and quantify antigen-specific T-cells in blood samples. In this assay, fluorophore labeled streptavidin beads are coated with biotin labeled MHC molecules and form a tetramer. The tetramers are incubated with PBMCs and peptide antigens. If these three form a complex, the fluorophore-labeled cells can be separated by a flow cytometer. Higher percentage of fluorophore positive complexes indicates a higher prevalence of antigen-specific T-cells (Altman et al., 1996). One study showed increased frequencies of T-cells against four novel epitopes in human pre- (pro-) insulin in new-onset patients compared with controls (Unger et al., 2011). Diab-Q-kit is essentially similar to the tetramer assay, however, the streptavidin beads are labeled with quantum dots instead of fluorophores.

The assay throughput for identifying and monitoring T-cell autoantigens is limited because of the intricate nature of these assays. T-cell activation needs the interaction between MHC molecules, peptides from antigens and TCRs. All three are important in activating autoreactive T-cells during T1D progression. The third factor such as epitopes of autoantigens, HLA genotypes of APCs or T-cell clones can be studied if the information of any of the other two was known. Continuously monitoring autoreactive T-cells can be used to track disease progression. These T-cell clones are also potential therapeutic targets in T1D.

2.3 Role of Viral Infection in T1D

2.3.1 Mechanisms of Virus Induced Diabetes

Viral infection is one of the most important environmental factors in T1D. It was implicated in the etiology of T1D more than 100 years ago (Knip and Simell, 2012). Viruses act in different mechanisms to induce T1D autoimmunity (Figure 2-5). Some



2- 5 Mechanisms of Virus Induced Diabetes
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viruses infect β-cells directly and cause β-cells cytolysis. In this case, β-Cells are destructed through the necrosis pathway. Infection of β-cells also leads to some indirect autoimmune effects including expression of viral antigens, cytokines, HLA molecules and alteration of β-cell antigens. All these lead to an increased inflammation signal. As a result, immune cells begin to recognize β-cells as non-self. More often, systemic viral

infection will activate immune response and breakdown immune tolerance. Some viral antigens show sequence similarity with known T1D-associated β-cell antigens. This is called molecular mimicry. The immune response to viral antigens cross reacts with β-cell autoantigens. All these different paths will lead to β-cell damage and clinical T1D (Jun and Yoon, 2004; Yoon, 1995).

2.3.2 Viruses Reported to Be Associated with T1D

A number of viruses have been implicated to be associated with T1D in either animal models or viral epidemiologic studies in human. These viruses encompassed different genome types (dsRNA, ssRNA(+), ssRNA(-), dsDNA). Different approaches were used to investigate T1D and viral associations. The most often reported T1D-associated viruses are: enterovirus, epstein-barr virus (EBV), human cytomegalovirus (HCMV), human endogenous retrovirus, mumps virus, rubella virus and rotavirus.

Enterovirus is ssRNA(+) virus that belongs to the family of *Picornaviridae*. There are 12 species and more than 100 serotypes under enterovirus genus (Oberste et al., 1999). Coxsackivirus (CV) is among one of the 12 species. Enterovirus infection affects millions of people worldwide. The symptoms of infection are usually mild. However, if viruses were spread to the central nervous system of newborns, it may cause severe problems. Complement fixation assay was used to measure the neutralization antibodies to seven coxsackvirus strains including A2, A5, A10, A16, B3, B4 and B5 in 123 patients with recent-onset T1D, 155 patients with two years' duration of T1D and 250 normal individuals in 1969. It was found that the titers of antibodies to coxsackievirus B (CVB), especially B4 strain, were higher in recent-onset patients than patients with longer

duration and healthy controls (Gamble et al., 1969). A follow-up study examined the diabetes incidence in individuals 5 years after they were tested positive for coxsackievirus B4 (CVB4) infection (Dippe et al., 1975). CVB4 strain was isolated from the pancreas of a child with sever diabetic ketoacidosis (Yoon et al., 1979). It provided a direct evidence of coxsackievirus infection in pancreatic cells. Enterovirus was shown to infect cultured β-cells (Ylipaasto et al., 2004). CVB4 infection can induce the development of antibodies to a 64 kDa autoantigen in mice (Gerling et al., 1991). CVB4 infection also induces cytokine production in pancreatic cell cultures through toll-like receptor 4 (Triantafilou and Triantafilou, 2004). In human pancreatic tissue, it was observed that CVB4 infection was specific to β-cells but not causing direct β-cell destruction. The infection induced inflammation mediated by NK cells (Dotta et al., 2007). Enterovirus is notorious in T1D at both the epidemiology and molecular level. More systematic clinical studies were performed to explore their associations. Enterovirus infection was found to be associated with the development of β-cell autoimmunity and the initiation of β-cell destruction in the Finnish Diabetes Prediction and Prevention (DIPP) study (Lonnrot et al., 2000). The same conclusion was reached from another study by detecting enterovirus RNA in blood samples (Oikarinen et al., 2011). A recent study surveyed neutralization antibodies to 41 different enterovirus serotypes in 183 DIPP AAb positive children and 360 AAb negative controls. The results support CVB1 is associated with induction of β-cell autoimmunity (Laitinen OH, 2014). Another study published by the same group further conformed CVB1 as a diabetogenic virus type (Oikarinen et al., 2014). However, two other prospective studies failed to show

the association of enterovirus infection and T1D (Fuchtenbusch et al., 2001; Graves et al., 2003). These two studies are the BABYDIAB study in Germany and the Diabetes Autoimmunity Study in the Young (DAISY) in US.

EBV, named as human herpesvirus 4 (HHV-4), is a dsDNA virus that belongs to the family of *Herpersviridae*. There are 8 species in this family. EBV infection is associated with cancers and autoimmune diseases including Hodgkin's lymphoma, Burkitt's lymphoma, systemic lupus erythematosus and multiple sclerosis (Maeda et al., 2009). EBV infection can induce fulminant onset of diabetes (Burgess et al., 1974b). PCR reaction was used to search for EBV genome in T1D patients (Foy et al., 1995; Foy et al., 1994). It was reported EBV infection was not associated with islet cell and insulin AAb seroconversion by profiling antibody response to EBV nuclear antigens (Elliott RB, 1995a). Antibody levels to viral capsid antigen (VCA) and early antigen (EA) was profiled by a commercial ELISA kit and it was found lower VCA IgG class antibody levels in T1D patients (Hyoty et al., 1991). EBV infection can stimulate cytokine production in the host (Hornef et al., 1995). EBV may be involved in T1D pathogenesis by the mechanism of molecular mimicry. It was reported that antibody reactivity to an epitope in one EBV protein was also presented in the human HLA-DQ8 protein (Parkkonen et al., 1994a).

HCMV, also known as human herpesvirus-5 (HHV-5), is a dsDNA virus that belongs to the family of *Herpersviridae*. It was reported that a diabetic case had congenital cytomegalovirus infection in 1979 (Ward et al., 1979). Using molecular probes specific for HCMV genome, the viral genome was found in 22% in diabetic

patients and 2.6% in healthy controls (Pak et al., 1988). Another study reported that there was no detectable cytomegalovirus DNA in the pancreas of recent-onset T1D patients (Elliott RB, 1995a). A peptide derived from the DNA-binding protein from HCMV can stimulate autoreactive T-cells. This peptide showed sequence similarity with GAD65 which is a major T1D-associated autoantigen (Roep BO, 2002).

Human endogenous retrovirus was implicated inT1D. Human endogenous retrovirus K18 encodes a superantigen which is a candidate autoantigen for T1D (Conrad et al., 1997). However, the following studies showed contradictory conclusions from this first report. The human endogenous retroviral strain was named as insulin dependent diabetes mellitus (IDDM)K (1,2)22 and it was not specific for T1D but also presented in healthy controls (Kim et al., 1999). The genomic DNA or mRNA of (IDDM)K (1,2)22 was not detectable in T1D patients (Muir A, 1999). Another study used reverse transcription-polymerase chain reaction (RT-PCR) to detect viral mRNA in six patients and controls and found no difference between these two groups (Jaeckel et al., 1999).

Mumps virus is a ssRNA(-) virus that belongs to the family of *Paramyxovirida*. Mumps virus can infect human pancreatic β-cell cultures as proved by immunohistochemical staining of mumps proteins (Prince et al., 1978). It was reported that the T1D patients showed a significantly lower prevalence and reduced titers of antibodies to mumps (Toniolo et al., 1985). IgA class antibody response to mumps virus was higher in T1D patients than healthy controls, while no difference was found in IgG or IgM responses (Hyoty et al., 1985). In the Italian Insulin-dependent Diabetes Registry

study, a significant association was found between mumps and rubella virus with T1D, but not measles virus (Ramondetti et al., 2012).

Besides, some studies reported the association between rotavirus and rubella virus infections with T1D. Many of the studies on the same virus generated conflicted conclusions. This may be due to the limited sample size, different methods to determine viral infections or geographical differences of sample locations. A systematic study is needed to evaluate the role of viral infections in T1D.

2.3.3 Methods for Studying Viral and T1D Association

To investigate the viral and T1D association, one way is to determine the prevalence of viral infections during T1D progression. Viral infection not only results in acute infection-related symptoms, the host innate and adaptive immune system work cooperatively to fight against infection. Anti-viral antibodies are produced during this course. Different approaches could be used to detect the presence of viral proteins, genome or anti-viral antibodies. The same method to detect AAbs can be used to detect anti-viral antibodies including RIA, ELISA, LIPS and luminex assay. There are some unique methods to detect anti-viral antibodies including plaque reduction neutralization assay and complement fixation assay.

Plaque reduction neutralization assay is a method to detect neutralizing antibodies in serum against a specific serotype of this virus. In this assay, a serum sample is mixed with a virus stock at different serial dilutions. After incubation, the serum and virus mixture is applied to a monolayer of the host cells of this virus on agar plates. If the sample has no neutralizing antibodies, the viruses in the mixture will infect the host cells

and form plaques on the monolayer. Conversely, if the sample has neutralizing antibodies that bind to the viral particles, it will be blocked from infecting host cells. As a result, the number of plaques will be reduced. The percentage of inhibition is calculated by dividing the number of plaques with serum incubation by without serum incubation. A high percentage of inhibition is considered positive for anti-viral antibodies. A recent study adapted this method to profile neutralizing antibodies to 41 enterovirus serotypes (Laitinen OH, 2014).

The complement fixation assay is another method to detect the presence of specific antibodies in serum samples. In this assay, a serum sample is incubated with a standard complement protein and viral antigens. If the serum sample has anti-viral antibodies, antibody-antigen complexes will be formed and destroy the complement component. When sheep red blood cell (sRBC) and anti-sRBC complex will be applied, since the complement that can react with anti-sRBC and sRBC complex is already depleted, the sRBC will stay intact and the reaction shows no red color. Conversely, if there are no anti-viral antibodies in the serum, the complement will not be depleted. It will react with anti-sRBC and RBC complexes and lyse the RBCs, resulting red color in the reaction. Viral antibodies to coxsackievirus, influenza and mumps virus were determined by this method (Gamble et al., 1969).

Some studies sought to detect viral protein in the pancreatic tissue by IHC staining. Others tried to detect viral genomes by PCR or IHC. The emerging NGS techniques opened new revenues to sequence the T1D samples for diabetogenic viral strains (Lee et al., 2013).

2.4 Protein Microarray

2.4.1 Overview

Protein array is defined as an array of proteins displayed on a tiny microscopic slide. The function and binding activity of displayed proteins can be analyzed in a high-throughput manner. The concept of protein array was first brought up by Roger Ekins in 1989 (Ekins, 1989). The first mature protein array platform was developed by Gavin MacBeath in 2000. He printed as many as 10,800 spots on one slide and demonstrated protein-protein interactions using purified proteins (MacBeath and Schreiber, 2000). Heng Zhu and his colleagues produced another mature protein array by purifying and printing 5,800 yeast proteins on slides in 2002 (Zhu et al., 2001). The binding activity of calmodulin and phospholipid to yeast proteins were investigated on this array. After that, more and more innovative protein array platforms were emerging. Based on their characteristics, they can be divided into three categories: analytical protein array, reverse phase protein array and functional protein array.

Analytical protein array is also known as capture array. Capture antibodies or aptamers that can bind specifically to proteins are printed. Protein mixture such as cell lysate is labeled with fluorescence and applied on the slides to determine and compare protein abundance in different samples (BB., 2005). It can be used to analyze proteins that are associated with disease status (Orchekowski et al., 2005). Some limitations of this technology include: epitope destruction during the process of labeling targeted protein; sensitivity, specificity and availability of high quality capture antibody; difficulty in detecting low abundance proteins.

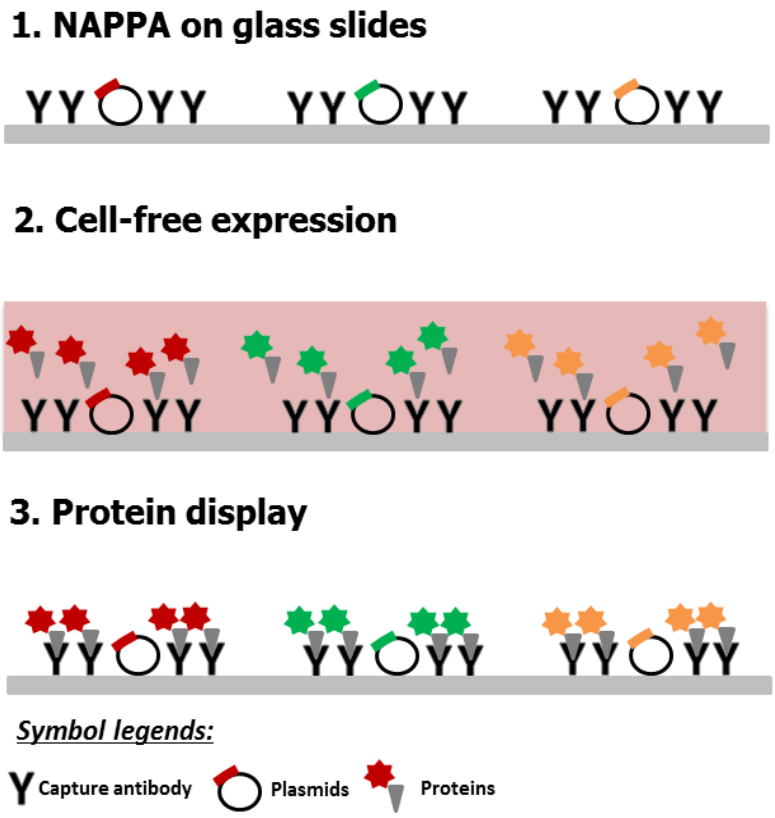
Reverse phase protein array is a platform that prints cell lysate or fractionated cell lysate on glass slides. Fluorescently labeled antibodies can be used to detect proteins of interest. Cloud Paweletz used this platform to identify molecular markers and pathway targets in patients during the progression of prostate cancer (Paweletz et al., 2001). This platform is also highly dependent on the availability of good antibodies.

Functional protein array is known as target protein array. Proteins are displayed on slides that allow for the investigation of their biochemistry activity including protein-protein, protein-DNA, protein-RNA, protein-drug interaction, protein enzymatic assay and protein translational modifications (PTMs). The first use of this platform is by Gavin MacBeath in 2000. Based on the source of printed proteins, the functional protein array could be further categorized as cell-dependent protein array and cell-free protein array. Cell-dependent protein array requires the expression and purification of individual proteins from a cell culture system such as bacteria, yeast, insect or mammalian cells. Cell-free protein arrays use an *in vitro* transcription and translation system to produce target proteins from their DNA templates. There are several classical cell-free protein array platforms. Protein *in situ* array (PISA) is platform that use PCR-generated DNA fragment to produce proteins by a cell-free protein expression system. Expressed proteins are spotted and immobilized on slide surface by a capture agent (He and Taussig, 2001). This method was adapted and modified. DNA is first printed on the slides before the cell-free expression reagent is printed. Proteins are captured *in situ* and can be detected by the specific antibodies (Angenendt et al., 2006). The DNA array to protein array (DAPA) was developed in 2007 by a group in UK (He et al., 2008). In this platform, DNA that

encodes proteins of interest is printed on a glass slide. A second slide is coated with capture agent. These two slides are assembled together with a cell-free expression reagent soaked membrane in the middle. The synthesized proteins can be captured by the capture agent on the other slide. DNA slides can be reused as long as it is not degraded. Nucleic Acid Programmable Protein Array (NAPPA) is another major cell-free proteins array. It will be introduced with details in next sections with high density (HD)-NAPPA.

2.4.2 Nucleic Acid Programmable Protein Array

NAPPA, Nucleic Acid Programmable Protein Array, was first described in 2004 (Figure 2-6). Instead of printing purified proteins, NAPPA is produced by printing cDNAs in a plasmid that is appended with a C-terminal GST tag together with an anti-GST capture antibody. Proteins are transcribed and translated from plasmid DNA and captured by the anti-tag antibodies. Proteins displayed on the slides can be used for functional studies such as binding assays (Ramachandran et al., 2004). In 2008, 1,000 unique human cDNAs was printed on NAPPA in duplicate on one slide. Proteins with variable sizes can be expressed, displayed with high day to day reproducibility (Ramachandran et al., 2008).



2 - 6 Nucleic Acid Programmable Protein Array (NAPPA)

NAPPA has several advantages over the traditional protein arrays: 1) Replaces printing proteins with the more reliable and less expensive process of printing DNA; 2) Avoids the need to express, purify and store proteins; 3) Displays better than 95% of sequence-verified full-length proteins. We have demonstrated its utility in assessing sero-reactivity to membrane proteins on NAPPA (Montor et al., 2009); 4) Protein display levels are consistent from protein to protein with 93% of proteins within two-folds of the mean. The binding between antibody and antigen is concentration dependent. On traditional protein-printed protein arrays, the amount of protein displayed on each spot

often varies as much as 3 orders of magnitude due to the naturally large variation in protein purification yields. This makes it difficult to interpret whether a weaker signal is due to low/no protein amount on the array or low/no antibody concentration in plasma sample. The consistent display of proteins on NAPPA arrays will make data interpretation much easier; 5) Assures protein integrity by using both mammalian expression machinery and chaperone proteins to synthesize and fold proteins.

NAPPA has a broad application in immunoprofiling of serological antibodies, protein-DNA and protein-protein interactions and PTMs. Humoral immune response to infectious agents including viruses and bacteria can be analyzed on NAPPA (Ceroni et al., 2010; Montor et al., 2009). NAPPA was widely used as an AAb biomarker discovery platform in cancers and autoimmune diseases (Anderson et al., 2015; Anderson et al., 2011; Anderson et al., 2010; Gibson et al., 2012; Wright et al., 2012). Recently, PTMs like AMPylation were profiled on NAPPA (Yu et al., 2014c).

2.4.3 High Density-NAPPA

HD-NAPPA is a new technology that helps NAPPA to achieve a much higher density (Takulapalli et al., 2012). HD-NAPPA is built on silicon nanowell substrates. Nanowells are generated on silicon substrates by different etching techniques including wet or plasma etching (Wiktor et al., 2015). The density of array increases with the decrease of well size. As a proof of concept, HD-NAPPA can reach as high as 24,000 features per slide. The regular HD-NAPPA has 14,000 spots per slide (Takulapalli et al., 2012). To mimic the glass surface of standard NAPPA, a thin film of silicon dioxide is thermally grown the surface of silicon nanowells. Aminopropyl-triethoxy-silane (APTES)

is coated on top to generate a monolayer for NAPPA chemistry. Printing mix is dispensed into individual wells by high-speed electronic Piezo printer. Using this platform, a lot less DNA is consumed compared with standard NAPPA. HD-NAPPA showed less diffusion, the capability of detecting antigen-antibody binding and protein-protein interaction (Takulapalli et al., 2012). HD-NAPPA has great potential in future applications of functional proteomic studies.

2.5 Rationale of This Dissertation Study

2.5.1 AAb Biomarker Discovery

AAbs are produced by autoreactive B-cells during T1D progression. The major T1D-associated autoantigen include insulin, GAD65, IA-2 and ZnT8 (Atkinson MA, 1990; Bonifacio et al., 1995a; Palmer et al., 1983; Wenzlau et al., 2007). A number of minor T1D-associated AAbs were identified by various approaches. The number of T1D-associated AAbs may be much higher than currently known. Diabetic individuals who are positive for ICA staining yet remain negative for the four known T1D-associated AAbs, indicating the existence of yet-to-be discovered AAbs (Wang J, 2007). Also, a proportion of patients are negative for both ICA and the known biochemical AAbs with no apparent difference in clinical performance compared with AAb positive T1D patients suggesting different disease subtypes (Hameed et al., 2011). One possible explanation is that AAbs in this group may target antigen proteins outside the landscape of the pancreatic islet, which give us a hint that a whole proteome screening may help to identify additional AAbs. In addition, some AAbs may appear at different stages of the disease such as the pre-diabetic period. Because their levels might not persist, they may not have been

detected in blood samples taken at the time of diagnosis or in clinically active patients. Some of these AAbs might be specific to certain individuals emphasizing the heterogeneous nature of the disease (e.g., different rates of progression, etiological factors, genetic backgrounds) (Achenbach et al., 2013; Achenbach et al., 2004). Furthermore, these AAbs might have been missed by the traditional assay methods used for AAb discovery. Additional AAbs may provide values in the following aspects:

(1) Identifying individuals at risk: Prevention of T1D will only be possible if individuals with high risk for progression to T1D (especially in the general population) can be identified. The incidence of T1D in the general population is around 22/100,000 in US. The majority of T1D cases are diagnosed in non-relatives, with 85% of new T1D cases occurring in individuals with no known family history (Hamalainen and Knip, 2002; Knip et al., 2010). Biomarkers are needed that can improve our prediction models and enable the selection of subjects with, for example, high 5-year risk of disease onset. Such markers could be deployed immediately to identify high risk subjects for intervention trials (Marino et al., 2011). Currently a large percentage of subjects based on our current risk prediction model might not develop an insulin requirement within 5 years, even among relatives of T1D subjects (Winter and Schatz, 2011). Identifying markers that are present prior to the development of our currently used AAbs could improve the risk prediction models.

(2) Stratifying patients with different clinical courses: Individuals who progress to T1D do so at different rates. The currently used AAb biomarkers do not explain the variations in progression rates towards clinical T1D. The mechanisms

regulating the progression rates of rapid or slow progressors in an AAb positive population are not well understood, yet they are crucial to ensuring that the most appropriate interventions are developed (Achenbach et al., 2013; Wasserfall and Atkinson, 2006). Once high risk individuals are identified, prevention strategies would benefit from additional biomarkers that provide a more detailed description of that individual's status with respect to disease progression rates.

(3) Improving our understanding of autoimmune destruction: The existence of the known T1D-associated AAbs indicates autoimmune destruction (Bonifacio and Ziegler, 2010). Yet we still lack a detailed understanding of the molecular mechanisms of T1D pathogenesis. Current T1D models explain the natural history but not mechanisms of the disease development. The etiopathogenesis remains elusive due to our limited ability to observe and monitor beta-cell destruction directly (Atkinson, 2012). An improved understanding of disease etiology, especially what events trigger the conversion from genetic high risk to serological high risk, will help strategies aimed at preventing or delaying disease progression. Finding biomarkers that are linked to these events or even mark them temporally, will clearly aid in this regard.

(4) Identifying cellular immune response antigens: Cellular immune response plays an important role in T1D development. Because the difficulties associated with the identification of T-cell autoantigens, assays measuring antibody responses to autoantigens are usually used as surrogates for identification of T-cell antigens with the assumption that antigens that are presented by B cells will also stimulate T cell responses. Therefore, the identification of autoantigens that can elicit autoantibodies may provide

candidates for research in their role in cellular immune response, which may be more relevant to immune destruction in T1D.

(5) Providing candidates for prevention trials: Autoantigens may provide candidates for developing T1D prevention strategies. Ongoing clinical trials are conducted by TrialNet to study the daily intake of oral insulin capsules or injections of GAD protein to delay the disease in at risk relatives of people with T1D or preserve insulin production respectively. DiaPep277, a synthetic peptide from human heat shock protein (Hsp60, a minor T1D autoantigen) was found to preserve endogenous insulin secretion up to 18 months (Raz et al., 2007). It is now in the phase III clinical trial. New autoantigens may also be fed into similar clinical trials.

However, the rate of discovering new AAbs has been limited by the throughput of the techniques used in the past. This suggests the need for new tools that can adequately test a broad antigen space, as well as the need to apply those tools to well-characterized samples. Ideally, such tools must be high-throughput and multiplexed to allow the generation of a comprehensive picture of antibody repertoire in many subjects at many time points. Yet, they must also be cost-effective to enable the discovery of leads for further confirmation. We believe the rapid advancement of "omics technologies" will help move this field forward. A protein array-based immunoproteomics approach is powerful to characterize humoral immunoprofile against human proteomes to identify novel T1D-associated AAbs. We believe that the identification of a panel of novel T1D-specific AAbs will help to connect the nodes in T1D etiology, provide candidates for detailed pathophysiology study and develop preventive approaches based on these targets.

2.5.2 Viral and T1D Association Study

Interplay between genetic, immunological and environmental factors contributes to T1D development (Eisenbarth, 1986; Herold et al., 2013), yet it is still poorly understood. Among various environmental factors, viral infections have attracted particular interest (Craig et al., 2013b; Roivainen and Klingel, 2010). One hypothesis holds that in susceptible individuals, the antiviral response leads to cross-reactivity against host antigens in the β-cells, ultimately leading to β-cell destruction. A number of viral genes have been found to share similar amino acid sequences with the known T1D-associated autoantigens (Roep BO, 2002). These findings provide support for the "molecular mimicry" theory of the role of viral infection in T1D development (Coppieters et al., 2012). An improved understanding of the contributions of different environmental risks (especially viral infections) may help devise novel prevention strategies in T1D.

Different approaches were applied to determine viral infection in T1D. The broad availability of DNA sequencing technologies in conjunction with the ease of amplifying nucleic acids has motivated DNA-based approaches to study the role of viral infection in T1D. RT-PCR is a traditional way to detect viral genomes from the stool samples or nasal swabs through the amplification of viral genes with gene-specific primers (Saito et al., 1989). While clearly very powerful, this approach demands that the nucleic acids are still persistent in the blood or tissue at the time of sample collection. In the context of the cross-reactivity hypothesis, many of the pathogenic events may occur after the virus is no longer present. Viremia can be as short as a few days (Lee et al., 2013). Moreover, the

high cost of NGS technology, and the need to test many samples make deep sequencing-based studies less amicable to epidemiological studies. Finally, the sensitivity of PCR-based detection also varies based on the choice of pathogen genes to be amplified and some mutations in the pathogen genomes also affect amplification efficiency. PCR is susceptible to false positive results due to contamination or non-specific amplification.

As noted above, the humoral response to viral antigens can be detected long after viremia has ended and the modern ability to test the immune response against the entire viral proteome will provide additional important clinical information about the individual's immune status beyond simply demonstrating a history of infection.

2.5.3 Advantages of NAPPA

Our NAPPA approach to profile AAbs and anti-viral antibodies is innovative by the following aspects:

(1) Our innovative protein array allows us to study the immune repertoire during T1D development. On this platform, we can: 1) assess the serological antibody profile to thousands of human and viral antigen proteins simultaneously and require as little as 4μl serum/plasma samples per array; 2) compare the sero-reactivity to known T1D-associated antigens on the same array; 3) track the antibody repertoire changes during the course of T1D progression; 4) profile both IgG and IgM or IgA antibodies in a multiplexed way.

(2) The consistent levels of proteins across the array give every protein a fair chance for detection by antibodies in serum. The issue of a broad dynamic range of protein concentrations in serum is not relevant here.

(3) The potential of linking multiple antigens to improve predictive value can be explored. It is highly possible that the prevalence of each of the novel AAbs will be lower than the known four, i.e., a relatively small fraction of patients may respond to each antigen. Thus, a biomarker test based on a single antigen would demonstrate poor sensitivity. An integrated panel of multiple T1D-specific antibodies will increase the predictive value.

(4) The identification of additional AAbs will improve our ability to understand their role in T1D pathogenesis from network and/or bioinformatics analysis.

(5) The identification of additional AAbs may potentially benefit proteomics studies on other platforms and search for these AAbs in serum.

From an "omics" perspective, AAb profile from this study can complement with other proteomic discoveries. The anti-viral antibody profile can be integrated with mirobiome and virome studies in T1D. The complete host humoral immunoprofile against human and viral proteomes will provide new insights into T1D pathophysiology.

CHAPTER 3

3 SEROLOGICAL ANTIBODY PROFILING OF TYPE 1 DIABETES BY PROTEIN ARRAYS

3.1 Abstract

The need for biomarkers that illuminate the pathophysiology of type 1 diabetes (T1D), enhance early diagnosis and provide additional avenues for therapeutic intervention is well recognized in the scientific community. A proteome-scale, two-stage serological autoantibody (AAb) screening followed by an independent validation study was performed. In the first stage, the immunoreactivity was compared between T1D cases and healthy controls against ~6,000 human proteins using the Nucleic Acid Programmable Protein Array (NAPPA). Genes identified with higher signal intensities in patients were challenged with a larger sample set during the second stage. Statistical analysis revealed 26 novel AAbs and a known T1D associated AAb. During validation, the presence of AAbs to dual specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2) was verified using the Luciferase Immunoprecipitation System (LIPS) assay (36% Sensitivity, 98% specificity). The area under the curve (AUC) for a combination of DYRK2 AAb (DYRK2A) and the classical T1D AAb IA-2 AAb (IA-2A) was 0.90 compared to 0.72 for DYRK2A and 0.64 for IA-2A alone. This is the first systematic screening for seroreactivity against large number of human proteins in T1D patients. The application of protein microarrays to identify novel AAbs in T1D, expanded the current T1D “autoantigenome” was demonstrated and it will help fulfill the goal of searching for novel biomarker candidates for T1D in the future.

3.2 Introduction

Early studies of diabetes demonstrated the seroreactivity of individuals with T1D to islet cells in pancreatic cryosections (Atkinson et al., 1990), suggesting an AAb-mediated autoimmune component in this disease. The identification and study of AAbs associated with T1D (Baekkeskov et al., 1990; Bonifacio et al., 1995a, b; Bottazzo et al., 1974; Palmer et al., 1983; Wenzlau et al., 2007), has confirmed their roles as biomarkers with value in diagnosis (Wasserfall and Atkinson, 2006), prognosis, patient treatment stratification (Christie et al., 2002), tolerizing therapies as well as providing insights into the pathophysiology of disease (Ludvigsson et al., 2008a). AAbs against insulin, glutamic acid decarboxylase 65 (GAD65), protein tyrosine phosphatase receptor N (IA-2) and zinc transporter member 8 (ZnT8) antigen proteins in T1D have a combined clinical performance enabling the detection of >90% of T1D (Wenzlau et al., 2007). The known T1D-associated AAbs are frequently used for research studies and increasingly used in clinical management for purposes such as identifying individuals with T1D risk, stratifying patients with different disease course and improving our understanding of autoimmune destruction (Achenbach et al., 2013; Bonifacio and Ziegler, 2010; Pozzilli, 2002). Despite this, known T1D associated AAbs against antigens like insulin and GAD65 are not diabetes specific. Insulin AAb (IAA) appears in insulin autoimmune syndrome while GAD65 AAb (GADA) appears in Stiff-Man Syndrome (Raju et al., 2005). In addition, many diabetic individuals positive for islet cell antibody (ICA) staining are negative for all known anti-islet AAbs (Wang et al., 2007), suggesting the existence of additional, yet-to-be-identified AAbs. Comprehensive identification of AAbs

targeted in T1D will help fully characterize the heterogeneity of disease represented in the "autoantigenome" that may enable enhanced diagnostics, personalized therapies and a fundamental understanding of diabetes pathology.

AAb discovery in T1D has been slow with the identification of four major AAbs over the past four decades. Previous AAb biomarkers were discovered based on either the known understanding of T1D pathogenesis (e.g., IAA) using radioimmunoassay (RIA) or immunoprecipitation of autoantigens from cell lysates by patient serum. With the development of genomics and bioinformatics, ZnT8 was identified as a major T1D AAb by analyzing pancreatic gene expression profiles followed by RIA (Wenzlau et al., 2007). No large-scale screening of individual proteins at the proteome level has been conducted to search for new T1D AAb biomarkers perhaps due to the lack of appropriate high-throughput proteomic techniques.

The advent of proteomics technologies like protein microarrays has provided attractive opportunities to profile AAb against a large number of human proteins in T1D. Protein microarrays in particular are an invaluable tool for simultaneous interrogation of thousands of proteins and possess immense potential as a non-biased discovery tool to identify AAbs targeting self-antigens. They have been used in a variety of systematic and organ-specific autoimmune diseases including autoimmune hepatitis (Song et al., 2010), rheumatoid arthritis (Hueber et al., 2005), multiple sclerosis (Quintana et al., 2008) as well as in various cancers including breast cancer (Anderson et al., 2011), lung cancer (Madoz-Gurpide et al., 2008), colon cancer and colorectal cancer (Babel et al., 2009; Nam et al., 2003).

Conventional protein microarrays printed from purified proteins, however, suffer from practical limitations including the substantial cost and time associated with purifying proteins from sizable libraries that can make large scale screening cost-prohibitive. The wide range of protein concentrations deposited and limited shelf lives due to the instability of purified proteins, can further compromise the array utility. Recent conceptual advances achieved with the NAPPA platform have circumvented these primary challenges associated with conventional protein microarrays by printing cDNA encoding plasmids instead of purified proteins (LaBaer and Ramachandran, 2005). Plasmids encoding genes with a C-terminal affinity tag are co-printed on the array along with anti-tag antibody allowing for the cell-free expression and immediate capture of thousands of functional proteins with a wide range distribution of protein sizes on glass slides (LaBaer and Ramachandran, 2005). Slides are stable at room temperature for at least one year and can be expressed at the time-of-use. NAPPA has been applied in a variety of AAb discovery studies such as breast cancer and arthritis (Anderson et al., 2011; Gibson et al., 2012).

In the present study, the first large scale use of protein microarrays in profiling of AAbs in T1D was reported by conducting a two-stage, sequential serological immunoreactivity screening and an independent validation study. The two stages of screening resulted in the identification of 26 candidate AAbs including ZnT8 ($p<0.005$, FDR<10%). Candidate genes were selected for further validation via LIPS assay in an independent serum set (Burbelo et al., 2005a). Using this assay, AAb to DYRK2 showed

36% sensitivity at 98% specificity, thus demonstrating the use of protein microarrays in the search for novel T1D associated AAbs

3.3 Experiments

3.3.1 Serum Samples

T1D serum samples included in the study were obtained from individuals diagnosed with T1D, according to American Diabetes Association (ADA) criteria, at College of Medicine, the University of Florida. Peripheral blood samples were drawn from the antecubital vein and serum was prepared according to a standardized protocol before freezing serum aliquots at -80^{o}C. Control samples were drawn and prepared in the identical fashion to T1D patients and selected to be age-matched to the patient set. Control samples were considered to be at low risk of T1D with absence of known T1D-associated AAbs and T1D family history. Independent sample sets were used in each stage of study. Neither patients nor control samples were known to have any other underlying autoimmune disease. All samples were collected with written informed consent, under the guidelines of the Institutional Review Boards (IRB) at the University of Florida, the Harvard Medical School and Arizona State University through the entire study. The detailed sample information is indicated in Table 3-1.

3 - 1 Sample Characteristics of Each Stage

		Total	Gender Male (%)	Age Mean±SD (Median)	Years from Diagnosis Mean±SD (Median)
First-stage screening	T1D	6.7%	50	17.6±10.4 (14.4)	6.7±8.4 (4.0)
	Healthy	13.3%	20	24.3±9.6 (23.4)	N/A
Second-stage screening	T1D	40.0%	74	21.1±11.3 (17.0)	5.5±7.7 (2.3)
	Healthy	20.0%	75	18.5±12.1 (15.5)	N/A
Validation Stage	T1D	46.7%	46	17.2±11.0 (13.0)	6.8±8.5 (3.5)
	Healthy	66.7%	46	17.7±11.5 (15)	N/A

3.3.2 Array Production and Quality Assessment

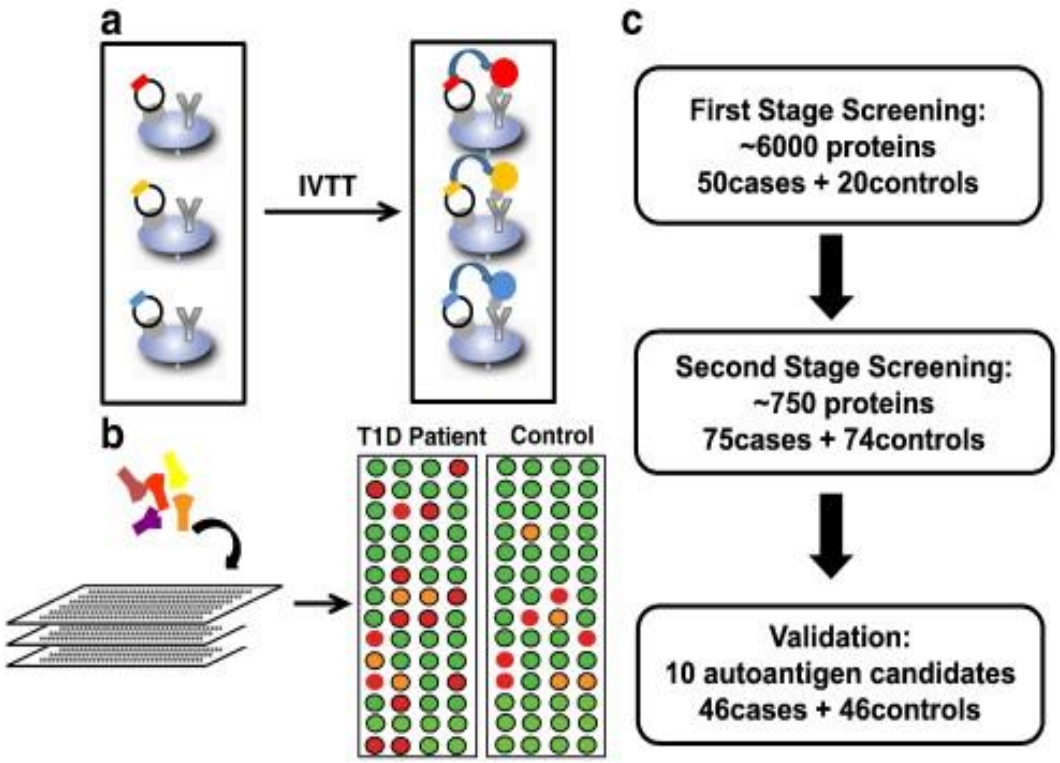
Human genes used in this study were obtained from DNASU (http://dnasu.asu.edu/DNASU/). NAPPA was produced and the quality of DNA printing and protein expression were controlled as previously described (Burbelo et al., 2005a; Miersch and LaBaer, 2011; Qiu and LaBaer, 2011; Sibani and LaBaer, 2011). The correlation of protein expression within one printing run or two printing runs was compared to determine the reproducibility of slides production.

3.3.3 Profiling of Serum AAbs

Serum AAb profiling on NAPPA was performed as previously described (Anderson et al., 2011). Briefly, once slides quality had been confirmed, expressed slides were challenged with serum samples, HRP-linked anti-human IgG secondary antibodies and developed by Tyramide Signal Amplification (TSA) (Clutter et al., 2010). All slides were scanned by a ScanArray ProScanArray HT scanner (Perkin Elmer) at the same settings. Individual spot signal intensities were quantified using Microvigene Image Analysis Software V2.9.9.2. Slide images were vetted to remove visually defective features (i.e. with scrapes, smudges, dust particles, or obvious contribution of signal from adjacent features).

3.3.4 Study Design and Data Analysis

Screening efforts were structured in two stages (Figure 3-1). In the first stage of screening, 50 cases and 20 control serum were used to screen >6,000 unique proteins across three arrays. Signal intensities were normalized by first subtracting the background signal estimated by the 25^{th} percentile of the negative control (printing mix alone without plasmid DNA) on each slide and then scaling data of each slide to the same overall median intensity and median absolute deviation. Normalized signal intensities were analyzed by comparing the 90^{th} percentiles of case and control samples using three statistical tests: Quantile regression (Koenker, 2001), Fisher's exact test and a binomial proportion test. The antigens that did not exhibit statistically significant ($p<0.05$) differential reactivity in cases versus controls according to any of the three tests were eliminated. The remaining antigens were ranked by the difference between the summed



3 - 1 Scheme of NAPPA and Strategy of AAb Screening
(a) Nucleic acid programmable protein array (NAPPA) - spotted plasmids are printed along with a capture antibody, expressed in situ using a cell free expression system, and the protein of interest is captured by an anti-tag antibody.
(b) Protein displaying-microarray slides are challenged with serum from either patient or control individuals. The difference in immunoreactivity is determined by tyramide-amplified signals from the HRP-labeled anti-human IgG secondary antibody. Antigens that repeatedly show a higher intensity in patients than controls are considered candidate T1D autoantigens. Red dots represent high reactivity, yellow dots represent medium reactivity; green dots represent low reactivity.
(c) Three stages of AAb discovery strategy: First stage screening: A large number of expressed antigen genes were challenged with a smaller number of sera samples to eliminate uninformative genes; Second stage screening: Antigens that exhibited higher responses among patient serum were challenged with a larger number of patient and control serum samples to scale down the number of candidates for validation; Validation stage: Top candidates from microarray analysis were validated by an independent luminescent immunoprecipitation (LIPS) method.

signals of cases versus controls above the 90th percentile of the controls. ~750 antigens from three arrays were advanced to the second stage screening.

In the second stage, 74 case and 75 control sera were screened using the ~750 antigen genes identified from the first stage. After background subtraction, Wilcoxon Rank-Sum Test and the partial AUC at 95% specificity (pAUC95) were applied in the analysis of normalized data to rank genes that have differential reactivity between patient and control group. The sensitivity and p-values of each candidate was generated. The rationale to include two statistical methods to analyze the second-stage screening data was to be more inclusive with autoantibody biomarker candidates for validation.

During the validation stage, the sensitivity and specificity of the combination of IA-2A and DYRK2A was investigated by constructing a classification rule using logistic regression (David W. Hosmer, 2013; Li et al., 2002; Rai et al., 2002). Leave one out cross validation was used to assess the performance of the logistic regression classifier. Specifically, disease status for each sample was predicted using a logistic regression classifier that was trained using the remaining samples. These cross validation predictions were then used to conduct receiver operator characteristic (ROC) analysis and calculate the AUC for the combination of IA-2A and DYRK2A.

3.3.5 Validation of Candidates by LIPS Assay

The liquid-phase, LIPS assay was used to verify a selection of candidate AAbs from the second stage screening (Burbelo et al., 2008). C-terminal renilla luciferase fusion products (Ag-cRuc) for each antigen were generated using a modified pRL-CMV vector (Promega), engineered to contain a gateway-compatible death cassette (available

at DNASU) and T7 promoter. Gateway compatibility enabled the rapid and accurate transfer of candidate genes from entry vectors into the expression vector. The incorporation of T7 promoter to pRL-CMV parental vector allows the choice of expression in either mammalian cells via the CMV promoter or *in vitro* by cell free expression via T7 promoter, thus increasing ease, speed and versatility. Where available, entry clones encoding the autoantigen candidate genes were used to generate the expression clones by a LR recombination reaction. In all other instances, inserts were cloned into a pDON221 entry vector by PCR amplification and homologous recombination using BP Clonase II (Invitrogen) and then into the pRL-CMV vector using LR Clonase II (invitrogen). Plasmid DNA encoding the antigen-cRuc was purified and sequence verified from single colonies and stored as glycerol stocks.

For expression, plasmid DNA encoding antigen-cRuc was mixed at a ratio of 2 µg/ 20 µL in rabbit reticulocyte lysate (RRL) supplemented with 10 µg/mL tRNA for protein expression at 30^{o}C for 90 min. Expression of each clone was verified by 1) luciferase activity of RRL-expressed antigen ($3X10^5$ to $1X10^6$ ALU / µL lysate) versus the same volume of no DNA control retic); 2) western blot using anti-renilla antibody or antigen specific antibody. For assay of serum responses, serum samples were diluted at a 1:10 ratio in buffer A (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100) and prepared in a 96 well plate. 50 µL diluted antigen-cRuc protein was incubated with 10 µL diluted serum sample and 4 0µL buffer A at 4 ^{o}C overnight with gently shaking at 500rpm on a plate shaker (Eppendorf). The antigen and serum mixture was transferred to a Millipore HTS filter plate (Millipore, Bedford, MA) with 5 of 30% slurry

protein A/G beads (Pierce Biotechnology, Rockford, IL) and incubated at RT for 2 hours. After intense wash with 8X of 100 µL buffer A each time and 2X of 200 µL PBS, the luminescent intensity of each sample was quantified in duplicate by reading on a Glomax Luminometer (Promega) with a 5 second integration time following automated injection of 50 µL of coelenterazine substrate. Jitter plots were then generated from log transformed raw luminescent signal intensities using R software to enable the comparison of patient and control population immunoreactivity to candidate autoantigens. Sensitivity for each AAb was calculated at the 98% specificity.

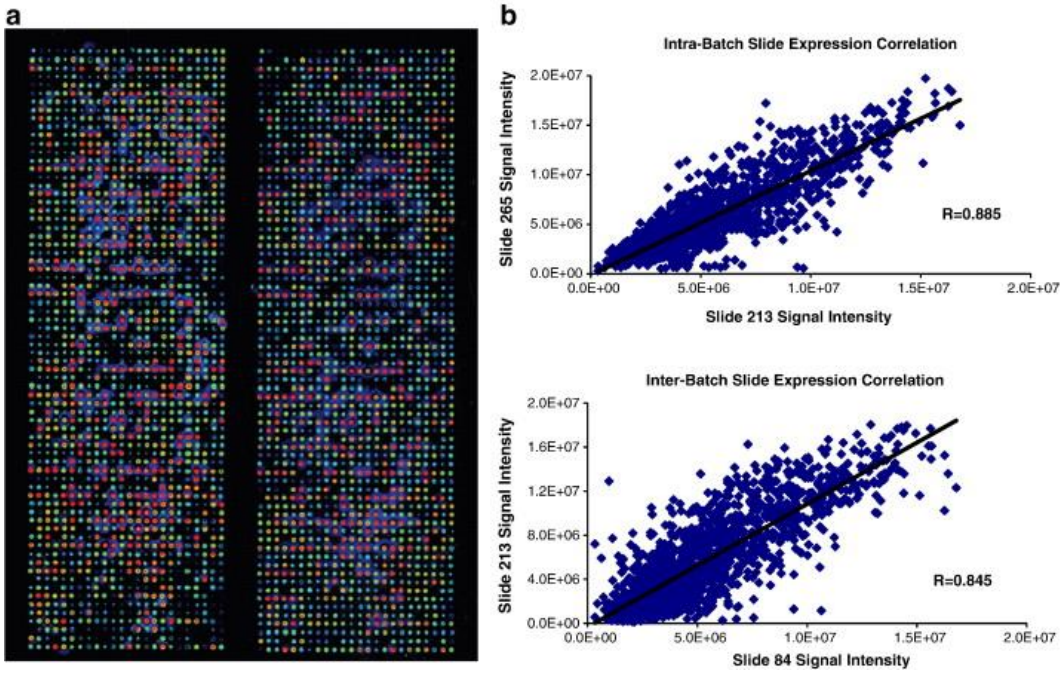
3.4 Results

3.4.1 Array Production and Quality Assessment

High quality NAPPA arrays comprised proteins displayed at high level are keys to a successful serum screening study. Prior to serum challenge, protein display on NAPPA was quality assessed by anti-tag antibodies, confirming the robustness of protein expression. Signal intensities for protein levels on the array were similar to those previously estimated to be in the average magnitude of 9 femtomoles per feature (Ramachandran et al., 2008). Importantly, scatter plots of signal intensities of anti-tag staining from replicate arrays revealed correlation coefficients of >0.84 from two randomly selected slides within a print run or between two print runs attesting the reproducibility of slide printing and protein display, further confirming the suitability for serum screening (Figure 3-2).

3.4.2 Screening Strategy

Screening efforts were structured in two stages. During the first stage, >6,000 unique proteins, printed across three arrays were challenged with serum from 50 T1D patients and 20 healthy controls. The goal was to facilitate the second stage by eliminating uninformative antigens. Thus any antigens that showed no immune response in either group (i.e., most antigens) or no appreciable difference in response were eliminated. During the second stage, ~750 candidate genes identified from the first stage were printed in duplicate and challenged with an independent sample set of 74 patients and 75 healthy controls to confirm the reproducibility of enhanced immunoreactivity and to further scale down the number of candidate AAbs for independent validation.



3 - 2 Reproducibility of Protein Expression on NAPPA
(a) Pseudo-colored image of anti-GST staining on two expressed slides to assess the level of protein display: slides were developed with a mouse monoclonal anti-GST to an epitope distinct from that used for antigen capture enabling assessment of protein expression, capture and display and confirming reproducibility.
(b) Plots of signal intensities from all spots for two arrays within or between printing batches showed R >0.84.

The advantages of this two-stage screening experimental design are three-fold: 1) It minimized the cost of screening against such a large initial group of antigens; 2) It ultimately reduced the logistical burden of handling large numbers of slides during screening and 3) It limited the potential for overfitting of the data.

In the first stage screening, statistical analysis revealed approximately 12% of these ~6,000 proteins displayed enhanced reactivity when challenged with patient sera as opposed to that from healthy controls. During the second stage, both the case and control

samples were independent of the samples used in the first stage. Raw screening data were produced and normalized after removing the within block and pin effects. Analysis of the normalized signal intensity by the Wilcoxon Rank-Sum Test identified 26 proteins whose median antigen reactivity was higher in patients than in controls ($p<0.005$) (Table 3-2).

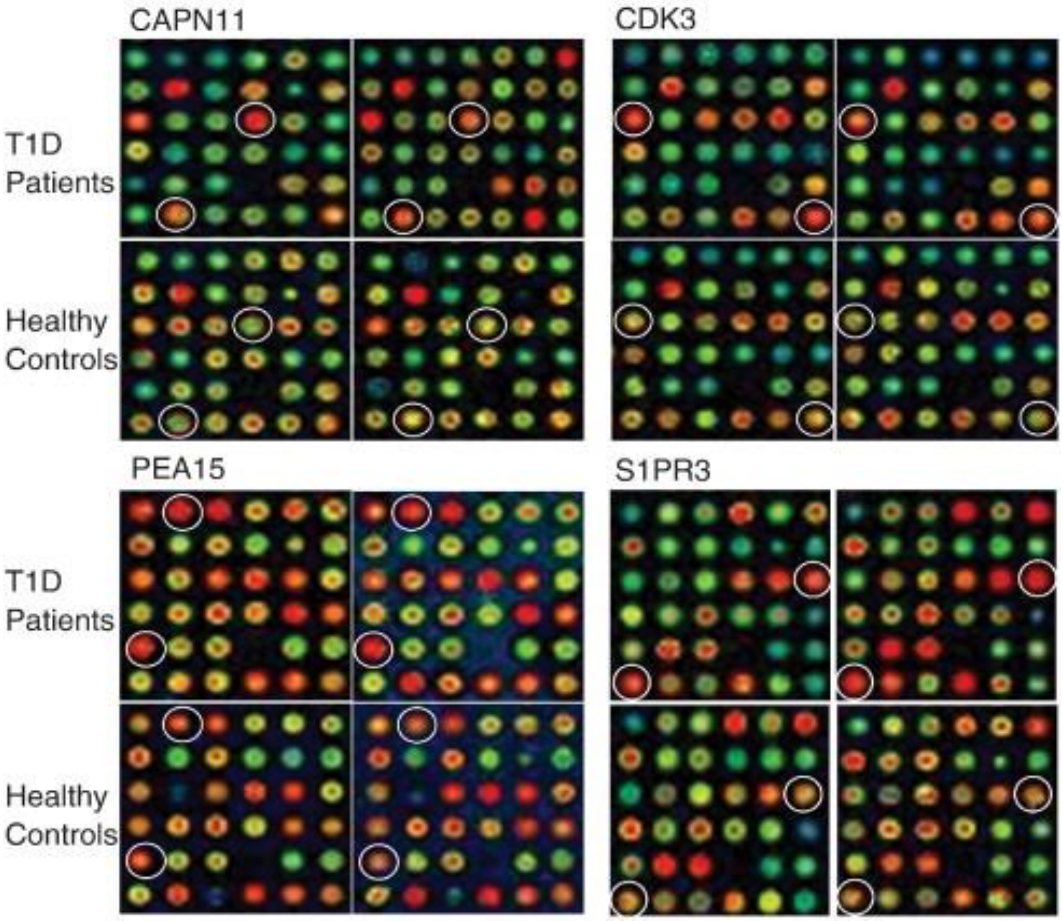
3 - 2 List of 26 Candidate AAbs by Wilcoxon Rank-Sum Test

Second Stage Screening Candidates Ranked by Wilcoxon Rank-Sum Test									
	PAUC-95	PAUC-95 p-value	PAUC-95 q-value	Median of Patients	Median of Controls	Wilcoxon Rank-Sum p-value	AUC	Sensitivity	Specificity
TBCA	0.0130	0.0000	0.0010	1.29	1.19	0.0000	0.72	32.00%	94.59%
DOM3Z	0.0064	0.0453	0.1920	0.27	0.21	0.0003	0.66	27.03%	94.59%
TTC4	0.0038	0.0382	0.1905	1.07	1.02	0.0004	0.66	9.33%	94.59%
Slc30A8-NC	0.0037	0.1229	0.2411	2.14	1.88	0.0004	0.66	16.00%	94.59%
LIMK2	0.0063	0.0139	0.1266	1.23	1.12	0.0005	0.66	20.00%	94.59%
CAPN11	0.0149	0.0000	0.0007	1.71	1.61	0.0005	0.66	36.49%	94.37%
TSPAN31	0.0043	0.0389	0.1905	0.96	0.91	0.0006	0.65	9.33%	94.52%
PEA15	0.0112	0.0000	0.0032	0.89	0.84	0.0006	0.65	25.33%	94.59%
CDK6	0.0046	0.0542	0.1967	1.02	1.00	0.0007	0.65	14.67%	94.59%
DYRK2	0.0046	0.0233	0.1647	0.81	0.73	0.0008	0.65	12.00%	94.52%
GPR120	0.0038	0.1187	0.2411	1.27	1.18	0.0011	0.64	17.33%	94.59%
POU2F1	0.0048	0.0808	0.2188	1.36	1.27	0.0014	0.64	18.67%	94.59%
CDK4	0.0062	0.0452	0.1920	1.75	1.61	0.0018	0.64	20.27%	94.44%
KCNK13	0.0077	0.0134	0.1266	1.11	1.06	0.0019	0.64	24.00%	94.52%
FOXM1	0.0025	0.2340	0.2901	0.81	0.77	0.0021	0.64	10.67%	94.59%
CDKN1A	0.0045	0.1307	0.2411	1.71	1.59	0.0022	0.64	23.29%	94.59%
HOXB7	0.0085	0.0022	0.0603	1.27	1.21	0.0025	0.63	22.97%	94.59%
ARF6	0.0006	0.5963	0.4706	1.24	1.11	0.0026	0.63	2.67%	94.59%
GTF2A1	0.0133	0.0000	0.0007	0.97	0.93	0.0033	0.63	26.67%	94.59%
HTR1E	0.0089	0.0033	0.0716	1.35	1.28	0.0034	0.63	28.00%	94.44%
IL13RA2	0.0081	0.0024	0.0603	1.01	0.99	0.0040	0.63	24.00%	94.44%
CDC2	0.0019	0.2352	0.2901	1.03	0.99	0.0040	0.63	5.33%	94.59%
SERPINH1	0.0060	0.0542	0.1967	1.10	1.08	0.0045	0.62	21.33%	94.52%
KLK3	0.0024	0.2166	0.2812	1.56	1.45	0.0046	0.62	10.67%	94.44%
TBRG4	0.0061	0.0082	0.1168	1.57	1.51	0.0048	0.62	15.07%	94.59%
ZFP64	0.0067	0.0137	0.1266	1.30	1.23	0.0048	0.62	18.92%	94.59%

Similarly, analysis of the normalized antibody-dependent signal intensity resulted in the identification of 25 proteins that displayed differential immunoreactivity when the statistical significance of the partial area under the curve at 95% specificity (pAUC95) was tested to be higher in T1D patients than controls ($p<0.005$) (Table 3-3).

3 - 3 List of 25 Candidate AAbs by pAUC Analysis

Second Stage Screening Ranked by PAUC95									
	PAUC-95	PAUC-95 p-value	PAUC-95 q-value	Median of Patients	Median of Controls	Wilcoxon Rank-Sum p-value	AUC	Sensitivity	Specificity
CAPN11	0.0149	0.0000	0.0007	1.71	1.61	0.0005	0.66	36.49%	94.37%
GTF2A1	0.0133	0.0000	0.0007	0.97	0.93	0.0033	0.63	26.67%	94.59%
ARG2	0.0130	0.0000	0.0010	1.29	1.19	0.0000	0.72	32.00%	94.59%
PEA15	0.0112	0.0000	0.0032	0.89	0.84	0.0006	0.65	25.33%	94.59%
SFRS7	0.0090	0.0003	0.0261	1.03	1.03	0.3008	0.52	22.67%	94.59%
LASS2	0.0090	0.0003	0.0261	1.19	1.16	0.0239	0.59	24.00%	94.59%
ZNF683	0.0090	0.0004	0.0290	1.88	1.83	0.0240	0.59	21.33%	94.44%
LGMN	0.0087	0.0005	0.0316	1.74	1.66	0.0061	0.62	21.33%	94.59%
STK32A	0.0083	0.0007	0.0374	1.25	1.17	0.0050	0.62	18.67%	94.52%
LATS1	0.0082	0.0007	0.0374	1.70	1.62	0.0216	0.60	18.67%	94.52%
TNRC5	0.0079	0.0009	0.0398	1.41	1.35	0.1402	0.55	18.92%	94.59%
ZBED1	0.0080	0.0011	0.0400	1.67	1.62	0.0401	0.58	17.81%	94.59%
APCS	0.0079	0.0011	0.0400	1.09	1.08	0.1326	0.55	22.97%	94.59%
CDK3	0.0090	0.0011	0.0400	1.76	1.69	0.1549	0.55	22.67%	94.59%
ZNF323	0.0078	0.0013	0.0437	0.83	0.85	0.3450	0.52	20.00%	94.52%
FOXA1	0.0084	0.0020	0.0603	1.17	1.15	0.0242	0.59	21.62%	94.52%
HOXB7	0.0085	0.0022	0.0603	1.27	1.21	0.0025	0.63	22.97%	94.59%
PDIA3	0.0068	0.0023	0.0603	0.92	0.92	0.6667	0.48	14.67%	94.59%
ZBTB25	0.0070	0.0024	0.0603	1.32	1.27	0.0190	0.60	15.49%	94.52%
IL13RA2	0.0081	0.0024	0.0603	1.01	0.99	0.0040	0.63	24.00%	94.44%
LOC91461	0.0076	0.0026	0.0624	1.32	1.28	0.0313	0.59	22.67%	94.44%
IKBKB	0.0072	0.0027	0.0624	0.90	0.92	0.2924	0.53	16.22%	94.59%
HTR1E	0.0089	0.0033	0.0716	1.35	1.28	0.0034	0.63	28.00%	94.44%
PRKCB1	0.0075	0.0039	0.0819	1.22	1.21	0.0870	0.56	21.62%	94.59%
CRH	0.0078	0.0045	0.0911	1.43	1.43	0.1973	0.54	21.33%	94.52%



3 - 3 Visual Comparison of Candidate AAbs

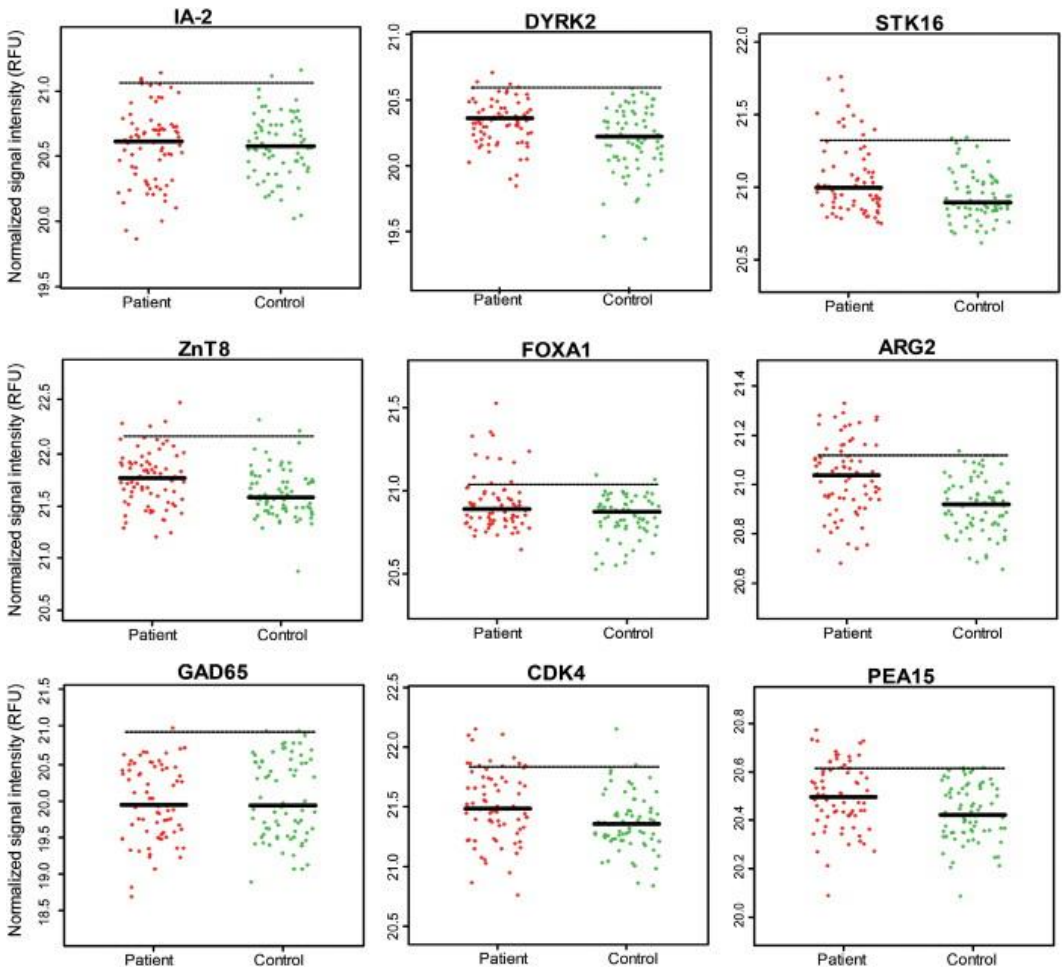
Array images challenged with serum sample from different patients that deemed positive by statistical analysis were adjusted to identical black and color thresholds to enable visual comparison. Differences were readily perceived upon visual examination of immunoreactivity of candidate features between patient and control serum-challenged slides. Intensity scale = red > orange > yellow >green.

To enable the visual comparison of immunoreactivity obtained from the second-stage screening, image scans of serum challenged slides were adjusted to be pseudo-colored in identical black and full color threshold scale. Slides with duplicate features designated as seropositive in different patients by statistical analysis were compared to a slide challenged with control serum with similar median signal intensities (Figure 3-3). Visually discernible differences for the antigens calpain-11 (CAPN11), CDK3, astrocytic phosphoprotein (PEA15) and sphingosine-1-phosphate receptor 3 (SIPR3) suggested that the normalization scheme employed did not distort the data in a manner that either 1) creates signal differentials that do not exist or 2) destroys true signal differentials. In order to provide a visual representation of signal intensities of candidate antigens across all samples, jitter plots were generated from normalized and log-transformed signal intensities to illustrate the comparison of immunoreactivity of known T1D associated AAbs as well as the novel ones in patient and control groups (Figure 3-4).

3.4.3 AAb Candidates Identified from the Second-stage Screening

Known T1D AAbs, such as ZnT8-NC (Slc30A8), GAD65 and IA-2, were included as positive controls on the second stage screening array. The display levels of these antigens were confirmed by anti-GST staining of arrays (data not shown). In this protein microarray assay, the median signal of known AAb to ZnT8-NC (Slc30A8) in patient group was significantly higher than the control group ($p<0.0004$) from the Wilcoxon Rank-Sum test (Table 3-2); however, none of the other three known AAbs to insulin, IA-2, or GAD65 exhibited statistically significant increases reactivity toward patient sera. The absence of immunoreactivity to those three known T1D associated

AAbs may reflect a differential performance of solid-phase immune-profiling techniques in the detection of some epitopes. Nevertheless, the additional 25 additional candidate AAbs besides ZnT8-NC (Slc30A8) were identified using the unbiased screening strategy. Some of these autoantigens are known to be expressed in the pancreas or enriched in islet cells



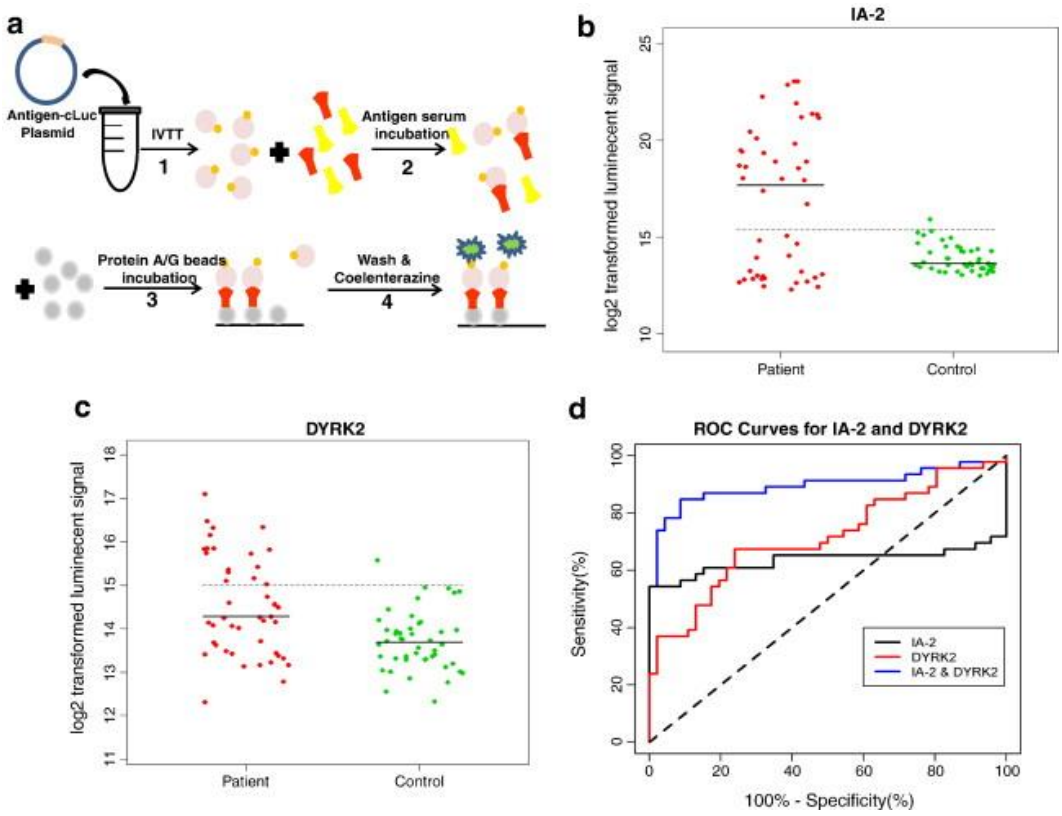
3 - 4 Jitter Plots of Serological Immunoreactivity to Candidate AAbs
Each dot represents the signal from one patient or control serum to the indicated antigens; the solid lines indicate median signal intensities of patient (n=74) or control group (n=75). The upper line across both patient and controls groups, indicates the 95th percentile of control signal.

such as PEA-15 and DYRK2 (Kuliawat et al., 2004; Miele et al., 2007).

3.4.4 Validation of Candidate AAbs

Candidate AAbs were selected based on the significance of differential reactivity (as indicated by p-value) and their potential roles in beta-cell physiology for validation in an independent serum set (46 patients and 46 controls) by LIPS assay (Burbelo et al., 2005a). LIPS assay was employed as a validation platform because it was a solution based assay platform orthogonal to protein array and the performance of LIPS on the known T1D associated AAbs (GAD65 and IA-2) was similar to the widely accepted RIA in clinical T1D studies. The published LIPS assay expression vector pRL-CMV was modified so that it was compatible with Gateway® Cloning, allowing us to transfer the candidate genes into the modified vector easily and to produce candidate antigens by the RRL IVTT system (Figure 3-5) consistent with the antigen production used throughout this study and in most reported RIA assays (Burbelo et al., 2009). The modified pRL-CMV vector was first tested on IA-2, a known T1D-associated AAb. LIPS assay confirmed IA-2 with a sensitivity of 54% at 98% specificity, comparable to the performance of LIPS assay and commonly used RIA assay reported (Burbelo et al., 2008).

Besides IA-2, nine sequence-verified candidate genes (PEA15, CAPN11, DYRK2, CDK1,CDK3, CDK4, CDK6, TBCA, GCK) including candidates from supplement statistical analysis (Table 3-2) and previous published T1D associated autoantigens were cloned into the modified pRL-CMV expression vector with c-terminal luciferase fusion and immunoreactivity to these antigens were measured by LIPS. Luminescent data for each sample collected were from the average of the replicate measurements. Average



3 - 5 Validation of Candidate AAbs Using LIPS Assay
(a) Schematic representation of LIPS assay.
(b,c) LIPS assay confirmed the performance of IA-2 and DYRK2 with 54% and 36% sensitivity at 98% specificity respectively. The solid lines represent the median signal of patient (n=46) and control group (n=46), the dash line indicates the 98% percentile of signal intensities from control group.
(d) ROC curves for IA-2 and DYRK2 alone and the combination of the two.

patient signals that exceed the 98th percentile of control signals were considered to exhibit positive reactivity.

One candidate AAb, DYRK2 showed 36% sensitivity at 98% specificity (Figure 3-5); whereas, the other nine candidates showed lower sensitivities (Data not shown). It is possible the failure to validate these latter candidates may be due to the platform difference. The performance of DYRK2 was confirmed by several repeated assays (Data

not shown). The addition of DYRK2A improved the AUC for IA-2A from 0.64 to 0.90. DYRK2A has an AUC of 0.72 on its own. The combination of DYRK2A and IA-2A has a sensitivity of 72% at 98% specificity.

3.5 Discussion

The rapid rise in diabetes incidence imposes a substantial economic burden on health care systems worldwide (Scully, 2012). Primarily affecting children and young adults, T1D is characterized by the progressive destruction of insulin producing pancreatic beta cells and imposes a lifelong responsibility to maintain glucose homeostasis through regular insulin injections. It is believed the autoimmune reactions mediated by B cells and T cells play an important role in disease pathogenesis and autoantibodies to self-proteins are good predictors for disease diagnosis and progression. Historically, four autoantigens (Insulin, GAD65, IA-2, ZnT8) have been reported as major targets of AAb with individual sensitivity more than 55%. The combination of these four AAbs detects >90% of T1D patients (Wenzlau et al., 2008). Nevertheless, the prevalence of individuals that are ICA positive but negative for all four known AAbs is not rare at all ages and thus provides impetus for the discovery of novel AAbs that are not yet identified (Hameed et al., 2011; Wang et al., 2007). Minor T1D-associated AAbs with low sensitivity and specificity such as carboxypeptidase-H (Castano et al., 1991), ICA69 (Pietropaolo et al., 1993), GM2-1(Dotta et al., 1997a), microsomal hepatic protein (Pehuet-Figoni et al., 2000), SOX13 (Kasimiotis et al., 2001), HSP70(Abulafia-Lapid et al., 2003), topoisomerase II (Chang et al., 2004), VAMP2 and NPY (Hirai et al., 2008a) were reported continuously and provide deeper understanding of T1D pathogenesis in addition to the four major ones. The above AAb biomarker discovery studies relied upon a targeted strategy and restricted inquiry to the landscape of the pancreas using low-throughput experimental techniques such as phage display, ELISA and RIA.

The value of AAbs in prediction and diagnosis of T1D is well-established; however, their pathogenic role is contentious (Baekkeskov et al., 2000). AAbs, generated during the influx of immune cells into the peri-islet space, are most often considered byproducts of a pre-existing underlying pathology, rather than a causative agent themselves. A comprehensive knowledge of the 'autoantigenome' in T1D will undoubtedly assist in generating and testing hypotheses aimed at answering questions regarding mechanisms of beta cell damage and the temporal expression patterns of autoantibodies that aid in disease characterization. A systematic profiling of serological AAbs will expand the current 'autoantigenome' in T1D and illuminate T1D pathogenesis.

To date, no unbiased, proteome scale and high-throughput AAb discovery studies have been published for T1D. The development and successful use of new discovery technologies such as protein microarrays offer a valuable opportunity to design non-biased, data-driven studies to profile serum AAbs to a large number of human proteins in a high-throughput manner. The innovative protein array platform, NAPPA, has been used successfully in identifying circulating antibodies in infectious diseases, autoimmune diseases and cancers (Anderson et al., 2011; Ceroni et al., 2010; Gibson et al., 2012). In this study, the establishment of a NAPPA protein array based high-throughput pipeline of AAb discovery in T1D is the basis of the success in identifying DYRK2 as a novel minor T1D-associated AAb.

The high-throughput discovery pipeline involved two large screening process followed by a targeted solution-based validation study. Although a known T1D-associated AAb-ZnT8 was among the final candidate list, neither GAD65 nor IA-2 were

identified presumably due to the differences in the manner in which responses to those antigens are typically measured. Nevertheless, several candidate AAbs identified are known to play critical roles in beta cell physiology and are associated with exocytotic machinery. PEA15 was implicated in the regulation of glucose induced insulin secretion through the inhibition of potassium channel expression and (Miele et al., 2007), STX6 has been shown to function in the process of endosomal maturity and its delivery to lysosomes and may further influence the secretory pathway in live pancreatic beta-cells (Kuliawat et al., 2004). Several ubiquitously expressed cyclin-dependent kinases (CDKs) were identified in the list including CDK3, CDK4 and CDK6. Studies in human and mice have revealed that beta cell replication and proliferation can be stimulated by the over-expression of either CDK4 or CDK6 without evidence of cell death, loss of function or aberrant differentiation (Fiaschi-Taesch et al., 2009; Marzo et al., 2008), but how they would be targeted in the pancreas as autoantigens is unclear. In addition, candidates that show no strong connection with diabetes such as S1PR3 and endoplasmic reticulum to nucleus signaling 2 (ERN2) were also identified. The diversity of functions of the identified AAb candidates demonstrated the power of the unbiased screening strategy.

To confirm these findings, an initial step was taken in validating the above AAb candidates in an independent serum sample set using an orthogonal liquid-phase assay originally developed by Peter D. Burbelo (Burbelo et al., 2009). To adapt this assay platform for compatibility with the IVTT system, the expression vector was modified and engineered with a Gateway™-compatible cassette to simplify the process of gene transfer and T7 promoter to enable antigen production in an RRL IVTT system. Candidate

antigens were selected for validation based on their classification performance and putative roles in beta-cell biology.

DYRK2 was validated with a 36% sensitivity at 98% specificity, further analysis indicate that DYKR2 complements IA-2 by a moderate increase in sensitivity, whereas it did not complement GAD65. IA-2A has a sensitivity of 54% at 98% specificity on its own. The widely accepted linear regression model was used to combine the two markers and generate the ROC curve. The addition of DYRK2A improved the AUC for IA-2A from 0.64 to 0.90. An important next step will be to validate the performance of the DYRK2A and IA-2A combination in future studies on independent sample sets.

The identification of minor AAbs like DYRK2 and future characterization of the correlation of their seropositivity with other clinical features may benefit other aspects of clinical patient management (e.g. stratification) and advance our understanding of disease etiology and heterogeneity. DRYK2 is a member from novel dual specificity protein kinase family and has been implicated in the glucose metabolic pathway as responsible for the phosphorylation and inactivation of glycogen synthase (Skurat and Dietrich, 2004). DYRK2 is highly expressed in the pancreas based on protein atlas data and has been implicated in the apoptosis pathway in response to severe DNA damage through the activation of the p53 signaling (Yoshida, 2008). Moreover, a Genome wide association study (GWAS) found that DYRK2 is significantly associated with type 2 diabetes in Mexican Americans (Hayes et al., 2007). Its role in T1D pathogenesis requires further investigation.

Other candidates from screening were not validated in the LIPS assay, which may reflect differences in the detection methods. Antigen conformation and / or binding characteristics of autoantibody markers to their target antigens that can be identified in a solid-phase assay may be very different from those identified in a liquid-phase assay. The original rationale for choosing a liquid phase validation platform included using a method that had previously been shown to work for two classical T1D AAbs and to ensure the markers reported can perform on two orthogonal validation platforms. It is expected that some markers will only perform well on one platform but not both. This may explain why some of the markers discovered on the arrays did not validate in the liquid phase assay. Some classical autoantibodies in T1D have been reported to behave differently on different platforms. For example, it has been reported that insulin antibody, a known T1D autoantibody, showed lower sensitivity, specificity and AUC in liquid phase assays compared with a solid phase ELISA assay (Schlosser et al., 2010; Yu et al., 2012). On the other hand, IA-2A performs better when measured by the liquid phase RIA than on the solid phase ELISA platform (Torn et al., 2008). It is possible that some conformational epitopes of antigen proteins might be blocked by the capture antibody in the array platform. In the liquid LIPS assay platform, these epitopes were exposed and recognized by serum autoantibodies. On the other hand, the array platform might display some epitopes that are not exposed in a liquid phase assay. Assessment of sero-reactivity to these candidates on different platforms such as the multiplexed self-assembling bead arrays on the luminex platform (Wong et al., 2009), plate-based programmable ELISAs

(Gibson et al., 2012) or targeted small content NAPPA arrays (Anderson et al., 2011) may shed light on the behavior of these antigens presented in different formats.

3.6 Conclusions

In conclusion, a discovery pipeline was developed that enables the first large scale proteome scale searching for AAb biomarkers in T1D on an innovative protein array platform using a two-stage screening strategy, resulting in the discovery of minor AAb-DYRK2 (36% sensitivity at 98% specificity), thus, demonstrating the applicability of NAPPA to AAb discovery in T1D and providing impetus to expand this technology to the whole human proteome with the established pipeline.

3.7 Acknowledgements

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CHAPTER 4

4 IDENTIFICATION OF NOVEL AUTOANTIBODIES IN TYPE 1 DIABETES BY PROTEIN ARRAYS

4.1 Abstract

Autoantibodies (AAbs) produced during the development of type 1 diabetes (T1D) are valuable in predicting risk, stratifying patients with different clinical courses, explaining disease pathogenesis and providing candidates for prevention trials. We performed a comprehensive profiling of humoral autoimmunity in T1D by human protein arrays and enzyme-linked immunoassay (ELISA). Analysis of reactive antigens revealed similar pathways were enriched in both patient and healthy control groups. At the individual antigen level, we identified and validated six AAbs with sensitivities ranged from 16% to 27% at 95% specificity. Their prevalence in T1D cases complements the major known T1D-assocaited AAbs and varies in different age subgroups. Our study enriched our knowledge of T1D "autoantigenome/autoantibody-ome" and provided new insights in T1D pathophysiology into unprecedented breadth and width.

4.2 Introduction

The rapid rise of incidence rate poses a huge challenge to find a cure for T1D (Onkamo et al., 1999). Upon diagnosis, more than 80-90% of pancreatic β-cells have been destroyed (Atkinson, 2012). While cellular immune response plays a dominant role in β-cell autoimmune destruction, antibodies against self-proteins, i.e. AAbs, are produced during disease progression. They were used for predicting T1D risk and selecting high risk subjects for prevention trials (Marino et al., 2011). Immune-therapeutic strategies were under development by targeting autoantigens recognized by AAbs (Ludvigsson et al., 2012).

AAbs to Insulin, GAD65, IA-2 and ZnT8 were identified as major T1D-associated AAbs (Baekkeskov et al., 1990; Bonifacio et al., 1995a; Palmer et al., 1983; Wenzlau et al., 2007). A number of minor AAbs were discovered by various approaches (de Graeff-Meeder et al., 1993; Hirai et al., 2008b; Kasimiotis et al., 2000; Pietropaolo et al., 1993; Winnock et al., 2001). The rate of discovering new AAbs has been greatly limited by the throughput of technologies. Phage displayed libraries were constructed from human or mouse pancreatic tissue and immunoprecipitated by T1D patient sera (Pietropaolo et al., 1993; Winnock et al., 2001). Immunoassays such as radioimmunoprecipitation (RIA) or ELISA are targeted approaches to test antigens one a time (de Graeff-Meeder et al., 1993; Hirai et al., 2008b; Kasimiotis et al., 2000). One hypothesis of β cell autoimmunity is the increased biosynthetic activity of proinsulin leads to the increase of misfolded proteins in the endoplasmic reticulum (ER) in β cells (Eizirik et al., 2009). When the ER faces sustained stress, the autoreactive cytotoxic T

cells that recognize abnormal epitopes will destroy β cells and release more antigenic epitopes to initiate a process called "epitope spreading" (Fonseca et al., 2009). The number of T1D-associated AAbs may be much higher than currently known. Diabetic individuals who are positive for Islet Cell Antigen (ICA) staining yet remain negative for the four known T1D-associated AAb biomarkers (Wang J, 2007). Also, a proportion of patients are negative for both ICA and the known biochemical AAbs with no apparent difference in clinical performance compared with AAb positive T1D patients suggesting different disease subtypes (Hameed et al., 2011). One possible explanation is that AAbs in this group may target antigen proteins outside the landscape of the pancreatic islet, which give us a hint that a whole proteome screening is needed to identify additional AAbs that will give us a complete picture of humoral autoimmunity in T1D.

However, we still have a limited understanding on global antibody response in T1D. A global profiling of AAbs and the identification of their eliciting autoantigens in T1D will aid in the construction of molecular pathways that explain the pathological events during autoimmunity and disease onset. The advent of the proteomics era opened new avenues to search for new AAbs in T1D. Phage immunoprecipitation sequencing (PhIP-Seq) is a technology that couples phage display with next generation sequencing (NGS). It was applied to screen peptide and AAb interactions and revealed a prematurely polyautoreactive phenotype in T1D patients (Larman et al., 2013). Serological proteome analysis (SERPA) is a method to identify patient specific antigen proteins using a proteomic approach (Massa et al., 2013). Protein microarrays provide an ideal tool to profile global antibody response to a large number of proteins in a high-throughput

manner in T1D. An innovative cell-free protein array platform named Nucleic Acid Programmable Protein Array (NAPPA) was used to screen 6,000 human proteins and identified a minor T1D-associated AAb (Miersch et al., 2013). Two additional novel AAbs were discovered by high-density protein microarrays in 2014 (Koo et al., 2014).

In this study, we performed a comprehensive search for T1D-associated AAbs by a screening-based approach using NAPPA including 10,000 human proteins and a knowledge-based approach using an individual immunoassay to test 126 pancreas enriched genes from literature and bioinformatics analysis. We performed network analysis and compared global antibody response in T1D patients and controls. Six AAbs were discovered and validated in independent sample set. Their value of complementary to the known T1D-associated AAbs and their prevalence in different age subgroups were analyzed.

4.3 Experiments

4.3.1 Study Samples

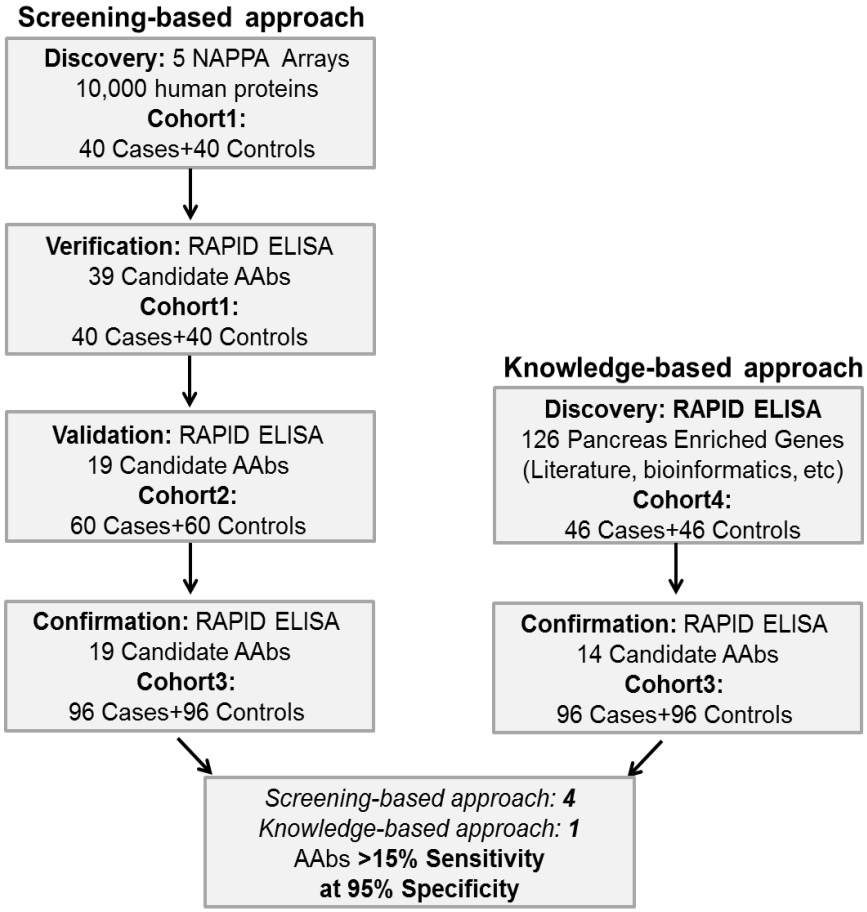
All samples were collected with written informed consent under the guidelines of the Institutional Review Boards (IRB) at the University of Florida. T1D samples were obtained from new-onset patients within three months of diagnosis according to the American Diabetes Association (ADA) criteria. Peripheral blood samples were drawn from the antecubital vein and serum was prepared before freezing as aliquots at -80^{o}C. Control samples were prepared in the identical fashion and selected to be age-gender matched to the patients. Controls had no T1D family history and were considered to be at low risk for T1D with absence of known T1D-associated AAbs except for two individuals are positive for GADA and one is positive for IA-2A. Neither patients nor control samples were known to have any other underlying autoimmune disease. Characteristics of study subjects including age, gender and status of known T1D-associated AAbs were presented as median, mean, standard deviation (SD) and percentage (Table 4-1).

4 - 1 Sample Characteristics of Each Cohort

	Screening Based				Knowledge Based			
Characteristics	**Cohort1**		**Cohort2**		**Cohort4**		**Cohort3**	
	T1DM	**HC**	**T1DM**	**HC**	**T1DM**	**HC**	**T1DM**	**HC**
Number of Subjects	40	40	*60	60	46	46	*96	96
Age								
Mean± SD	15.0±6.5	15.0±6.6	14.0±7.6	14.2±7.4	14.0±8.4	14.0±8.1	14.0±7.3	14.0±7.1
Median	13	13	12	12	11	12	12	12
Gender								
Male (%)	(14)35.0%	(14)35.0%	(28)47.5%	(29)48.3%	(20)43.5%	(21)45.7%	(41)43.2%	(42)43.8%
GADA positive	(36)90.0%	0	(50)83.3%	(2)3.3%	(40)87.0%	(2)4.3%	(82)85.4%	(2)2.1%
IA-2A positive	(26)65.0%	0	(40)66.7%	(1)1.7%	(31)67.4%	(1)2.2%	(62)64.6%	(1)1.0%

4.3.2 Study Design

This study adapted screening-based and knowledge-based approaches to search for novel AAbs in T1D (Figure 4-1). In the screening-based approach, we profiled AAbs against 10,000 human proteins in cohort 1 (40 cases and 40 controls) on 5 arrays sets during the discovery stage. We selected 39 AAbs to verify their differential sero-reactivity in cohort 1 by ELISA.19 AAbs had >10 sensitivity at 95% specificity during the verification stage. They were tested in an independent set of cohort 4 (60 cases and 60 controls) and further confirmed in cohort 3 (96 cases and 96 controls) by ELISA. In the knowledge-based approach, 126 pancreas enriched genes were selected by literature search (Hirai et al., 2008b; Ludvigsson et al., 2012; Wenzlau et al., 2007) and bioinformatics analysis. AAbs to these proteins were profiled in cohort 4 (46 cases and 46 controls) by ELISA. 14 AAbs showed >10% sensitivity at 98% specificity were confirmed in cohort3.



4 - 1 Study Design

(1) Screening-based approach: i. Discovery: samples from cohort 1 were screened against 10,000 human proteins across 5 NAPPA array sets; ii. Verification: 39 candidate autoantigens were verified using samples from cohort1 by RAPID ELISA; iii. Validation: 19 candidate autoantigens were validated using samples from cohort2 by RAPID ELISA; iv. Confirmation: 19 candidate autoantigens were confirmed using samples from cohort3 by RAPID ELISA.

(2) Knowledge based approach: i. Discovery: 126 pancreas enriched genes (literature, bioinformatic) were tested in samples from cohort4 by RAPID ELISA; ii. Confirmation: 14 candidate autoantigens were tested in samples from cohort3 by RAPID ELISA.

4.3.3 NAPPA Production and Quality Assessment

10,000 human genes obtained from DNASU (http://dnasu.asu.edu/DNASU/) (Seiler et al., 2014) were printed on 5 array sets (GST1, GST2, GST3, GST4 and Flag) (Miersch and LaBaer, 2011). Plasmids encoding human proteins have either a C-terminal glutathione S-transferase (GST) or N-terminal Flag fusion tag in frame with the protein. Proteins were expressed by an *in vitro* transcription and translation (IVTT) system. Expressed proteins with fusion tag can be easily captured on slides by the co-printed anti-tag antibodies (Ramachandran et al., 2008). Known T1D-associated AAbs (Insulin, IA-2, GAD65 and ZnT8) were printed as positive controls for serum profiling. Both DNA printing and protein display on NAPPA was quality assured as described (Miersch et al., 2013).

4.3.4 Profiling of AAbs on NAPPA

As described (Bian et al., 2015), printed slides were blocked in SuperBlock Buffer (Thermo Fisher Scientific, MA) at room temperature (RT) for 1 hour (hr) on the shaker. Then slides were rinsed five times with deionized (DI) water and placed in a metal slide rack (Amazon, WA) for drying by centrifugation at 1,000 rpm for 3 min at RT. 160 μL human HeLa cell lysate-based protein expression system (Thermo Fisher scientific, MA) was injected into HybriWell (Grace BIO-LABS, OR) sealed slides and incubated in the oven (EchoTherm, CA) at 30°C for 1.5 hrs for protein expression and 15°C for 30 min for protein capture. Expressed slides were washed 10 times with water and dried by centrifugation at 4°C, 1,000 rpm for 3 min. Slides were placed in the hybridization chamber of HS 4800™ Pro hybridization station (Tecan, Männedorf,

Switzerland) and programed with 1 h blocking with 5% milk-PBST (0.2% Tween), 16 hrs of 160 µL 1:50 diluted serum at 4°C followed by 1 hr incubation of 160 µL 1:500 diluted Alexa Fluor 647® Goat Anti-Human IgG (Jackson ImmunoResearch Laboratories, PA). Slides were washed, dried and scanned by Tecan scanner under consistent settings. A pool sample was prepared by mixing equal volumes of samples from each patient and control. The pool sample was run as technical replicates on every serum screening day.

Normalized signal intensity was generated using the raw signal intensity divided by the medium signal intensity of each array. Strong sero-reactivity resulted in saturated signals of the local spot with diffusion to the neighboring spots. The presence of diffusion was defined as a ring. Differences in ring counts between the case and control group were used as selection criteria for candidate AAbs for ELISA verification.

4.3.5 Rapid Antigenic Protein in situ Display (RAPID) ELISA

As described (Bian et al., 2015), 96-well ELISA plates (Corning Life Sciences, CA) were coated with 50 µL 10 µg/mL anti-GST antibody (GE Healthcare, PA) in coating buffer (0.5 M carbonate bicarbonate buffer, pH 9.6) overnight at 4°C. On the next day, coated plates were washed five times with 100 µL PBST and blocked with 100 µL 5% milk-PBST for 1.5 hrs. Meanwhile, 40 ng/µL protein encoding plasmid was expressed in the human HeLa cell lysate-based protein expression system at 30°C for 1.5 hrs in the oven. Expressed proteins were diluted in milk-PBST at 1:50. 50 µL diluted antigen was captured in each well at RT for 1 hr on a shaker at 500 rpm. Plates were washed with five times of PBST. Each well was incubated with 50 µL 1:200 diluted

serum at RT for 1 hr, washed again and incubated with 50 µL 1:10,000 diluted HRP labeled Goat Anti-Human IgG (Jackson ImmunoResearch Laboratories, PA) at RT for 1 hr. Plates were incubated on a shaker at 500 rpm. Finally, the plates were washed and incubated with 50 µL 1-Step Ultra TMB - ELISA Substrate for 10 min (Thermo Fisher scientific, MA) for detection and 50 µL 2M sulfuric acid to stop the reaction. OD450 was measured by Envision® Multilabel Reader (PerkinElmer, MA).

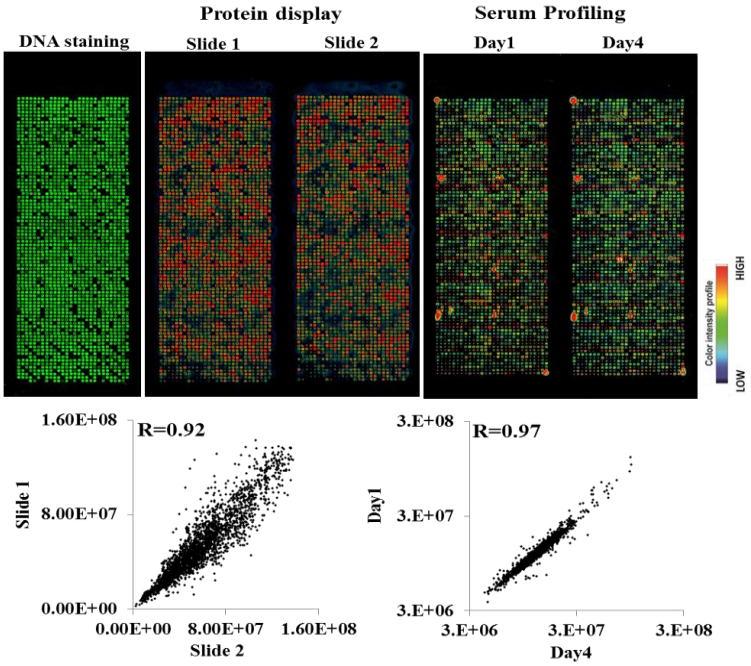
4.3.6 Statistical Analysis

Network analysis was conducted on antigens that showed sero-positivity in at least two individuals (either cases or controls) using Cytoscape version 3.2.0. The pathway function were enriched as modules and annotated by ReactomeFIPlugIn. The size of the nodes indicates the frequency of individual antigens with sero-positivity. The color of the nodes indicates the percentage of sero-positivity in cases. Sensitivity and specificity of candidate AAbs were determined by RAPID ELISA. Heatmaps were generated in MultiExperiment Viewer version 4.9 (http://www.tm4.org/mev.html). Graphs and plots were drawn in GraphPad Prism 6.

4.4 Results

4.4.1 Quality of Protein Array and Serum Profiling

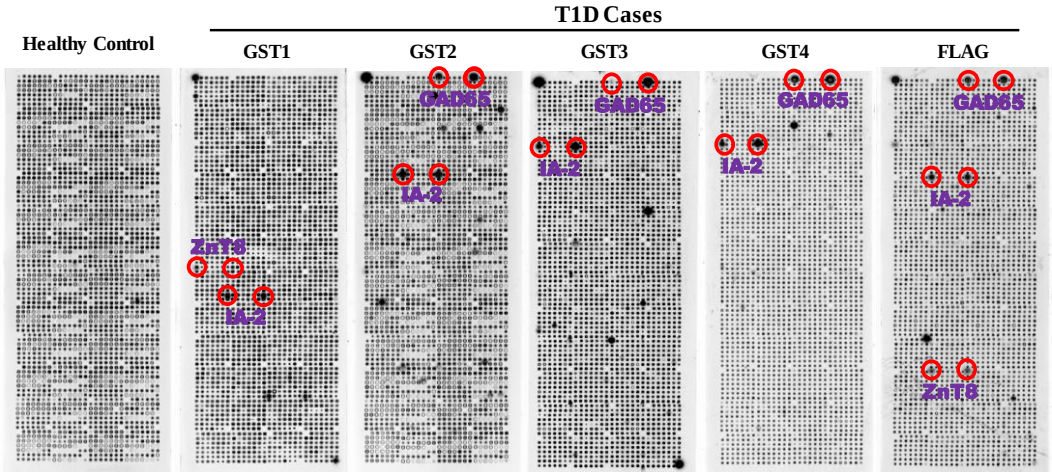
We constructed five array sets (GST1, GST2, GST3, GST4 and Flag) with ~2,000 features on each array. Pico-green staining showed DNA printed on the array was uniform. The correlation coefficient of protein display and serum profiling was 0.92 and 0.97, respectively. The high reproducibility of protein display demonstrated the high quality of our protein arrays. The high reproducibility of serum profiling proved the robustness of our serum screening practice (Figure 4-2).



4 - 2 Quality of DNA Staining, Protein Display and Serum Profiling on NAPPA
(A) Representative slide images of DNA staining, protein display and serum profiling. DNA staining is pesudo-colored in green, protein display and serum profiling is pesudo-colored in rainbow color (Blue, yellow and red indicate low, medium and high signal intensity).
(B) Scatter plot of signal intensities of two protein display slides on the left and two serum profiling slides on the right.

4.4.2 Screening-based Approach by NAPPA

Sero-reactivity to known T1D-associated AAbs (IA-2, GAD65 and ZnT8) was visually distinguishable from other antigens which showed negative sero-reactivity on the same array. The healthy control sample did not show any responses to any of these antigens (Figure 4-3), clearly proving the validity of our array platform in profiling serological AAbs in T1D.



4 - 3 Sero-reactivity to Known T1D-associated Autoantigens on NAPPA
Slide images showed the apparent sero-reactivity to the known T1D-associated autoantigens (IA-2, GAD65 and ZnT8) on five NAPPA array sets (four printed with genes with GST tag, one printed with genes with flag tag).

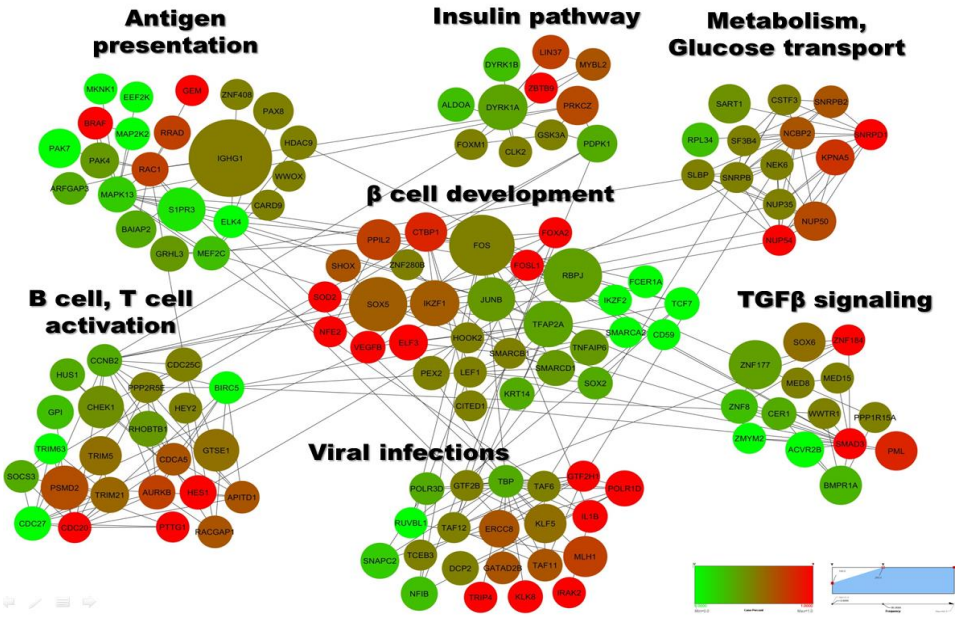
We selected 39 candidate AAbs based on their sero-reactivity on protein arrays for verification by RAPID ELISA in the same sample set used in screening (cohort1). 19 candidates were confirmed by ELISA with >10% sensitivity at 95% specificity (Table 4-2).

4 - 2 Statistics of Candidates from Screening-based Approach

	Cohort1		Cohort2		Cohort3	
Antigen	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
PTPRN2	0.25	0.95	0.20	0.95	0.22	0.95
MLH1	0.15	0.95	0.33	0.95	0.27	0.95
MTIF3	0.15	0.95	0.20	0.95	0.25	0.95
PPIL2	0.20	0.95	0.18	0.95	0.19	0.95
NUP50	0.15	0.95	0.17	0.95	0.16	0.95
TOX4	0.20	0.95	0.12	0.95	0.13	0.95
FIGN	0.13	0.95	0.12	0.95	0.13	0.95
C9orf142	0.18	0.95	0.05	0.95	0.11	0.95
ZNF280D	0.13	0.95	0.08	0.95	0.11	0.95
HES1	0.10	0.95	0.15	0.95	0.11	0.95
TOX	0.30	0.95	0.07	0.95	0.09	0.95
CTBP1	0.25	0.95	0.08	0.95	0.09	0.95
POLR1D	0.15	0.95	0.07	0.95	0.09	0.95
TAF11	0.15	0.95	0.03	0.95	0.08	0.95
PHF15	0.15	0.95	0.05	0.95	0.07	0.95
STMN3	0.10	0.95	0.05	0.95	0.06	0.95
TCEAL4	0.13	0.95	0.03	0.95	0.05	0.95
FILIP1	0.10	0.95	0.03	0.95	0.04	0.95
BANK1	0.10	0.95	0.02	0.95	0.02	0.95

4.4.3 Network Analysis of Sero-positive Proteins on NAPPA

1,204 antigens showed sero-positivity in at least one of the cases or controls. Network analysis was conducted on the 558 antigens that showed sero-positivity in at least two individuals (either cases or controls) using Cytoscape. It generated 21 modules including 135 nodes and 274 edges. Although no difference was found between cases and controls at the pathway level, representative pathways enriched were β-cell development, class I MHC mediated antigen processing & presentation, B-cell activation, T-cell



4 - 4 Network Analysis of Sero-positive Proteins on NAPPA
The size of the nodes indicates the frequency of individual antigens with sero-positivity. The color of the nodes indicates the percentage of sero-positivity in cases.

receptor signaling pathway, viral infections, TGFβ signaling, metabolism, glucose transport and insulin pathway (Figure 4-4).

4.4.4 Knowledge-based Approach by RAPID ELISA

In the knowledge-based approach, AAbs to126 pancreas enriched genes selected by literature search and bioinformatics analysis were profiled by RAPID ELISA in cohort4. The aim of this step is to discover the sero-reactive antigens and to scale down the number of proteins for confirmation in cohort3. 14 candidate AAbs that has >10% sensitivity at 98% specificity in cohort4 were further selected for validation in cohort3 (Table 4-3).

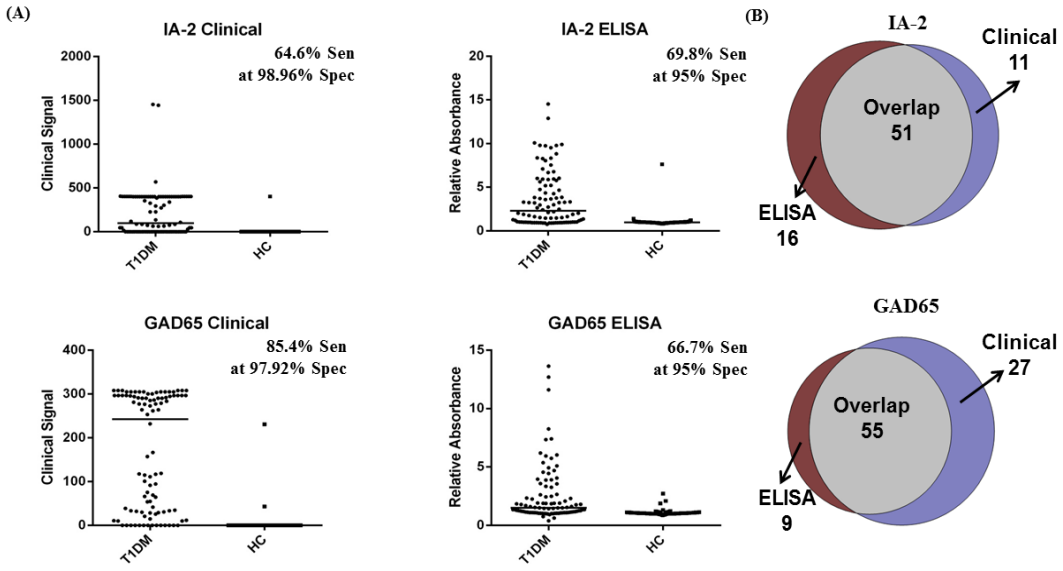
4 - 3 Statistics of Candidates from Knowledge-based Approach

	Cohort4		Cohort3	
Antigen	Sensitivity	Specificity	Sensitivity	Specificity
QRFPR	0.11	0.98	0.20	0.95
CTRC	0.13	0.98	0.15	0.95
SNX6	0.13	0.98	0.15	0.95
SYTL4	0.17	0.98	0.13	0.95
ELA2A	0.11	0.98	0.13	0.95
IGRP	0.11	0.98	0.11	0.95
PAX6	0.11	0.98	0.11	0.95
HMGN3	0.20	0.98	0.10	0.95
STXBP1	0.22	0.98	0.09	0.95
REG3G	0.15	0.98	0.09	0.95
SCG5	0.13	0.98	0.06	0.95
RCBTB2	0.13	0.98	0.05	0.95
ASB9	0.13	0.98	0.04	0.95
PPY	0.13	0.98	0.02	0.95

4.4.5 RAPID ELISA as an Individual Immunoassay to Profile AAbs

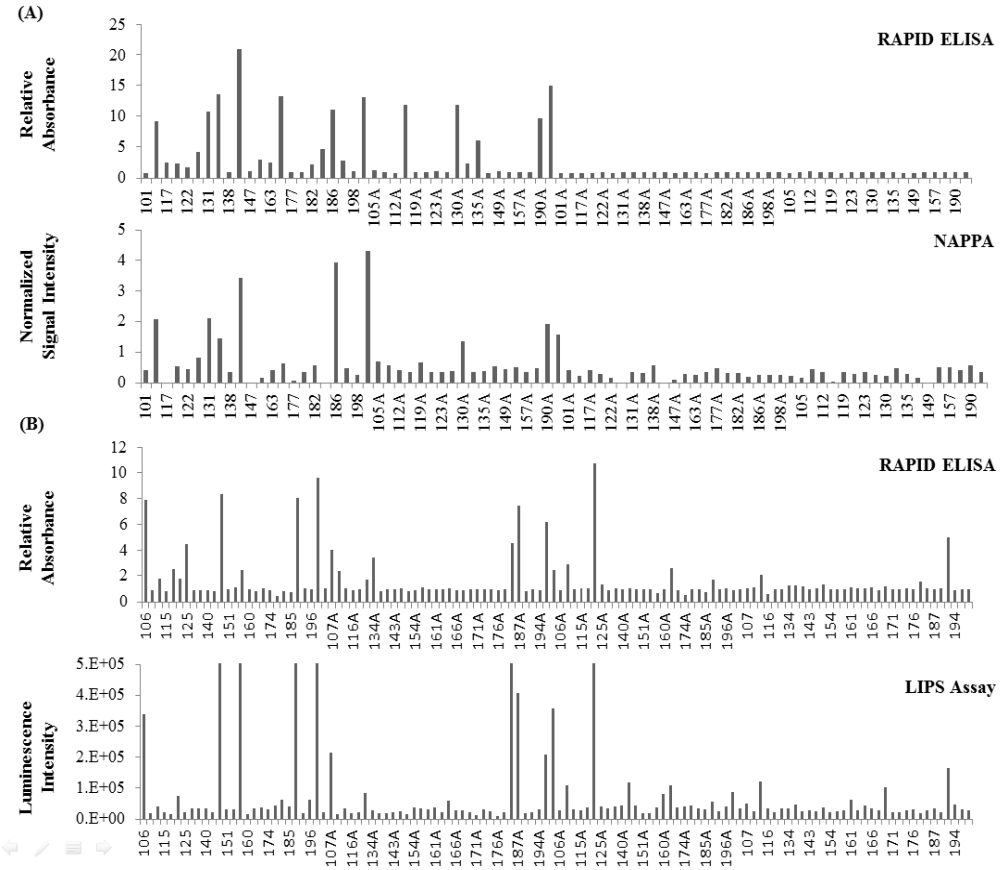
Protein microarray (NAPPA) offers a great high-throughput screening tool to profile AAbs against thousands of antigen proteins. RAPID ELISA is more suitable to profile AAbs to a smaller number of candidates using a large number of serum samples in a plate format. Before applying RAPID ELISA as an individual immunoassay to test and validate AAb candidates, we compared its performance with an RIA based clinical assay in measuring antibody response to known T1D-associated AAbs (IA-2 and GAD65). The clinical assay achieved 64.6% sensitivity at 96.96% specificity for IA-2A and 85.4% sensitivity at 97.92% specificity for GADA. Our in-house RAPID ELISA obtained 69.8% and 66.7% sensitivity at 95% specificity for IA-2A and GADA, respectively (Figure 4-5). Using the above cutline, 51 and 55 subjects were positive by both assays for IA-2A and

GADA, respectively. 16 subjects were positive only by ELISA and 11 were only positive by clinical assay for IA-2A. 9 subjects were positive only by ELISA and 27 were positive only by clinical assay for GADA (Figure 4-5).



4 - 5 Sero-reactivity to IA-2 and GAD65
(A) Jitter plots of sero-reactivity of IA-2 and GAD65 by clinical test and ELISA.
(B) Venn diagram shows the overlap of individuals with positive sero-reactivity to IA-2 or GAD65 in clinical test and ELISA.

In addition, we compared the sero-reactivity to IA-2 by RAPID ELISA and NAPPA in cohort1. The relative absorbance of RAPID ELISA and normalized signal intensity of NAPPA agreed well (Figure 4-6). We further compared RAPID ELISA with LIPS assay which was used in our previous publication (Miersch et al., 2013). AAb response to NUP50 was measured in cohort2. The relative absorbance of RAPID ELISA and luminescent signal of LIPS assay matched well (Figure 4-6). RAPID ELISA also has

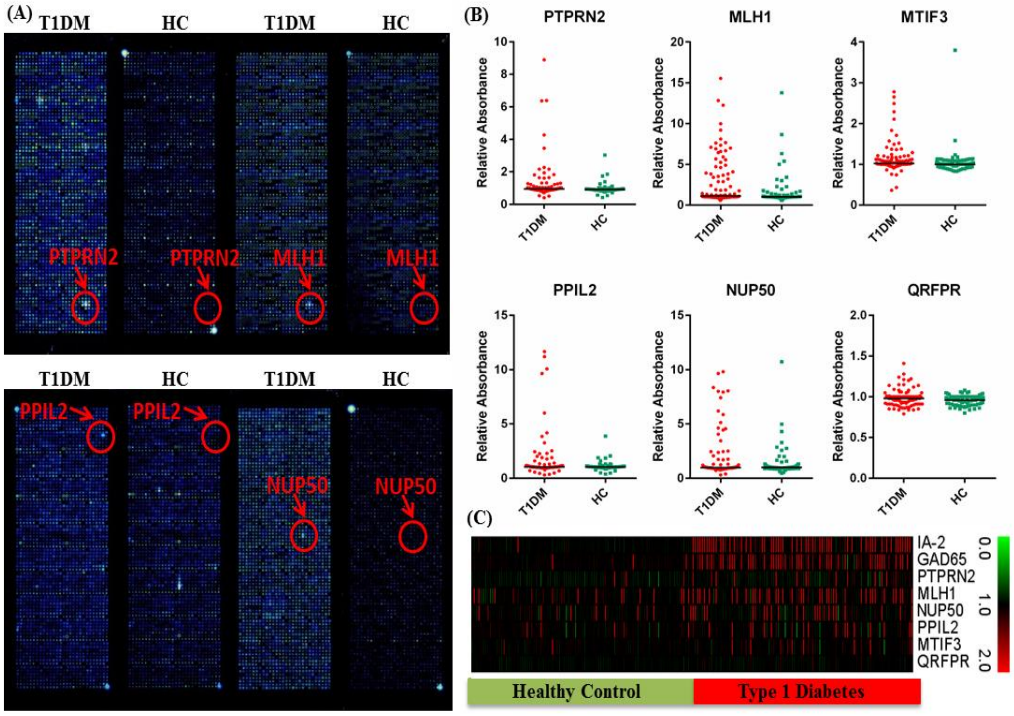


4 - 6 Comparison of RAPID ELISA with NAPPA and LIPS assay
(A) Bar plots of sero-reactivity to IA-2 measured by RAPID ELISA and NAPPA in cohort1.
(B) Bar plots of sero-reactivity to NUP50 measured by RAPID ELISA and LIPS Assay in cohort2.

high intra-assay and inter-assay reproducibility (data not shown). Due to the high sensitivity in detecting serum AAbs and the large overlap of sero-positivity with the clinical assay, NAPPA and LIPS assay, RAPID ELISA was proved as a superb individual immunoassay to profile AAbs.

4.4.6 Novel AAbs from Two Approaches

In the knowledge-based approach, 7 out of 15 candidates showed >10% sensitivity at 95% specificity in the independent sample set (cohort3). Of note, AAb to



4 - 7 Novel AAbs from Two Approaches
(A) Visual representative images of sero-reactivity to PTPRN2, MLH1, PPIL2 and NUP50 in one case-control pair.
(B) Jitter plots of sero-reactivity in cohort3.
(C) Heatmap of sero-reactivity in cohort3.

QRFPR, also named G-protein coupled receptor 103, had 20% sensitivity (Table 4-3). In the screening-based approach, AAbs to five antigens (PTPRN2, MLH1, MTIF3, PPIL2 and NUP50) showed >15% sensitivity at 95% specificity cohort3 (Table 4-2).

Among these, PTPRN2/IA-2β is an isoform of a major T1D-associated AAb (PTPRN/IA-2) (Lu et al., 1996). Although it was described a long time ago, the fact that we detected it in our non-biased screening further proved the power of our protein array platform in searching for AAb biomarkers. Representative slide images showed the apparent sero-reactivity to PTPRN2, MLH1, PPIL2 and NUP50 on NAPPA in the case sample but not in the control (Figure 4-7). The antibody response to MTIF3 was not strong on the array which may be due to the lower titer of this antibody in the serum which is consistent with the observation by RAPID ELISA. Notably, PPIL2 was printed on two array sets (GST1 and GST3) and showed up on both sets (data not shown). Jitter plots and heatmap were generated based on the relative absorbance data of RAPID ELISA in cohort3 (Figure 4-7).

The cellular functions of six AAbs were listed in Table 4-4. PTPRN2 and QRFPR were implicated in the biology of T1D (Granata et al., 2014; Lu et al., 1996). MTIF3 gene contains one of the established BMI-associated loci on obesity-related traits in French population (Goumidi et al., 2014).

4 - 4 Cellular Functions of Six AAbs

Gene	Tissue Specificity	Subcellular Location	General Funcion
PTPRN2	Brain and pancreas	Cytoplasmic vesicle	Transmembrane receptor
MLH1	Lymphocytes, spleen, testis, thyroid,	Nucleus	DNA repair
MTIF3	Highest in thymus, pancreas and testis	Mitochondrion	Translation initiation
PPIL2	Highest in thymus, pancreas and testis	Nucleus	Chaperone, ubiquitin ligase activity
NUP50	Ubiquitous.	Nucleus	Nuclear pore complex component
QRFPR	Pancreas	Membrane	Neuopeptide Y receptor activity

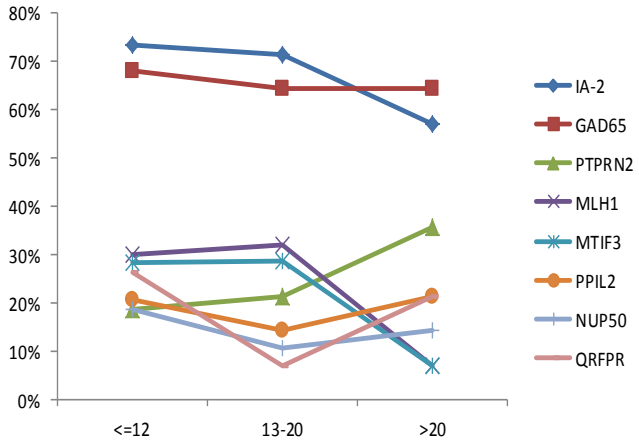
4.4.7 Characteristics of Identified AAbs

The complementary of novel AAbs to the known T1D-associated AAbs was evaluated. The prevalence of novel AAbs ranged from 13.43% to 29.85% of the 29 IA-2A-negative T1D patients. Similarly, the prevalence was 9.38% to 25.00% of the 32 GADA-negative T1D patients (Table 4-5).

4 - 5 Prevalence of Identified AAbs in IA-2A and GADA Subgroups

	IA-2A positive n=67	IA-2A Negative n=29	GADA Positive n=64	GADA Negative n=32
Prevalence of anti-PTPRN2	26.87%(18)	10.34%(3)	21.86(14)	21.86%(7)
Prevalence of anti-MLH1	29.85%(20)	20.69%(6)	28.13%(18)	25.00%(8)
Prevalence of anti-MTIF3	23.88%(16)	27.59%(8)	28.13%(18)	18.75%(6)
Prevalence of anti-PPIL2	17.91%(12)	20.69%(6)	23.44%(15)	9.38%(3)
Prevalence of anti-NUP50	13.43%(9)	20.69%(6)	15.63%(10)	15.63%(5)
Prevalence of anti-QRFPR	19.40%(13)	20.69%(6)	18.75%(16)	9.38%(3)

The prevalence of AAbs among three age subgroups (<=12, 13-20 and >20 years) was investigated in the cases of cohort3. Antibody response to IA-2 and GAD65 remain high (57%-74%) in three age subgroups with slight decrease in individuals older than 20. Sero-reactivity to MLH1 and MTIF3 showed a dramatic decrease in age group (>20 years). The prevalence of AAbs to PTPRN2, PPIL2, NUP50 and QRFPR were increased in >20 age group (Figure 4-8).



4 - 8 Prevalence of Identified AAbs in Age Subgroups

4.5 Discussion

To our knowledge, this is the most comprehensive study that profiled the global humoral response in T1D. We accomplished this by a screening-based approach using NAPPA including 10,000 human proteins and a knowledge-based approach using ELISA to test 126 pancreas enriched genes.

In the screening-based approach, sero-reactivity of the known T1D-associated AAbs was apparently distinguishable on the arrays probed with a T1D patient sample from a healthy control sample. However, the number of antigens that showed positive sero-reactivity was nearly the same between the patient and control groups. Network analysis revealed sero-positive antigens were enriched in pathways like β-cell development, class I MHC mediated antigen processing & presentation, B-cell activation, T-cell receptor signaling, viral infections, TGFβ signaling, metabolism, glucose transport and insulin signaling. Overall, there was no difference in the pathways enriched between cases and controls. One possible explanation is autoimmune destruction in T1D is a global effect rather than targeting specific pathways. The major known T1D-assocaited AAbs were not enriched in the same signaling pathway, either. In healthy controls without T1D, proteins in different pathways were more or less sero-positive by the accumulation of historical humoral responses.

In addition to array-based profiling to search for candidate T1D specific antibodies, we have also performed a comprehensive assessment of antigens overexpressed in pancreas at the gene level. Of the four major known T1D-assocaited AAbs, insulin is highly expressed in pancreatic β-cells that regulate metabolic hemostasis.

GAD65 is expressed primary in the cytosol of neuroendocrine cells that distributed in the islet and neurons. IA-2 is localized at the membrane of insulin secretory granules (Lu et al., 1996). ZnT8 was discovered by analyzing genes that are highly expressed in pancreas (Wenzlau et al., 2007). Besides the protein array based screening approach, we also profiled AAbs to protein candidates that are highly expressed in pancreas. We relied on a RAPID-ELISA platform as an individual assay to assess the performance of individual antigens including antigen candidates from screening-based approach and antigens enriched in pancreas from bioinformatics analysis. Like NAPPA, RAPID-ELISA also uses *in vitro* produced antigens to circumvent the challenges of human antigen production *in vivo*. The results of RAPID ELISA agreed well with liquid-based clinical RIA assay and LIPS assay (Figure 4-5).

Using ELISA, we were able to confirm that 19 AAbs had a performance of >10% sensitivity at 95% specificity. 5 were validated with performance >15% sensitivity at 95% specificity in an independent sample set (Table 4-2). The identification of PTPRN2 as the best performer proves the validity of our discovery approach. PTPRN2 belongs to the same family of PTPRN (IA-2) and has been reported before. IA-2β/PTPRN2 was directly linked with β cell biology (Lu et al., 1996). MLH1, a DNA mismatch repair protein, was frequently mutated or deleted in cancer (Bronner et al., 1994). MTIF3 is a mitochondrial translation initiation factor and NUP50 is a nuclear pore complex protein. They both the have highest expression in thymus, pancreas and testis. MTIF3 was implicated in obesity-related traits in French population (Goumidi et al., 2014). NUP50 and PPIL2 were rarely described in T1D. QRFPR, also known as GPR103, it is a G-

protein-coupled receptor and enriched in the membrane of pancreas islet cells. GPR103 contains seven transmembrane domes and functions as the ligand of RFamide peptides. Binding of RFamide peptides with GPR103 promote the survival of pancreatic β-cells and human pancreatic islets, but different peptides exert opposite effects on insulin secretion (Granata et al., 2014).

In addition, AAbs to a dozen of other antigen proteins showed >10% sensitivity at 95% specificity (Table 4-3). SNX6 is on the membrane of cytoplasmic vesicle and involved in intracellular trafficking. SYTL4 is participated in exocytosis (Jacobsson G, 1994). IGRP/G6PC2 is an enzyme that hydrolyzes glucose-6-phosphate to glucose in the ER (Arden et al., 1999). It was reported as the target of pathogenic $CD8^+$ T cells in T1D (Lieberman et al., 2003). This is the first time of describing it as an AAb target.

Our study clearly demonstrated the success of using a data driven immunoproteomics approach to profile global humoral response in T1D. There are two previous reports on T1D AAbs using protein arrays, including one from our group and another from a group in Korean (Koo et al., 2014; Miersch et al., 2013). We examined three reported AAbs (anti-DYRK2, anti-EEF1A1 and anti-UBE2L3) by RAPID ELISA in cohort3. None of them showed reproducible sensitivity as previous report (data not shown). The inconsistent result may be due to the differences in the study samples. In this study, we used samples from new-onset patients of 4-31 years old with a median age of 11. Cases and controls were pair-matched. In our previous paper, the subjects were older and with longer duration (>6 years) of T1D. In the study published on *Diabetes* Journal (Koo et al., 2014), the average age of T1D patients used in discovery phase is 42 years

old with a standard deviation of 16 years. As T1D is a juvenile autoimmune disease and T1D patients need insulin treatment as soon as diagnosis, we believe samples from subjects with younger age and shorter duration of disease will be better for AAb study.

4.6 Conclusions

We successfully profiled the global humoral response in T1D. A comprehensive search for novel AAbs biomarkers in T1D using a screening-based approach by NAPPA and a knowledge-based approach by ELISA was performed. Six AAbs were identified and validated with sensitivities ranged from 16% to 27% at 95% specificity. Their prevalence in T1D cases complement to the major known T1D-assocaited AAbs and varied in different age subgroups.

4.7 Acknowledgements

I would like to thank the funding support from Juvenile Diabetes Research Foundation (JDRF): 5-2005-117, 17-2007-1045 and 5-2012-537.

CHAPTER 5

5 IMMUNOPROTEOMIC PROFILING OF ANTI-VIRAL ANTIBODIES IN NEW-ONSET TYPE 1 DIABETES USING PROTEIN ARRAYS

5.1 Abstract

The rapid rise in the incidence of type 1 diabetes (T1D) suggests the potential involvement of environmental factors including viral infections. The association between viral infections and T1D was evaluated by profiling anti-viral antibodies using a high-throughput immunoproteomics approach in new-onset T1D patients. A viral protein array was constructed comprising the complete proteomes of seven viruses reported to be associated with T1D and additional open reading frames (ORFs) from other common viruses. Sero-reactivity to 646 viral antigens was assessed in 42 new-onset T1D patients and 42 age-gender matched healthy controls (mean age 12.7 years, 50% males). Prevalence of anti-viral antibodies agreed well with the infection rates of the corresponding virus based on epidemiological studies. Antibody positive rate of Epstein-Barr virus (EBV) was significantly higher in cases than controls (OR 6.6; 95% CI 2.0-25.7) while the other viruses did not differ between the two groups. The EBV and T1D association was significant in both genders and age subgroups (<=12 and >12). These results suggest a potential role of EBV in T1D development. The innovative immunoproteomic platform is useful for understanding the role of viral infections in T1D and other disorders where associations between viral infection and disease are unclear.

5.2 Introduction

T1D is a chronic heterogeneous disease characterized by the progressive autoimmune destruction of pancreatic β cells. The incidence of T1D is rising by an average of 3-5% in recent years that cannot be fully explained by genetic predisposition alone (Onkamo et al., 1999). Moreover, the concordance rate for developing T1D among monozygotic twins is around 40% (Kumar et al., 1993). Hence, it is likely that environmental factors play a significant role during T1D development (Eringsmark Regnell and Lernmark, 2013). Among various environmental factors considered relevant to T1D are those of nutrition and psychosocial factors; yet, viral infections have attracted particular interest (Craig et al., 2013a; Roivainen and Klingel, 2010).

Although there are a number of studies indicating viral effects on T1D pathogenesis, the exact mechanistic explanations for how viruses contribute to T1D etiology are still unknown. Viral infection or presence may act as a longitudinal factor during the process of the induction of single islet antibody, or the simulation from single islet antibody to multiple islet antibodies, or the progression from β cell autoimmunity to clinical onset of T1D (Schneider and von Herrath, 2014a). Several studies reported that the initial development of autoantibodies and progression to multiple autoantibodies occurred at an early age. Subsequently, individuals progress to clinical T1D at different paces during which viral infections may act as an accelerator (Chmiel R, 2015; Parikka et al., 2012). For example, enterovirus infection was shown to increase progression to clinical onset in the Diabetes and Autoimmunity Study in the Young (DAISY) study (Stene et al., 2010). Given the complexity to understand the role of viral infections in

T1D, it is valuable to tackle this important scientific question by assessing sero-reactivity to many viruses/viral antigens in many samples collected longitudinally, from birth to disease onset.

Many viruses have been implicated in T1D in both animal models and humans with varying levels of evidence. Historically, the prevalence of viral infections in T1D was explored by genomic and immunological approaches that only support the study of one viral protein or one type of virus at a time (Banatvala et al., 1985; Gamble et al., 1969). Viral DNA or mRNA was detected by PCR or *in situ* hybridization (ISH) in a relatively low-throughput manner (Foy et al., 1995; Pak et al., 1988). At the protein level, immunohistochemical (IHC) staining and electron microscopy was used to stain and observe viral proteins (Dotta et al., 2007; Ylipaasto et al., 2004). Both ISH and IHC require the use of pancreatic sections from rare pancreatic tissue followed by tedious sample processing procedures. Many serological studies investigated the presence of antibodies to viruses. M-antibody enzyme-linked immunosorbent assay (ELISA) has been a classic way to profile IgM antibodies in T1D patients (Banatvala et al., 1985). The plaque assay, which measures the presence of neutralization antibodies against the whole virus, is another method to profile serological antibodies to specific viral serotypes (Laitinen OH, 2014; Oikarinen et al., 2014). The complement fixation test utilizes complement activation and the lysis of red blood cells to indicate the presence of certain viruses (Gamble et al., 1969). Recent advances in next generation sequencing (NGS) technology have opened new venues for studying the role of viral infection in T1D development (Lee et al., 2013).

Despite these efforts, the understanding of the association between viral infections and T1D development is still not clear. A lack of quantitative and high-throughput technologies has limited the ability to study the role of viral infections in disease comprehensively. Conflicting reports have stemmed from observations based on limited sample sizes (Craig et al., 2013a). Previous studies focusing on single viral protein or single viral species fail to provide a complete picture of infection and antibody responses at the systems level. Protein microarrays provide an ideal tool for multiplexed screening of specific antibodies in sera against thousands of different viral proteins printed on a standard microscope slide.

The aim of this study was to assess the prevalence of anti-viral antibodies to 646 viral proteins from 23 T1D-related and other common viruses in new-onset T1D patients and age-sex matched healthy controls. By examining antibody responses to hundreds of individual viral antigens at the proteome level, the goal is to provide a complete picture of infection at a dimension never achieved before. Antibody positive rates of studied viruses were determined and compared between T1D cases and controls. Specific antibody responses on the array were validated by a confirmatory ELISA. The past onset nature of T1D samples may prevent us from drawing conclusions of whether the virus is pathological in induction of β cell autoimmunity or acceleration of clinical T1D. Nonetheless, this is first comprehensive study of antibody response to a large number of viral species at the individual viral protein level in T1D. The successful application of protein array platform to a large number of samples collected longitudinally before or

after seroconversion will provide a better understanding of viral infection and T1D development.

5.3 Experiments

5.3.1 Serum Samples

All samples were collected with written informed consent with the approval of Institutional Review Boards (IRB) at the University of Florida. Serum samples were obtained from new-onset T1D patients within three months of diagnosis. Control samples were prepared in the identical fashion as T1D patient samples and selected to be age-sex matched. Control samples were tested to be negative for major known T1D autoantibodies (AAbs) (IA-2A, GADA and ZnT8A). Peripheral blood samples were drawn from the antecubital vein and serum was prepared and stored as aliquots at -80^{o}C. All individuals were free of other autoimmune diseases at the time of collection. The sample information is shown in Table 5-1.

5 - 1 Sample Characteristics

Characteristics	New Onset Patients (n=42)	Healthy Controls (n=42)
Age		
Mean$\pm$ SD	12.64$\pm$4.94	12.83$\pm$4.96
Median	11.5	12
Gender		
Male(%)	(21)50%	(21)50%
GADA positive	(33)78.57%	0
IA-2A positive	(18)54.54%	0
ZnT8A positive	(20)47.62%	0

5.3.2 Selection of Viral Strains

Based on literature mining, viruses from seven different genera that have been implicated in T1D, which include Enterovirus (Coxsackievirus B4), Human endogenous retrovirus K (IDDMK1,2-22), Rubivirus (Rubella virus), Rubulavirus (Mumps virus),

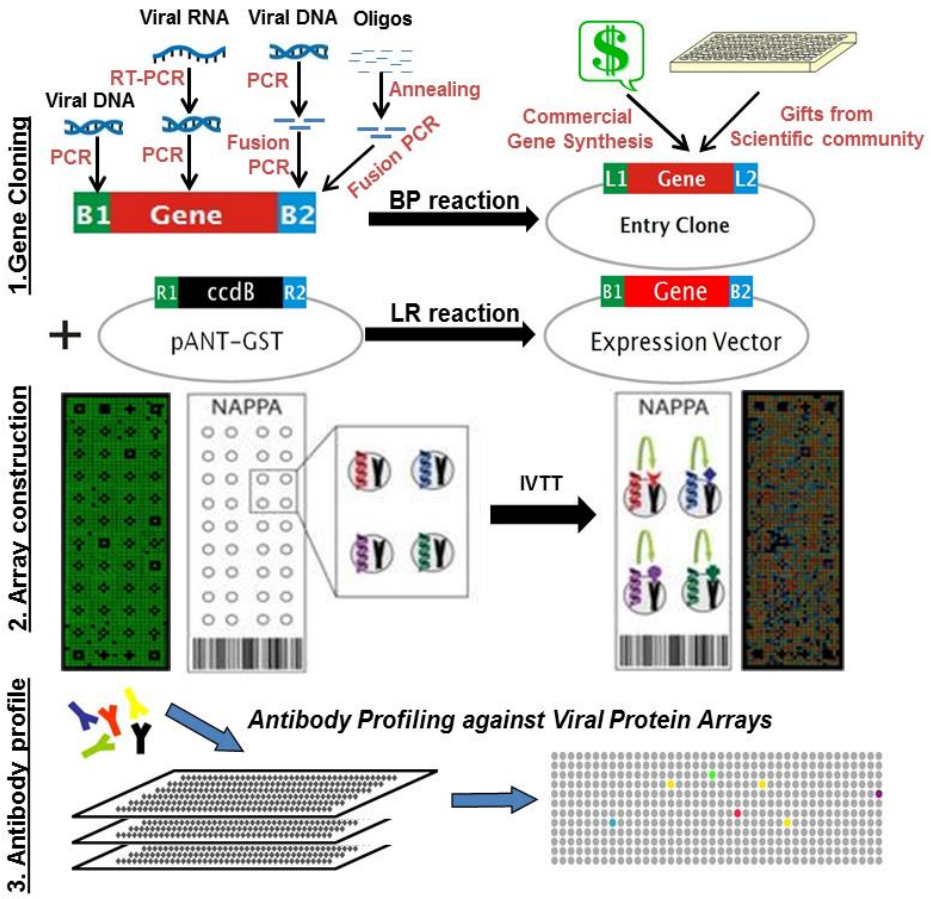
Rotavirus (Rotavirus A), Cytomegalovirus (Human cytomegalovirus) and Lymphocryptovirus (Epstein-Barr virus) were identified. These viruses encompassed a variety of genome types (ssRNA+, ssRNA-, dsRNA and dsDNA). Selection of viral strain from each genus was based on their relevance to T1D as well as the availability of viral genome template (Conrad et al., 1997; Craig et al., 2013a; Forrest et al., 1969; Foy et al., 1995; Gamble et al., 1969; Honeyman et al., 2000; Pak et al., 1988; Yoon et al., 1979). Additional viral genes were from other common viruses to further enrich in the collection (Table 5-2).

5 - 2 Characteristics of Viruses

Virus Species	Abbre.	Family	Genome	ORF clones	% of complete ORFeome
*Human Cytomegalovirus	HCMV/HHV-5	*Herpesvirinae*	dsDNA	164	100%
*Esptein-Barr virus	EBV/HHV-4	*Herpesvirinae*	dsDNA	85	100%
*Coxsackievirus B	CVB	*Picornaviridae*	ssRNA+	12	100%
*Rubella virus	RUBA	*Togaviridae*	ssRNA+	6	100%
*Mumps virus	MuV	*Paramyxoviridae*	ssRNA-	9	100%
*Human endogenous retrovirus K	HERK	*Retroviridea*	ssRNA-	4	100%
*Rotaviruses	RV	*Reoviridae*	dsRNA	12	100%
Hepatitis B virus	HBV	*Herpesviridae*	dsDNA	10	100%
Human Papillomavirus16	HPV16	*Papillomaviridae*	dsDNA	8	100%
Human Papillomavirus18	HPV18	*Papillomaviridae*	dsDNA	8	100%
Chikungunya virus	CHIKV	*Togaviridae*	ssRNA+	9	100%
Semliki Forest virus	SFV	*Togaviridae*	ssRNA+	9	100%
Sindbis virus	SINV	*Togaviridae*	ssRNA+	9	100%
Influenza A virus (H1N1)	n/a	*Orthomyxoviridae*	ssRNA-	10	100%
Influenza A virus (H3N2)	n/a	*Orthomyxoviridae*	ssRNA-	10	100%
Varicella-zoster virus	VZV	*Herpeviridae*	dsDNA	68	93.10%
Simian virus 40	SV40	*Polyomaviridae*	dsDNA	6	85.71%
Vaccinia virus	VACV	*Poxviridae*	dsDNA	167	74.89%
Yellow fever virus	YF	*Flaviviridae*	ssRNA+	11	71.42%
Measles virus, vaccine strain	MeV,vaccine	*Paramyxoviridae*	ssRNA-	5	62.50%
Measles virus, WT strain	MeV,WT	*Paramyxoviridae*	ssRNA-	5	62.50%
Adenovirus	n/a	*Adenoviridae*	dsDNA	16	42.10%
Tioman virus	n/a	*Paramyxoviridae*	ssRNA-	3	37.50%

5.3.3 Viral Genes Cloning

All viral genes were first cloned into the pDONR221 Gateway compatible donor vector (Life technologies, CA). Different approaches were used based on the available resources (viral genome type, gene characteristics, et al). Viral genes were directly amplified by PCR if the viral DNA or cDNA templates were available. Viruses of ssRNA+ genome type, such as coxsackievirus, were first reverse-transcribed into cDNA before PCR amplification. Some viral genes are not continuous across the viral genome, for example, UL89 from human cytomegalovirus (HCMV). Each gene fragment was amplified separately and then fused together by PCR elongation to obtain full-length viral genes. Genes without available templates, such as the superantigen gene from the IDDMK1,2-22 strain, were produced using de novo gene synthesis. Many Gateway compatible viral entry clones were obtained from the scientific community (Figure 5-1). Viral genes in the pDONR221 vector were transferred to a T7 promoter based *in vitro* expression vector pANT7_cGST by LR reaction (Festa et al., 2013). All viral genes cloned in this vector have a C-terminal glutathione S-transferase (GST) fusion tag in frame with the protein. The sequence verified clones were stored in the plasmid repository (http://dnasu.org/DNASU/) (Seiler et al., 2014).



5 - 1 Study Design

1. Gene cloning: various approaches were used to capture viral genes into protein array compatible expression vector.

2. Array construction: protein arrays were constructed as previous described and quality assured.

3. Antibody profiling: antibodies to viral proteins were profiled between T1D case and healthy control group.

5.3.4 Nucleic Acid Programmable Protein Array (NAPPA) Production

Using the standard protocol (Miersch et al., 2013; Miersch and LaBaer, 2011), 1200 ng/μL plasmid DNA was co-printed with rabbit anti-GST polycolonal antibody (GE Healthcare, PA), BSA (bovine serum albumin) and bissulfosuccinimidyl suberate (BS^3) protein cross-linker (Thermo Fisher Scientific, MA) as printing mix using Genetix QArrayer (Genetix, UK). Each viral gene was printed in duplicate on each slide. Human immunoglobulin (Ig)G, IgA and IgM were printed to show successful detection by secondary antibodies. Plasmid encoding a known T1D AAb (IA-2 protein) was printed as a positive control for T1D samples. Empty spots, spots printed with printing buffer without plasmid DNA, spots printed with printing buffer with plasmid encoding a hemagglutinin (HA)-fusion protein and anti-GST capture antibody were negative controls. HA-fusion protein can be expressed but not captured by anti-GST capture antibody to serve as negative control for non-specific capturing. NAPPA has been successfully applied in AAb biomarker discovery and protein post-translational modifications studies (Takulapalli et al., 2012; Yu et al., 2014c).

5.3.5 Array Quality Assessment

As previously described (Qiu and LaBaer, 2011; Ramachandran et al., 2008), plasmid DNA on the array was stained by Quant-iT™ PicoGreen® dsDNA Assay Kit (Life technologies, CA). For protein display, every four slides were blocked in 30 mL SuperBlock Blocking Buffer (Thermo Fisher Scientific, MA) at room temperature (RT) for 1 hour (hr) on the shaker. Then slides were rinsed five times with deionized (DI) water and placed in a metal slide rack (Amazon, WA) for drying by centrifugation at

1000 rpm for 3 min at RT. 160 µL human HeLa cell lysate-based protein expression system (Thermo Fisher scientific, MA) was injected into HybriWell (Grace BIO-LABS, OR) sealed slides and incubated in the oven (EchoTherm, CA) at 30°C for 1.5 hrs for protein expression and 15°C for 30 min for protein capture. Viral proteins were produced with a C-terminal GST fusion tag and captured in situ by co-printed anti-GST capture antibody on the array. The use of a tag at the C-terminus ensures that captured proteins have been fully translated. Each slide was incubated with 5% milk-PBST (0.2% Tween-20), 3 mL 1:200 diluted anti-GST monoclonal antibody (Cell Signaling Technology, MA) and 3 mL1:500 diluted horseradish peroxidase (HRP) labeled goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, PA) for 1 hr at RT on the shaker, respectively. Slides were washed three times with 5% milk-PBST, 5 min each time in between. Finally, slides were washed with PBST and rinsed with DI water. Each slide was incubated with 500 µL 1:50 diluted tyramide signal amplification (TSA) buffer (PerkinElmer, MA) to generate fluorescent signals. Then slides were washed, dried and scanned by Tecan scanner (Männedorf, Switzerland).

5.3.6 Anti-viral Antibody Profiling

Anti-viral antibodies were profiled on the HS 4800™ Pro hybridization station (Tecan, Männedorf, Switzerland). Proteins were expressed as described. Expressed slides were placed in the hybridization chambers and programmed with 1 hr of blocking with 5% milk-PBST, 16 hrs of incubation with 160 µL 1:50 diluted serum at 4°C followed by 1 hr of detection with 160 µL 1:500 diluted Alexa Fluor 647® Goat Anti-Human IgG or 160 µL 1:300 diluted Alexa Fluor 647® Goat Anti-Human IgA and DyLight™ 549-

conjugated Goat Anti-Human IgM (Jackson ImmunoResearch Laboratories, PA) at RT. Slides were washed, dried and scanned by Tecan scanner (Männedorf, Switzerland). A pooled sample was prepared by mixing equal volumes of individual samples. The pooled sample was run on every serum screening day to show day-to-day technical reproducibility.

Strong antibody response resulted in saturated signals of the local spot with diffusion to the neighboring spots. Serum dilution was determined by the optimal sensitivity with minimum diffusion. The presence of diffusion was defined as ring. To determine the signal intensities for the rings, the median intensity in between the ring spot and the neighboring spot was quantified as the raw ring intensity. The median intensity in between the ring spot and the second closest spot (excluding the spot area) was quantified as the background ring intensity. The net ring intensity was calculated by subtracting the background ring intensity from the raw ring intensity. The data was only extracted for the spots with rings as ring positive net intensity data. The maximum of the ring positive net intensity for duplicates of each viral antigen was used in quantitative analysis.

5.3.7 RAPID (Rapid Antigenic Protein *In situ* Display) ELISA

96-well ELISA plates (Corning Life Sciences, CA) were coated with 50 µL 10 ng/mL anti-GST antibody (GE Healthcare, PA) in coating buffer (0.5 M carbonate bicarbonate buffer, pH 9.6) overnight at 4°C. On the next day, coated plates were washed five times with 100 µL PBST and blocked with 100 µL 5% milk-PBST for 1.5 hrs. Meanwhile, 40 ng/µL viral protein encoding plasmid was expressed in the human HeLa

cell lysate-based protein expression system at 30°C for 1.5 hrs in the oven. Viral antigen was diluted in milk-PBST at 1:200. 50 µL diluted antigen was captured in each well at RT for 1 hr on a shaker at 500 rpm. Plates were washed with five times of PBST. Each well was incubated with 50 µL 1:1000 diluted serum at RT for 1 hr, washed again and incubated with 50 µL 1:10,000 diluted HRP labeled anti-human secondary antibody (Jackson ImmunoResearch Laboratories, PA) at RT for 1 hr. Plates were incubated on a shaker at 500 rpm. Finally, the plates were washed and incubated with 50 µL 1-Step Ultra TMB - ELISA Substrate for 10 min (Thermo Fisher scientific, MA) for detection and 50 µL 2M sulfuric acid to stop the reaction. OD_{450} was measured by Envision® Multilabel Reader (PerkinElmer, MA).

5.3.8 Statistical Analysis

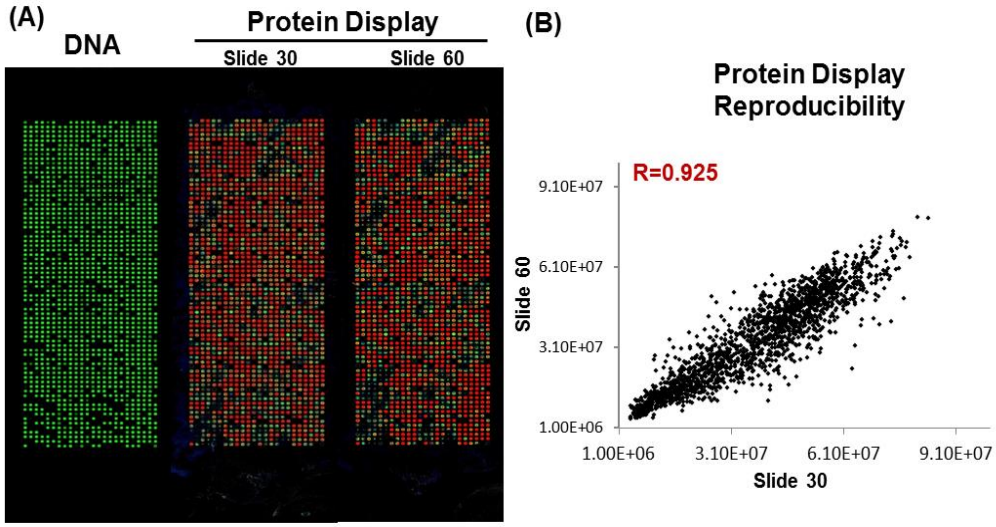
Sample information is presented as proportions, medians and ranges. Positive antibody response to viral antigen was defined by the presence of a ring in at least one of the replicates of that protein. Positive antibody response to viruses in each individual was defined by the positivity of antibody response to at least one viral antigen from the entire viral proteome. Odds ratios (ORs) and exact 95% confidence intervals (CIs) were calculated for viruses and T1D association using conditional likelihood estimation and Fisher's exact test (Breslow and Day, 1980). Exact Wilcoxon rank sum tests were applied to the maximum of the ring positive net intensity data to determine the significance of antibodies to individual viral proteins between cases and controls. The Benjamini-Hochberg procedure was used to adjust p-values to account for multiple testing. These tests were performed using the coin package in R 3.0.3. Exploratory subgroup analyses of

the association between sero-positivity to viral proteins and three known T1D AAbs (GADA, IA-2A or ZnT8A), gender and age subgroups (<=12 and >12) were performed. P-value<0.05 were considered significant. Heatmaps were generated in MultiExperiment Viewer version 4.9 (http://www.tm4.org/mev.html). Graphs and plots were generated in GraphPad Prism 6.

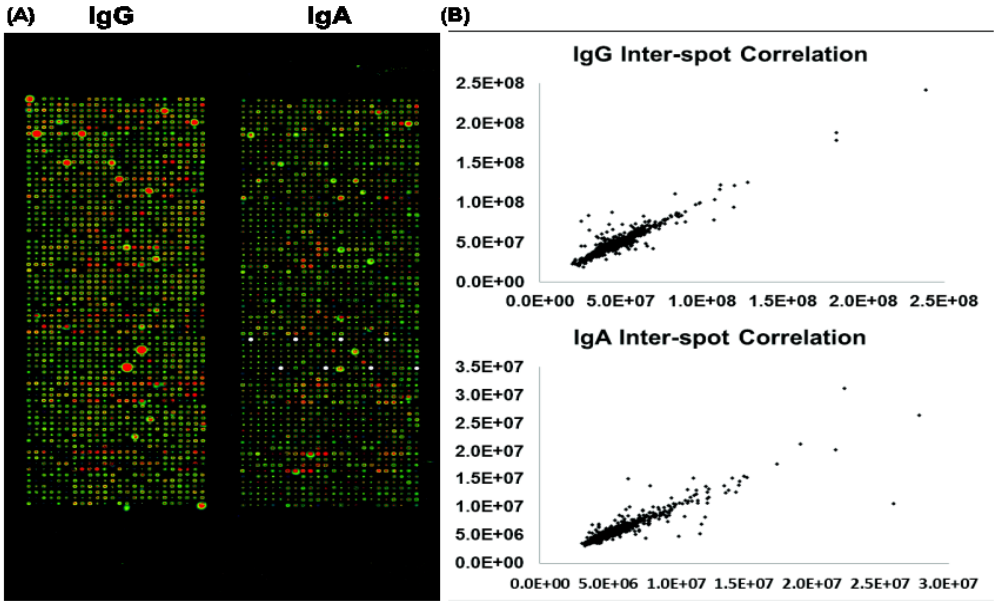
5.4 Results

5.4.1 Viral Protein Array Production and Quality Assessment

A high-throughput pipeline (Figure 5-1) was established and cloned 292 ORFs from seven viruses reported to be associated with T1D and 354 ORFs from other common viruses into the protein array compatible expression vector (Table 5-2). Among these, 15 out of 23 viral strains had 100% ORFs from the viral genome. The high coverage of viral ORFs and the diversity of viral strains would help us to fulfill the goal of a systematic survey of anti-viral antibodies in new-onset T1D patients. After printing, plasmids on the array were stained by PicoGreen that showed uniform DNA quality across the slide (Figure 5-2). The fluorescent signals of protein display were pseudo-



5 - 2 Quality of DNA and Protein Display
(A) Visual representative images of DNA and protein display, green indicates pico-green staining of DNA printed on the array, red indicates the antibody detection of protein display on the array; (B) Scatter plot of the reproducibility of protein display between two slides.



5 - 3 Inter-spot Correlation of IgG and IgA Profiling
(A) Representative images of IgG or IgA class antibody response on NAPPA probed by the pool sample;
(B) Scatter plots of inter-spot correlation of IgG or IgA class antibody response.

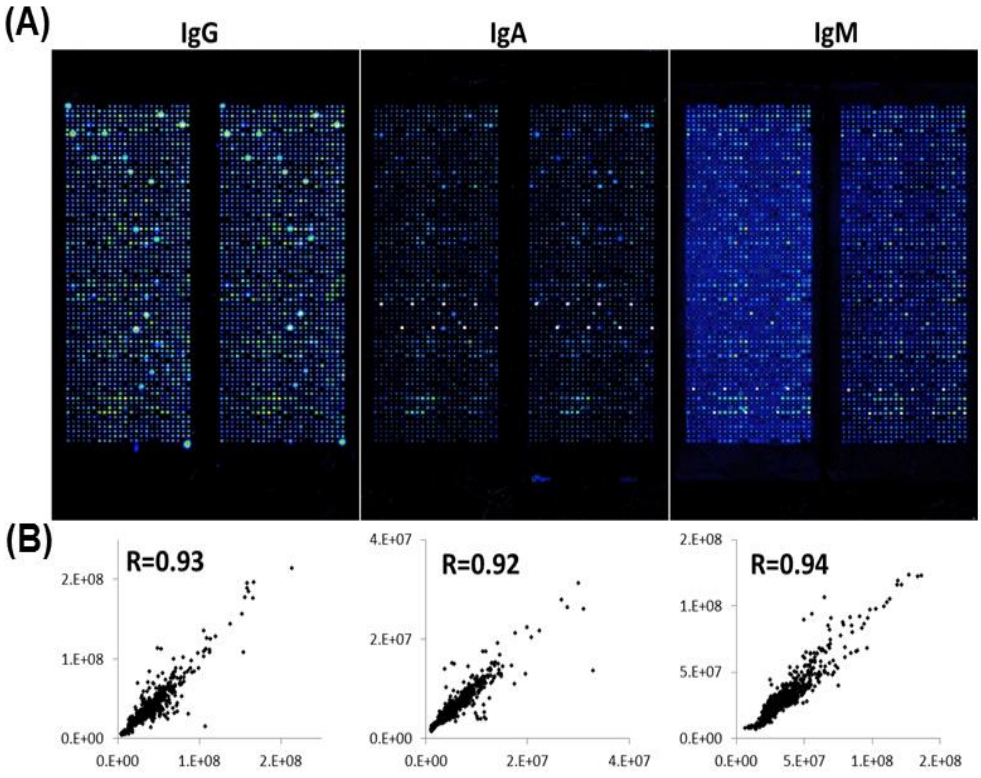
colored with a rainbow color scheme. The correlation coefficient of signal intensities of protein display as detected by the anti-GST tag antibody between two slides was above 0.92 (Figure 5-2).

5.4.2 Profiling of Anti-viral Antibodies

Quality assured slides were challenged with serum samples from 42 new-onset T1D patients and 42 age-gender matched healthy controls, with median ages of 11.5 and 12, respectively. IgG was profiled on a set of 84 slides, IgA and IgM were profiled simultaneously on another slide set. Arrays were probed with the pooled sample to assess intra and inter-array reproducibility. The inter-spot correlation coefficients for IgG and

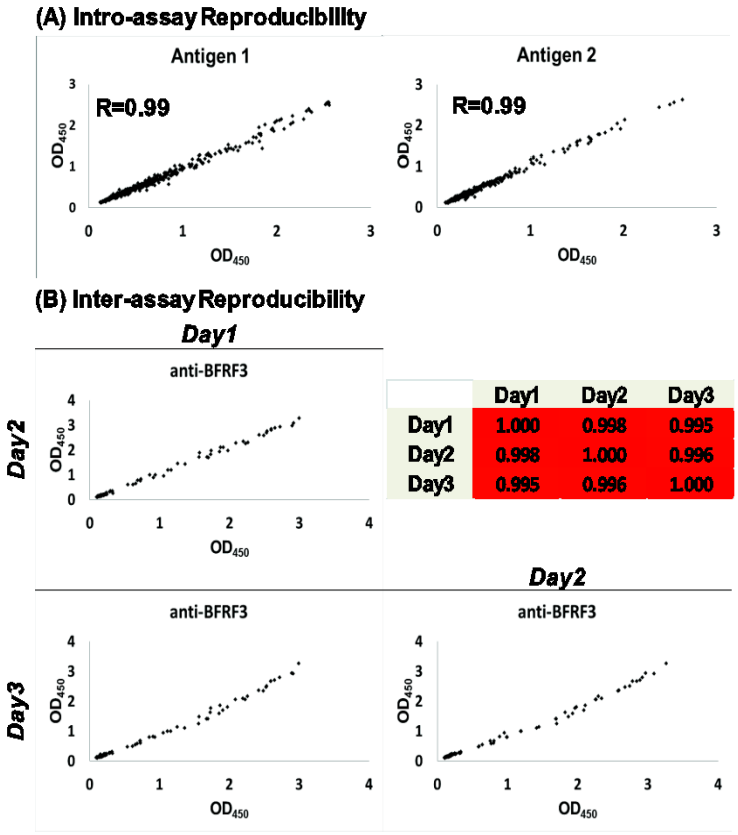
IgA profiling of duplicate spots on the same array were 0.94 and 0.91, respectively (Figure 5-3).

The inter-array correlation coefficients of IgG, IgA and IgM profiling of duplicate arrays on different days ranged from 0.92 to 0.94 (Figure 5-4). The high inter-spot precision on one slide and day-to-day reproducibility on different slides demonstrated the robustness of the serum screening practice and good control of assay quality.



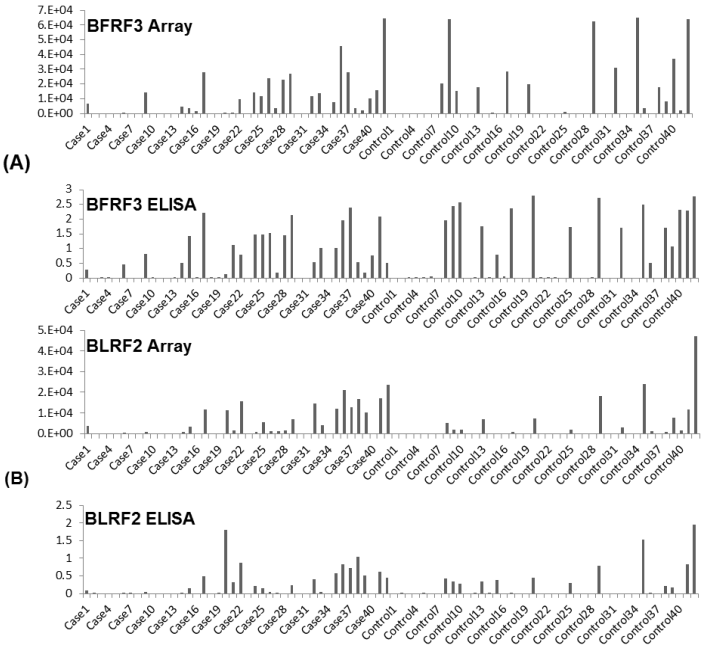
5 - 4 Reproducibility of Anti-viral Antibody Profiling
(A) Representative images of IgG, IgA and IgM sero-reactivity of a pooled sample on two slides from two serum screening days.
(B) Scatter plot and correlation coefficients of IgG, IgA and IgM sero-reactivity of a pooled sample on two slides from two serum screening days.

RAPID-ELISA was used to confirm the sero-reactivity obtained on the viral protein arrays. RAPID ELISA is an established in-house developed immunoassay. Like NAPPA and several well-established clinical assays for T1D autoantibodies, it relies on a cell free system to produce antigens. The same viral antigen-encoding plasmids used on the array can be directly applied in RAPID ELISA. The intra-assay and inter-assay reproducibility is typically 0.99 (Figure 5-5).



5 - 5 Reproducibility of RAPID ELISA
(A) Intra-assay reproducibility presented by antibody profiling against two antigens on duplicated wells on the same day.
(B) Inter-assay reproducibility presented by scatter plots of day-to-day reproducibility of anti-BFRF3 (EBV) profiling on three different days, their correlation coefficients were listed in table.

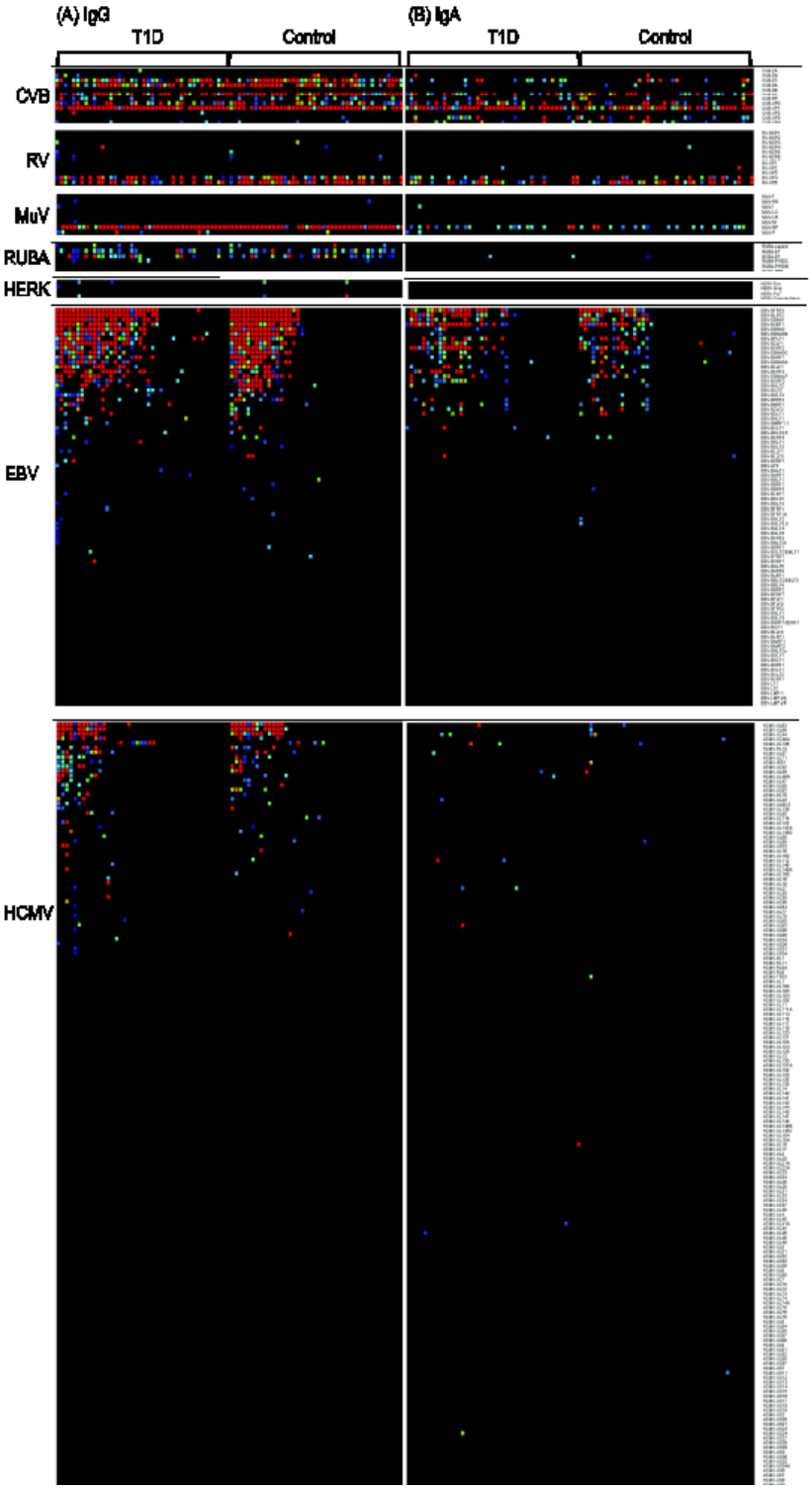
Because of the ease in developing a RAPID ELISA to any antigen in the collection, this allowed us to confirm the performance of a smaller number of candidate antigens with many samples reproducibly, quickly and affordably. RAPID ELISA data was consistent with clinical radioimmunoprecipitation assay (RIA) and the luciferase immunoprecipitation systems (LIPS) assay data (data not shown). Given its easy adaptability, high reproducibility and comparability to established clinical assays, it was chosen as a confirmatory platform to validate the signals on the array. The ring positive net intensities of two EBV antigens, BFRF3 and BLRF2, obtained on the viral protein arrays agreed well with ELISA, further proving the validity of this innovative array platform in profiling anti-viral antibodies (Figure 5-6).



5 - 6 Concordance of Antibody Response to Viral Proteins
(A) BFRF3 and (B) BLRF2 from Epstein-Barr virus (EBV).

5.4.3 Differential Anti-viral Antibody Response on Arrays

A protein array approach enables the assessment of different sero-reactivity patterns for all the proteins from the same virus. Heatmaps of IgG and IgA sero-reactivity to viral proteins from the seven viruses previous associated T1D were generated using the ring positive net intensities (Figure 5-7). IgG antibody response to VP1 from coxsackievirus B (CVB), nucleoprotein from mumps virus (MuV) and influenza A virus were detected in almost every individual in both case and control group, which suggests they are the immunodominant antigens upon viral infection. IgA sero-reactivity was generally a subset of antigens that also showed IgG sero-reactivity and weaker than IgG sero-rectivity for the same viral protein, however, VP3 and VP4 from CVB were both more reactive for IgA response than IgG. Although the secondary antibody can clearly detected control IgA and IgM spots simultaneously on our arrays, no positive serological IgM reactivity was observed. This may be due to the lack of acute infection when the samples were drawn and low titer of IgM concentration in the serum.



5 - 7 Heatmaps of IgG and IgA Sero-reactivity

Sero-reactivity to viral proteins from the seven viruses previous associated T1D.

5.4.4 Higher Frequency of Antibody Response to EBV in T1D Patients

The apparent antibody positive rate of each virus between the case and the control groups was compared. As a sensitive criterion, a positive antibody response to a virus in each subject was defined by a positive antibody response to at least one viral antigen from the viral proteome. By this criterion, the prevalence of antibody positive rate obtained on the array is shown in Table 5-3.

5 - 3 Frequencies of Antibody Responses to Studied Viruses

Virus	T1D (n)	T1D (%)	HC(n)	HC(%)	Odds Ratio	95% CI	p-Value
*HCMV	24	57.1%	23	54.8%	1.1	0.4 to 2.8	1.000
*EBV	37	88.1%	22	52.4%	6.6	2.0 to 25.7	*0.018
*CVB	42	100.0%	42	100.0%	0.0	0.0 to Inf	1.000
*RUBA	22	52.4%	25	59.5%	0.8	0.3 to 2.0	1.000
*MuV	41	97.6%	40	95.2%	2.0	0.1 to 123.8	1.000
*HERK	3	7.1%	2	4.8%	1.5	0.2 to 19.3	1.000
*RV	27	64.3%	32	76.2%	0.6	0.2 to 1.6	1.000
HBV	1	2.4%	0	0.0%	Inf	0.0 to Inf	1.000
HPV16	2	4.8%	1	2.4%	2.0	0.1 to 123.8	1.000
HPV18	4	9.5%	0	0.0%	Inf	0.7 to Inf	1.000
CHIKV	1	2.4%	0	0.0%	Inf	0.0 to Inf	1.000
SFV	1	2.4%	0	0.0%	Inf	0.0 to Inf	1.000
SINV	0	0.0%	0	0.0%	0.0	0.0 to Inf	1.000
Influenza A virus(H1N1)	41	97.6%	42	100.0%	0.0	0.0 to 39.0	1.000
Influenza A virus(H3N2)	37	88.1%	40	95.2%	0.4	0.0 to 2.6	1.000
VZV	37	73.8%	27	64.3%	1.6	0.6 to 4.5	1.000
SV40	1	2.4%	0	0.0%	Inf	0.0 to Inf	1.000
VACV	1	2.4%	2	4.8%	0.5	0.0 to 9.8	1.000
YF	0	0.0%	0	0.0%	0.0	0.0 to Inf	1.000
MeV,vaccine strain	14	33.3%	14	33.3%	1.0	0.4 to 2.7	1.000
MeV,WT strain	31	73.8%	35	83.3%	0.6	0.2 to 1.8	1.000
Adenovirus	34	81.0%	36	85.7%	0.7	0.2 to 2.6	1.000
Tioman virus	0	0.0%	0	0.0%	0.0	0.0 to Inf	1.000

It agreed well with the background rates of infection determined by epidemiology studies where the sera were sampled. For example, the prevalence of HPV infection was

low while the frequencies of influenza A viruses were high in the study subjects. Notably, the antibody positive rate of EBV was more frequent in new-onset T1D cases than in healthy controls (88% VS 52%; OR 6.6, 95% CI 2.0-25.7; p-value=0.018) while none of the other viruses showed difference between the two groups (Table 5-3). Sero-positivity against EBV was higher in cases than controls among both genders and age subgroups (>=12 and <12) (Table 5-4).

5 - 4 Sero-positivity of EBV in Gender and Age Subgroups

Subgroup	T1D	HC	Odds Ratio	95% CI	p-Value
Female (n=21)	85.71%	57.14%	4.500	1.01 to 20.11	0.049
Male (n=21)	90.48%	47.62%	10.450	1.93 to 56.64	0.007
Age(<=12) (n=24)	83.33%	50.00%	5.000	1.61 to 19.07	0.019
Age(>12) (n=18)	94.44%	55.56%	13.600	1.48 to 125.32	0.021

Many immunodominant EBV proteins were detected based on antibody-mediated immunity such as BFRF3 and BLRF2 (viral capsid antigen/VCA), BZLF1 (early antigen/EA) and EBNA1 (nuclear antigen). On average, each case has antibody response to 8 EBV proteins while each control has antibody response to 6 EBV proteins.

The association of antibody response to individual viral protein and subgroups of T1D patients was assessed. IgG antibody response to IA-2 was statistically significantly higher in T1D patients (p=0.007) confirming the detection of known T1D autoantigens on the protein array platform. However, of all the 646 viral proteins on the array, none of them exhibited statistically significant differences between cases and controls. Next, the association of antibodies against all viral proteins with the sero-positivity to three known T1D autoantigens (IA-2, GAD65 and ZnT8) was analyzed. No proteins exhibited

statistically significant associations with any of the three known T1D autoantigens or gender. Only one protein, IgG antibody response to the NP protein from influenza A virus (H1N1) was significantly ($p=0.007$) higher in age (>12) than age (<=12) subgroup of T1D patients (data not shown).

5.5 Discussion

This is the first study that examined anti-viral antibodies to individual viral proteins at the proteomic level in new-onset T1D patients. It was accomplished by using an innovative and flexible protein array platform, resulting in a study with several unique strengths suggesting higher a frequency of EBV-specific immune response in T1D.

By examining antibody responses to hundreds of individual viral antigens, the role of viral infections in T1D development was analyzed at a dimension never achieved before. Previously, publications focused on the detection of antibodies to whole viruses or a limited number of viral proteins (Banatvala et al., 1985; Gamble et al., 1969). Such studies might underrepresent the prevalence of viral infections because some patients may not produce antibodies that respond to those antigens. Even some commercial ELISA kits cannot achieve 100% sensitivity in detecting viral infections (Tramper-Stranders et al., 2006). The examination of all proteins for each virus affords the opportunity to look for associations not only with viral infection but with immune responses to specific viral antigens. By focusing on the host immune response, the missing events due to the transient nature of detecting viral DNA/RNA was avoided, which may only be present briefly during infection (Lee et al., 2013).

High-throughput and low sample consumption are great advantages of the array platform over conventional one antigen-at-a-time immunoassays. Using this platform, only 3.2 µL sera were needed to probe thousands of antigens on the slide. On average, each antigen consumes as little as 0.002 µl of serum sample, which is significantly less

than individual assay such as enzyme immuno assay (EIA) or RIA that consumes a few microliters per antigen per assay.

This protein array platform also offers great opportunity for multiplexed detection of different immunoglobulin classes and flexibility to include any genes of interest into the study design. Typically, IgG is considered the most common and durable response, providing information of historical infections, IgA indicates the mucosal immunity and IgM correlates with acute infection. On the other hand, the emerging NGS techniques provide powerful opportunities to uncover new viral/microbiome strains that associated with T1D (Lee et al., 2013; Mejia-Leon et al., 2014). Any proteins with known gene sequences can be included in the innovative array platform to assess their sero-reactivity. Therefore, it is straightforward to incorporate metagenomics findings of T1D-relevant viruses/pathogens into the design of the viral/pathogen protein arrays to study their sero-reactivity. This combination of antibody immunoprofiles and viral metagenomics in T1D will lead to a better understanding of viral infections in T1D.

The prevalence of subjects with positive antibody response to the studied viruses (Table 5-3) agreed well with the infection rate of the corresponding virus from epidemiological studies in the United States (Table 5-5) (Balfour et al., 2013; Butel, 2012; Dunne et al., 2007; Eisenhut et al., 1999; Nwanegbo et al., 2004; Staras et al., 2006; Vogetseder et al., 1993; Weinbaum et al., 2008), proving the validity of the array platform in detecting historical viral infections. In addition to infection, antibody responses to viruses may also depend on the vaccine history, vaccine efficacy, antibody

specificity and detection methods (JD Cherry, 2013; Latner et al., 2014). Unfortunately, the vaccination history for these de-identified samples was not available.

5 - 5 Infection Rate and Vaccination Information of Studied Viruses

Virus	Infection Rate	Vaccine
HCMV	36.3% - 58.9% (US)	NA
EBV	50 - 89% (US)	NA
CVB	52,812 detections between 1970-2005	NA
RUBA	no longer endemic (US)	Yes
MuV	no big outbreaks (US)	Yes
HERK	0% (US)	NA
RV	3 million cases per year (US)	NA
HBV	804,000 - 1.4 million (US)	Yes
HPV16	1% (US)	Yes
HPV18	0.125% (US)	Yes
CHIKV	0% (US)	NA
SFV	0% (US)	NA
SINV	0.1% (German)	NA
Influenza virus	4 - 5600 cases per week (US)	Yes
VZV	1 million per year (US)	Yes
SV40	0% - 4.2% (US)	NA
VACV	0% (US)	NA
YF	0% (US)	NA
MeV	50 - 350 cases per year (US)	Yes
Adenovirus	37% (US)	NA
Tioman virus	0% (US)	NA

These results clearly demonstrate the importance of taking a systems approach to assess the relationship between infection and chronic disease. A significant association between antibody response to EBV and new-onset T1D patients (88% VS 52%; OR 6.6, 95% CI 2.0-25.7; p-value=0.018) was found. Unfortunately, none of the EBV proteins showed a statistical difference between cases and controls which further emphasized the importance to take a systematic view to examine every individual protein from EBV.

Similarly, immunoassays based on the whole viral particles only detect responses against viral capsid antigens. Of note, the EBV and T1D association is not depended on gender or age subgroups (Table 5-4).

Connections between EBV and T1D date back to 1974 (Burgess et al., 1974a). Earlier studies focused on detecting low-grade temporary viral infections using PCR or serological antibody in a small sample size (Elliott RB, 1995b; Foy et al., 1994). It was reported EBV infection was not associated with islet cell and insulin AAb seroconversion (Elliott RB, 1995b). Antibody levels to VCA and EA was profiled by a commercial ELISA kit and found lower VCA IgG class antibody levels in T1D patients (Hyoty et al., 1991). This apparent difference in response to EBV may arise from several potential reasons. One possibility may be differences in the methods for determining viral infections. This study profiled antibodies to every individual EBV protein, whereas this earlier study focused on antibody responses to several EBV proteins. If only one protein or several proteins from VCA or EA from the data was considered, none of them showed significant higher prevalence in cases than controls (Table 5-6). There were also differences in sample source: the sample used in this study was obtained in US compared to Finland for the previous study. Viral infections are both seasonal and regional. Differences of viral infections in the general population may be different based on sample locations.

5 - 6 Antibody Responses to VCA and EA Antigens of EBV

Gene Symbol	Antigen Class	Case	Control	Odds ratio	95% CI	p value
BFRF3	VCA	0.62	0.40	2.3897	1.0 o 2.7	0.0513
BLRF2	VCA	0.57	0.38	2.1667	0.9 to 51.2	0.0824
BXLF1	EA	0.05	0.05	1	0.1 to 7.5	1
BGLF5	EA	0.02	0.00	3.0723	0.1 to 77.6	0.4957
BMRF1	EA	0.00	0.00	1	0.0 to 51.6	1
BZLF1	EA	0.45	0.29	2.0652	0.8 to 5.1	0.1159

The mechanism through which EBV might contribute to the pathogenesis of T1D remains uncertain. However, several possible scenarios can be envisioned. First, EBV may be spread from circulating infected B cells to pancreatic tissue, resulting in local antiviral immune responses. Second, EBV infection induces the release of cytokines, which promotes the maturation of immune cells to enhance their cytotoxicity (Hornef et al., 1995; Williams et al., 2005). Third, EBV infection may trigger a cross reactive autoimmune response through molecular mimicry of viral antigens and host proteins (Parkkonen et al., 1994b). These hypotheses need further evaluation. Recent evidence showed viral infection may play a role in accelerating the progression from β cell autoimmunity to clinical T1D (Stene et al., 2010). The antibody responses detected in this work indicated past infections. As the samples used in this study were from post-onset T1D patients, further investigation of longitudinal samples is needed to determine whether infection is important during seroconversion or progression to clinical diagnosis of T1D. The successful application of the array platform in detecting antibodies to thousands of viral antigens provided a better tool to investigate this problem.

Historically, coxsackievirus is the most frequently cited T1D-related virus. The E2 strain of CVB was selected in this study because it was first isolated from the pancreas of a child with diabetic ketoacidosis (Yoon et al., 1979). However, no difference in the apparent history of infection (OR 1, p-value=1.000) was found, which is consistent with the recent work by Hyöty H et al. using a neutralizing antibody assay to investigate 41 different enterovirus serotypes (Oikarinen et al., 2014). The other five viruses previously reported to be related with T1D did not show prevalence difference between cases and controls.

Despite its virtues, the present study has some limitations. This study samples were new-onset T1D patients with relative small sample size and not matched for HLA genotypes. Viral antibodies from new-onset patients may provide limited information for the involvement of these viral infections/antibodies during the progression from initiation of autoimmunity to the onset of T1D symptoms. The relatively small sample size also prevented us from drawing strong statistical conclusions. Furthermore, the HLA genotype is important in modulating the immune responses during viral infection. In addition, only one of the most relevant viral strains based on previous publications was investigated in this study. The infection rate between viral serotypes varies and specific serotypes may have different roles in association with T1D (Laitinen OH, 2014; Oikarinen et al., 2014).

5.6 Conclusions

In summary, the use of viral protein array platform in profiling anti-viral antibodies was successfully demonstrated. A preliminary study was completed and found a potential link between EBV infection and T1D development. The success of this work established the utility of a flexible high-throughput multiplexed platform to profile a large number of longitudinal samples including time points surrounding "seroconversion" and clinical diagnosis. A comprehensive understanding of antibody responses to thousands of viral antigens in these longitudinal samples and samples from HLA-matched healthy controls will greatly improve our knowledge of the role of viral infection in T1D development.

5.7 Acknowledgements

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CHAPTER 6

6 ANTI-VIRAL ANTIBODY PROFILING BY HIGH DENSITY PROTEIN ARRAYS

6.1 Abstract

Viral infections elicit anti-viral antibodies and have been associated with various chronic diseases. Detection of these antibodies can facilitate diagnosis, treatment of infection and understanding of the mechanisms of virus associated diseases. In this work, anti-viral antibodies were assayed using a novel high density-nucleic acid programmable protein array (HD-NAPPA) platform. Individual viral proteins were expressed *in situ* directly from plasmids encoding proteins in an array of microscopic reaction chambers. Quality of protein display and serum response was assured by comparing intra- and inter-array correlation within or between printing batches with average correlation coefficients of 0.91 and 0.96, respectively. HD-NAPPA showed higher signal to background (S/B) ratio compared with standard NAPPA on planar glass slides and ELISA. Antibody responses to 761 antigens from 25 different viruses were profiled among patients with juvenile idiopathic arthritis (JIA) and type 1 diabetes (T1D). Common as well as unique antibody reactivity patterns were detected between patients and healthy controls. HD-viral-NAPPA will enable the study of host-pathogen interactions at unprecedented dimensions and elucidate the role of pathogen infections in disease development

6.2 Introduction

Viral infections not only elicit acute symptoms but have been implicated in a variety of chronic illnesses including autoimmune diseases and cancers (Fujinami et al., 2006; Moore and Chang, 2010). One direct consequence of viral infections is the elicitation of antibodies against viral proteins (Odumade et al., 2011). Detection of these antibodies can facilitate diagnosis and treatment of viral infections (Odumade et al., 2011). It can further help elucidate the roles of viral infections and the role of specific viral antigens in disease development (Cepok et al., 2005).

ELISA is a traditional method to study anti-viral antibodies; however, it is typically limited to only one or a few protein antigens and often requires significant investment in optimizing antigen production (Athmaram et al., 2013). Even within the same virus, different antigens display markedly different immunogenicity. Sero-reactivity to these antigens may correlate with different clinical parameters and have different clinical utility (Schaade et al., 2001). The opportunity to gather the information of antibody responses to an entire viral proteome will enable the understanding of the relationship between individual anti-viral antibody responses and clinical parameters and measurements. It is very common that more than one virus has been epidemiologically associated with certain diseases (Jun and Yoon, 2004). Comprehensive studies of complete viral proteomes for multiple viruses are impractical with the traditional one-antigen-at-a-time approach. An assay platform which can examine responses to whole proteomes of many viruses could generate a comprehensive overview of responses to

viral infections, providing biological log files of past infections, and possibly unveil viral associations with autoimmune diseases or cancers.

Protein arrays provide an ideal tool to profile antibodies in blood against thousands of proteins on a microscopic format (Sutandy et al., 2013). Traditional protein array technology is based on expressing, purifying and printing thousands of different proteins. This is scientifically challenging and labor intensive. NAPPA circumvented these inherent problems by printing plasmids with cDNA encoding each protein instead of the proteins themselves (Ramachandran et al., 2008). NAPPA has been successfully used in novel autoantibody biomarker discovery and protein functional studies (Anderson et al., 2011; Ceroni et al., 2010; Gibson et al., 2012; Miersch et al., 2013; Wright et al., 2012; Yu et al., 2014b).

Standard NAPPA involves just-in-time *in situ* protein expression from printed cDNAs using *in vitro* transcription and translation (IVTT)-coupled cell lysates (Miersch and LaBaer, 2011; Qiu and LaBaer, 2011; Ramachandran et al., 2008). Expressed tagged proteins are captured on a planar glass surface by co-printed anti-tag antibodies. When feature densities are increased, mRNAs and proteins from one feature start to diffuse to the neighboring features during protein expression resulting in mixed protein display (Takulapalli et al., 2012). Diffusion prohibits standard NAPPA from achieving densities higher than 2,500 features per array. HD-NAPPA overcame these challenges by expressing and capturing proteins in arrays of isolated sealed ‘nanowells’ to prevent diffusion and cross-talk between spots (Takulapalli et al., 2012). The utility of HD-

NAPPA has been demonstrated in commercial antibody target detection and protein-protein interactions (Takulapalli et al., 2012).

Connections between viral infections and JIA and T1D were supported at the epidemiological, serological and molecular levels (Franssila and Hedman, 2006; Jun and Yoon, 2004). Parvovirus B19 (PB19) and coxsackievirus B4 (CVB) were isolated directly from the synovial tissue of a patient with severe arthritis and the pancreas of a child with diabetic ketoacidosis, respectively (Dijkmans et al., 1988; Yoon et al., 1979). PCR, *in situ* hybridization (ISH) and immunohistochemistry (IHC) staining were employed to detect viral genomes or proteins among JIA and T1D patients (Foy et al., 1994; Pak et al., 1988). Other immunological methodologies, including plaque, ELISA and complement fixation assays, were applied to measure antibodies specific to viral antigens from various biological samples such as serum, plasma and synovial fluid (Banatvala et al., 1985; Gamble et al., 1969; Oikarinen et al., 2014). All these immunoassays depended on the detection of anti-viral antibodies to the intact whole virus or a limited number of viral proteins. This precluded us from acquiring a complete picture of viral infections in JIA and T1D (Clarris, 1978; Craig et al., 2013b).

To characterize the advantages and demonstrate the utility of HD-viral-NAPPA to document past viral infections, anti-viral antibodies to 761 viral antigens from 25 different viruses were profiled in the two most common juvenile autoimmune diseases: JIA and T1D. HD-viral-NAPPA enabled studying anti-viral antibodies in JIA and T1D patients at unprecedented breadth and depth. HD-NAPPA showed high reproducibility of protein display and serum profiling on HD-viral-NAPPA. HD-viral-NAPPA greatly

improved sensitivity in detecting anti-viral antibodies compared to standard NAPPA and ELISA. Unique and common signatures of anti-viral antigen antibodies were found. It was clearly demonstrated that HD-viral-NAPPA is a flexible, sensitive and high-throughput platform enabling quantitative measurements of anti-viral antibody levels in infectious and autoimmune diseases.

6.3 Experiments

6.3.1 Serum Samples

T1D samples were collected at the University of Florida with written informed consent and approval of institutional review board (IRB) at the University of Florida. Peripheral blood samples were obtained from T1D patients diagnosed within three months according to the American Diabetes Association (ADA) criteria. Serum was prepared and stored as aliquots at -80°C. T1D controls were age/gender matched family members of patients and tested to be negative for the known T1D autoantibodies (GADA, IA-2A and ZnT8A). JIA samples were collected at Queen's University of Belfast with the Office for Research Ethics Committees Northern Ireland (ORECNI) approval (ORECNI 408/03). JIA patients and JIA patients with uveitis were matched with disease subtypes and antinuclear antibodies (ANA) status in addition to age/gender. Healthy controls were only age/gender matched to JIA patients or JIA patients with uveitis. Uveitis is the inflammation of the uvea which is regarded as a severe symptom in JIA patients (Thurn, 1988). The sample information of T1D and JIA is characterized in Table 6-1 and 6-2.

6 - 1 Sample Characteristics of T1D Sample Set

Characteristics	New Onset Patients (n=20)	Healthy Controls (n=20)
Age		
Mean± SD	13.2±4.96	13.3±4.94
Median	13.5	13.5
Gender		
Male(%)	(14)70%	(14)70%
GADA positive	(14)70%	0
IA-2A positive	(14)70%	0
ZnT8A positive	(11)55%	0

6 - 2 Sample Characteristics of JIA Sample Set

Characteristics	JIA (n=10)	JIA with Uveitis (n=10)	Healthy control (n=10)
Age			
Mean± SD	5.83±3.86	4.78±3.43	6.17±6.46
Median	3.86	3.51	2.71
Gender			
Male(%)	(1)10%	(1)10%	(1)10%
Disease Subtype			
Oligo	7	7	NA
Ext.Oligo	1	1	NA
Poly	2	2	NA
ANA status			
negative	(5)50%	(5)50%	NA

6.3.2 HD-viral-NAPPA Fabrication

6.3.2.1 DNA Preparation

Viral genes were cloned into the T7-based mammalian expression vector pANT7_cGST (Cormier et al., 2011; Festa et al., 2013; Yu et al., 2014a). All genes were sequence verified and are publicly available at https://dnasu.org/DNASU/ (Seiler et al., 2014). Plasmid DNA was extracted and quality assured as described (Miersch and

LaBaer, 2011; Qiu and LaBaer, 2011). DNA concentration was normalized to 100 ng/µL before printing.

6.3.2.2 Silicon Nanowell (SiNW) Substrate Preparation

All SiNW substrates were fabricated at Arizona State University Center for Solid State Electronics Research (CSSER). The detailed procedure for nanowell production was described in (Takulapalli et al., 2012). Briefly, isotropic wet etching was used to produce circular nanowells with flat surface at the bottom. Nanowells were 250 µm in diameter and 70 µm in depth. The etched silicon wafers were diced to yield the SiNW substrates the same size as a standard microscope slide. A silicon dioxide layer was thermally grown on the surface and later coated with a 3-Aminopropyltriethoxysilane monolayer for NAPPA chemistry.

6.3.2.3 Piezoelectric Printing in Nanowells

HD-viral-NAPPA was printed by au302 piezoelectric dispensing system (Engineering Arts LLC, Tempe, AZ) with integrated alignment system. "On the fly" non-contact dispensing with 16-pin dispensing head was used to dispense DNA/printing mix at 175 mm/sec speed. Each nanowell was filled with 1,200 picoliters of printing mix followed by 300 picoliters of DNA. Each SiNW substrate was equally divided into four sub-arrays. Each viral gene was printed in duplicate within the sub-arrays. Printed arrays were stored desiccated in a nitrogen atmosphere at room temperature until use.

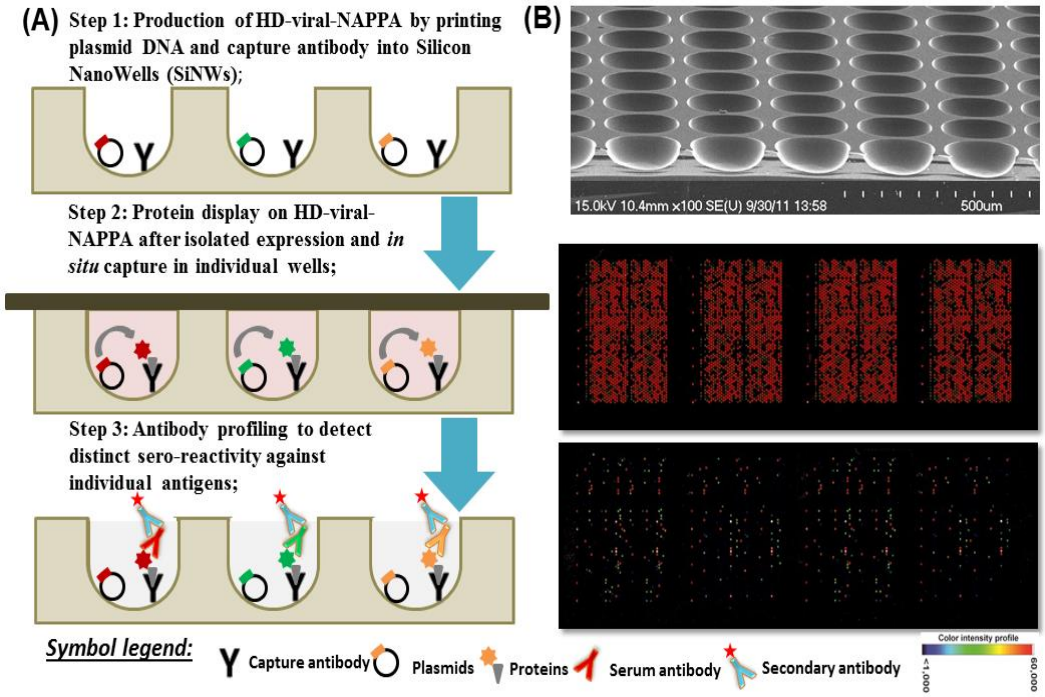
6.3.3 Protein Expression

After printing, SiNW substrates were blocked using SuperBlock TB (Thermo Scientific, Waltham, MA) for 30 min. The substrates were rinsed, dried and placed in an

airtight chamber (Takulapalli et al., 2012) with a flexible film above the substrates. Air in the chamber was removed by vacuum from a fluid gap between the substrate and the flexible film. Human HeLa cell lysate-based IVTT system was injected into the fluid gap by syringe thus filling nanowells with lysate. Excess reagent was swept away from the substrate by flowing pressurized viscous liquid over the flexible film. Individual nanowells were thus sealed into isolated reaction chambers by the flexible film and pressurized viscous liquid. The chamber was placed in an incubator (EchoTherm chilling incubator, Torrey Pines Scientific, Carlsbad, CA) for protein expression at 30^{o}C for 2 hrs and capture at 15^{o}C for 1 hr. Displayed proteins were detected with a monoclonal anti-GST antibody (Cell signaling Inc., Danvers, MA) and Alexa Fluor® 647 goat anti-mouse IgG (H+L) (Life technologies, Carlsbad, CA). Substrates were washed, dried and scanned by Tecan PowerScanner™ (Tecan Group Ltd, Männedorf, Switzerland).

6.3.4 Serum Profiling on HD-viral-NAPPA

The scheme of antibody profiling on HD-viral-NAPPA was shown in Figure 6-1. Following protein expression, substrates were blocked in 5% milk-PBST (0.2% Tween) for 1 hr and later incubated with serum samples in proplate 4-well tray set (Grace Bio-Labs, Bend, OR) at 4^{o}C overnight. Next day, the substrates were washed and detected by Alexa Fluro® 647-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA). Finally, substrates were washed, dried and scanned as described.



6 - 1 Antibody Profiling on HD-viral-NAPPA
A. Step 1: Production of HD-viral-NAPPA by printing plasmid DNA and capture antibody into Silicon NanoWells (SiNWs); Step 2: Protein display on HD-viral-NAPPA after isolated expression and *in situ* capture in individual wells; Step 3: Antibody profiling to detect distinct sero-reactivity against individual antigens.
B. Images of a SiNW substrate by scanning electron microscopy (top), proteins displayed on HD-viral-NAPPA as detected by anti-GST antibody (middle), and sero-profiling on HD-viral-NAPPA (bottom).

6.3.5 RAPID ELISA

RAPID ELISA was performed to verify the sero-reactivity to viral proteins (Anderson et al., 2008). Briefly, each well of 96-well ELISA plates (Corning life science, Union City, CA) was coated with 50 µL 10 µg/mL anti-GST antibody (GE Healthcare Life Sciences, Pittsburgh, PA) in coating buffer (0.5 M sodium bicarbonate buffer, pH 9.6) at 4°C overnight. Next day, coated plates were washed with PBST and blocked with

5% milk-PBST (0.2% Tween) for 1.5 hrs. Meanwhile, 40 ng/μL plasmids encoding viral antigens were expressed in the human HeLa cell lysate-based IVTT system at 30°C for 1.5 hrs. Viral proteins were diluted 1:200 in milk-PBST and captured in the wells. Then plates were washed with PBST, incubated with serum samples diluted at 1:1,000, washed with PBST again and incubated with peroxidase AffiniPure goat anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, the plates were detected by 1-Step Ultra TMB - ELISA Substrate (Thermo scientific, Waltham, MA) for 10 min and then 2 M sulfuric acid was used to stop the reaction. OD_{450} was measured by Envision Multilabel Reader (Perkin Elmer, Waltham, MA).

6.3.6 Statistical Analysis

Sample characteristics including age, gender and known autoantibody status were presented as proportion, median and mean with standard deviations. Signal intensities of protein display and serum profiling were extracted by Array-Pro Analyzer (MediaCybernetics, Rockville, MD). Raw signal intensities of protein display were log transformed before comparing their intra-array, intra-batch and inter-batch correlations. To calculate the S/B ratios on the array platform, the background signal was represented by the median of the raw signal intensities of all the antigens on an array probed with a serum sample. For ELISA, the background signal was represented by the median signal of all the antigens for a serum sample probed on the same day. Wilcoxon rank-sum test was used to test the difference of sero-reactivity among subgroups in the JIA sample set. Heatmaps were generated in MultiExperiment Viewer version 4.9 obtained from

http://www.tm4.org/. Bar graphs and plots were generated in GraphPad Prism 6 (GraphPad software, La Jolla, CA).

6.4 Results

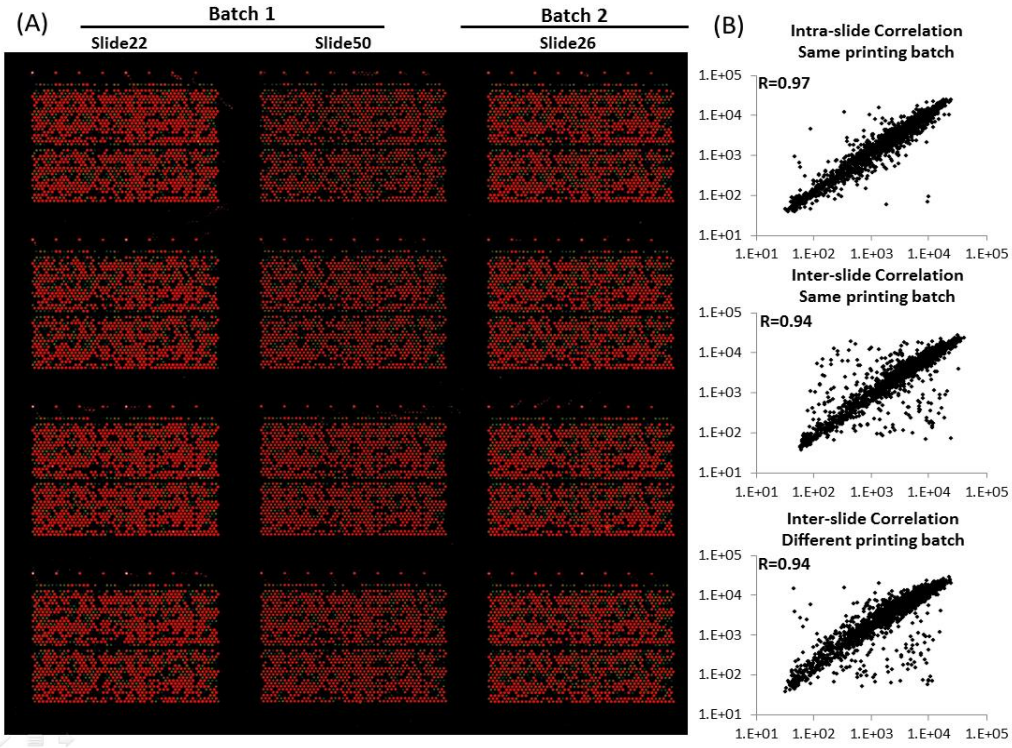
6.4.1 Quality of Protein Display on HD-viral-NAPPA

761 viral genes from 25 viral strains were collected to build the HD-viral-NAPPA platform (Table 6-3). Among these, 16 viral strains had 100% of viral genes available in this collection. During printing, each array was divided into four sub-arrays and each viral gene was printed in duplicate on each sub-array.

To confirm the quality of protein display on HD-viral-NAPPA, protein display levels for all ~1,500 features on two sub-arrays from the same printing batch and one sub-array from a separate printing batch was compared. Representative array images and scatter plots of signal intensities of protein display are shown in Figure 6-2. The average intra-array, intra-batch and inter-batch correlation coefficients of protein display calculated by the log transformed raw signal intensity were 0.92, 0.91 and 0.91, respectively.

6 - 3 List of Viruses in This Study

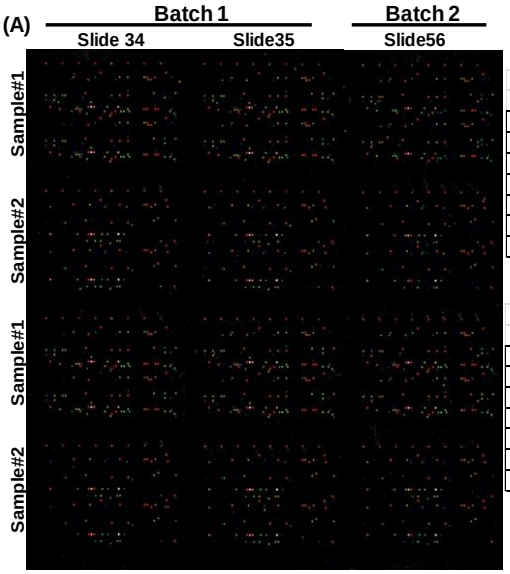
Virus Species	Abbre.	Family	Genome	ORF clones	% of complete ORFeome
Human Cytomegalovirus	HCMV/HHV-5	*Herpesvirinae*	dsDNA	164	100%
Esptein-Barr virus	EBV/HHV-4	*Herpesvirinae*	dsDNA	85	100%
Coxsackievirus B	CVB	*Picornaviridae*	ssRNA+	12	100%
Rubella virus	RUBA	*Togaviridae*	ssRNA+	6	100%
Mumps virus	MuV	*Paramyxoviridae*	ssRNA-	9	100%
Human endogenous retrovirus K	HERK	*Retroviridea*	ssRNA-	4	100%
Rotaviruses	RV	*Reoviridae*	dsRNA	12	100%
Parvovirus B19	B19	*Parvoviridae*	ssDNA	6	100%
Hepatitis B virus	HBV	*Herpesviridae*	dsDNA	10	100%
Human Papillomavirus16	HPV16	*Papillomaviridae*	dsDNA	10	100%
Human Papillomavirus18	HPV18	*Papillomaviridae*	dsDNA	9	100%
Chikungunya virus	CHIKV	*Togaviridae*	ssRNA+	9	100%
Semliki Forest virus	SFV	*Togaviridae*	ssRNA+	9	100%
Sindbis virus	SINV	*Togaviridae*	ssRNA+	9	100%
Influenza A virus (H1N1)	n/a	*Orthomyxoviridae*	ssRNA-	10	100%
Influenza A virus (H3N2)	n/a	*Orthomyxoviridae*	ssRNA-	10	100%
Varicella-zoster virus	VZV	*Herpeviridae*	dsDNA	91	93.10%
Simian virus 40	SV40	*Polyomaviridae*	dsDNA	6	85.71%
Vaccinia virus	VACV	*Poxviridae*	dsDNA	167	74.90%
Yellow fever virus	YF	*Flaviviridae*	ssRNA+	11	71.42%
Measles virus, vaccine strain	MeV,vaccine	*Paramyxoviridae*	ssRNA-	5	62.50%
Measles virus, WT strain	MeV,WT	*Paramyxoviridae*	ssRNA-	5	62.50%
Herpes simplex virus 1	HSV-1	*Herpesviridae*	dsDNA	83	61.90%
Adenovirus	n/a	*Adenoviridae*	dsDNA	16	42.10%
Tioman virus	n/a	*Paramyxoviridae*	ssRNA-	3	37.50%



6 - 2 Quality of Protein Display on HD-viral NAPPA
(A) Visual representative images of HD-viral NAPPA protein display on slides within the same printing batch and across different printing batches.
(B) Scatter plot of log transformed signal intensities for protein display on slides within the same printing batch and across different printing batch.

6.4.2 Quality of Serum Profiling on HD-viral-NAPPA

The work flow of serum profiling on HD-viral-NAPPA includes the production of HD-viral-NAPPA, protein display and antibody profiling to detect sero-reactivity against individual antigens. To demonstrate the quality of serum profiling, the same pair of T1D patient and control samples were applied on two arrays from the same printing batch and one array from a separate printing batch. Four sub-arrays of each array (1,522 features, 761 unique viral genes) were probed twice with the patient sample and twice with the



(B)

Sample#1		Batch	1	1	1	1	2	2
		Slide	34	34	35	35	56	56
Batch	Slide	Subarray	1	3	1	3	1	3
1	34	1	1.00	0.98	0.99	0.98	0.94	0.99
1	34	3	0.98	1.00	0.98	0.96	0.94	0.97
1	35	1	0.99	0.98	1.00	0.98	0.96	0.98
1	35	3	0.98	0.96	0.98	1.00	0.93	0.98
2	56	1	0.94	0.94	0.96	0.93	1.00	0.93
2	56	3	0.99	0.97	0.98	0.98	0.93	1.00

Sample#2		Batch	1	1	1	1	2	2
		Slide	34	34	35	35	56	56
Batch	Slide	Subarray	1	3	1	3	1	3
1	34	1	1.00	0.93	0.99	1.00	0.97	0.97
1	34	3	0.93	1.00	0.93	0.93	0.91	0.93
1	35	1	0.99	0.93	1.00	0.99	0.97	0.97
1	35	3	1.00	0.93	0.99	1.00	0.97	0.97
2	56	1	0.97	0.91	0.97	0.97	1.00	0.97
2	56	3	0.97	0.93	0.97	0.97	0.97	1.00

	Lowest	Highest	
0.8			1.0

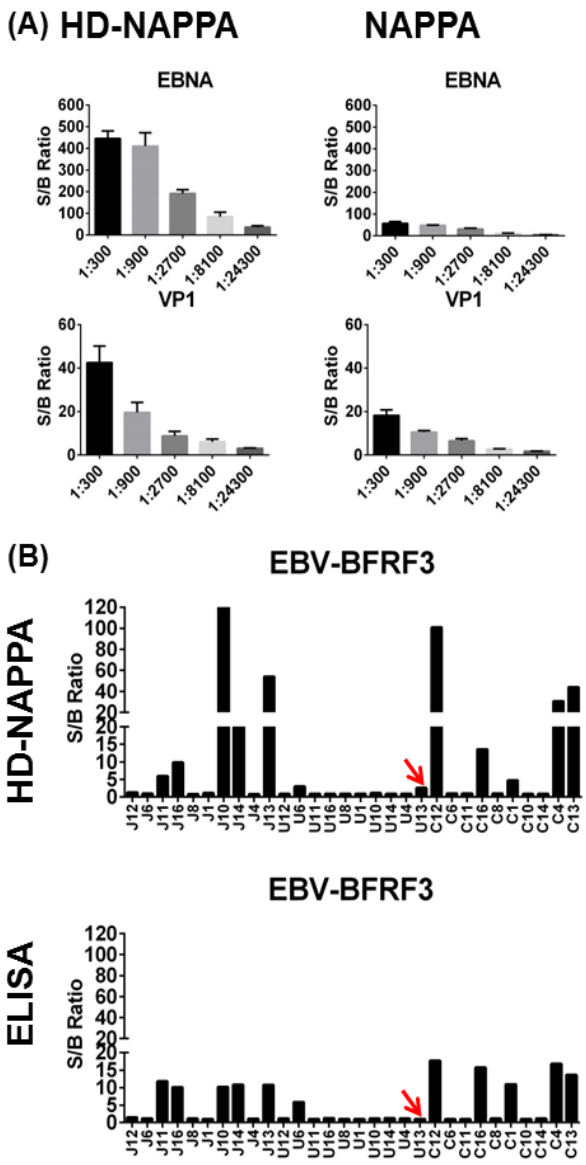
6 - 3 Quality of Serum Profiling on HD-NAPPA
(A) Visual representative images for serum profiling on two arrays from the same printing batch and one array from a different printing batch.
(B) Correlation coefficients for intra-array and inter-array correlation within the same printing batch, inter-array correlation across different printing batch. Sample#1 is a T1D patient sample, sample#2 is a healthy control sample.

control sample. The average correlation coefficients of intra-array, intra-batch and inter-batch for both samples were all above 0.95. The results were highly reproducible as shown by the representative array images and the correlation coefficients between sub-arrays in Figure 6-3.

6.4.3 Higher Sensitivity in Detecting Antibodies on HD-NAPPA

Assay sensitivity of sero-reactivity on HD-NAPPA and standard NAPPA was compared. The same set of 190 unique genes was printed on both glass, using the standard NAPPA method (Miersch and LaBaer, 2011; Ramachandran et al., 2008), and the SiNW surface, using the HD-NAPPA method (Takulapalli et al., 2012). A serum

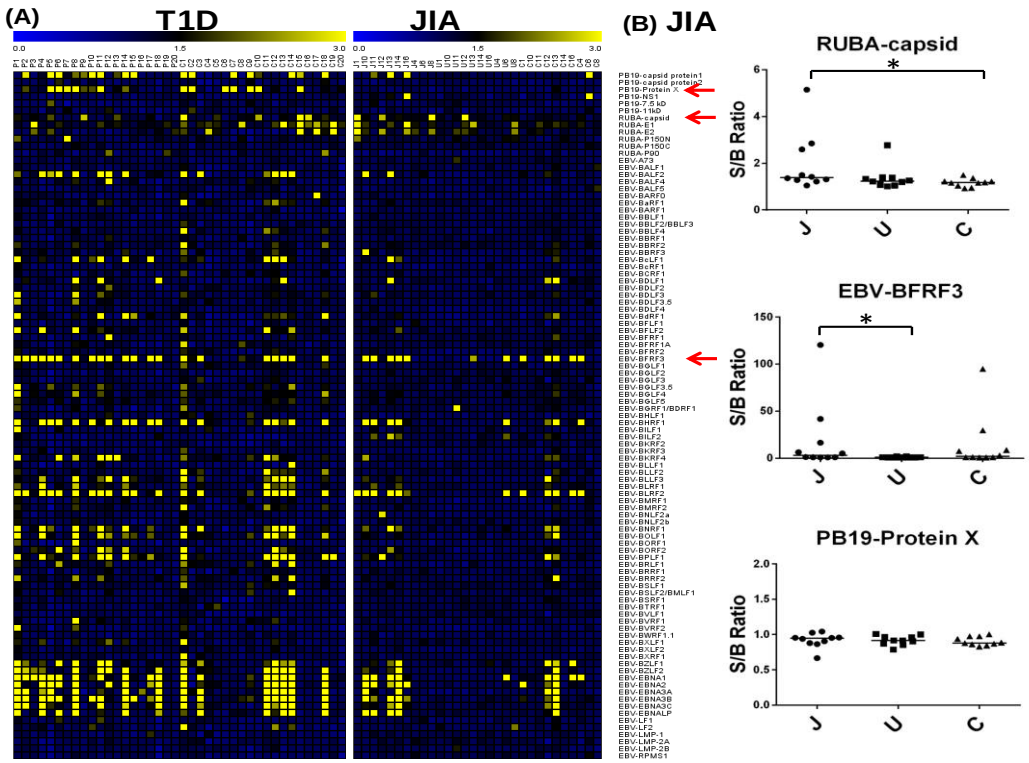
sample was applied at three-fold serial dilutions from 1:300 to 1:24,300 on both platforms. The S/B ratio was used to assess sero-positivity, which was the signal of any feature divided by the median signal on the same array, because the majority of features on the array were considered non-responses (see Materials and methods). An increase in the S/B ratio was found as high as 9 times for anti-EBNA1 response from Epstein-Barr virus (EBV) at 1:900 dilution and more than 2 times for anti-VP1 (CVB) response at 1:300 dilution when comparing HD-NAPPA with standard NAPPA (Figure 6-4). Sero-reactivity to the BFRF3 antigen of EBV among the JIA sample set was profiled on HD-viral-NAPPA and by ELISA to compare their ability to measure positive sero-reactivity. Overall, sero-positivity for all 30 samples agreed well on both platforms except for sample U13, which showed S/B >2 on HD-viral-NAPPA but was not distinguishable from background by ELISA (red arrows on Figure 6-4). Additional tests are needed to further confirm the sero-positivity. Sample J10 had more than 12 times higher S/B ratios on HD-viral-NAPPA than that of ELISA. The wide S/B ratio range made HD-viral-NAPPA a more sensitive immunoassay platform in distinguishing sero-positive from sero-negative samples than standard NAPPA or ELISA (Figure 6-4).



6 - 4 Higher Sensitivity in Detecting Anti-viral Antibodies on HD-NAPPA
(A) S/B ratio of sero-reactivity to EBNA1 (EBV) and VP1 (CVB) at serial dilutions on HD-NAPPA and NAPPA.
(B) S/B ratio of sero-reactivity to BFRF3 (EBV) among JIA sample set on HD-NAPPA and ELISA.

6.4.4 Anti-viral Antibodies of Three Common Viruses in JIA

Sero-reactivity to 761 viral antigens from 25 viruses by HD-viral-NAPPA using JIA and T1D samples (Table 6-3). The T1D samples included 20 new onset patients and 20 healthy controls. The percentage of males with T1D was 70%, which is slightly higher than the expected distribution, probably due to sampling error, but was appropriately matched with controls. The JIA samples included 10 patients, 10 patients with the symptom of uveitis and 10 healthy controls. In the case of JIA, the percentage of males in the cases was 10%, reflecting the well-documented gender disparity of JIA, which primarily affects females (Thierry et al., 2014). The controls for JIA were appropriately gender matched. Among 25 viral strains, PB19, rubella virus (RUBA) and EBV are the most reported viruses associated with JIA. The heatmaps of sero-reactivity to the three viruses for both diseases were generated (Figure 6-5). The power of a proteomic approach in discovering autoimmune disease-associated viral infections is the opportunity to test all possible viral antigens to see which one gives the best response. This could not be achieved by a more conventional one-antigen-at-a-time approach. In the JIA sample set, sero-reactivity to the capsid protein (RUBA) was significantly higher in JIA patients than healthy controls by Wilcoxon rank-sum test ($p<0.05$; Figure 6-3B); whereas, no difference of sero-reactivity to other proteins (E1, E2 and P150) from RUBA was observed. JIA patients with uveitis had less sero-reactivity to most EBV proteins compared to the other subgroups. In the T1D sample set, the prevalence of antibodies to EBV proteins was higher in patients than controls (Figure 6-5).

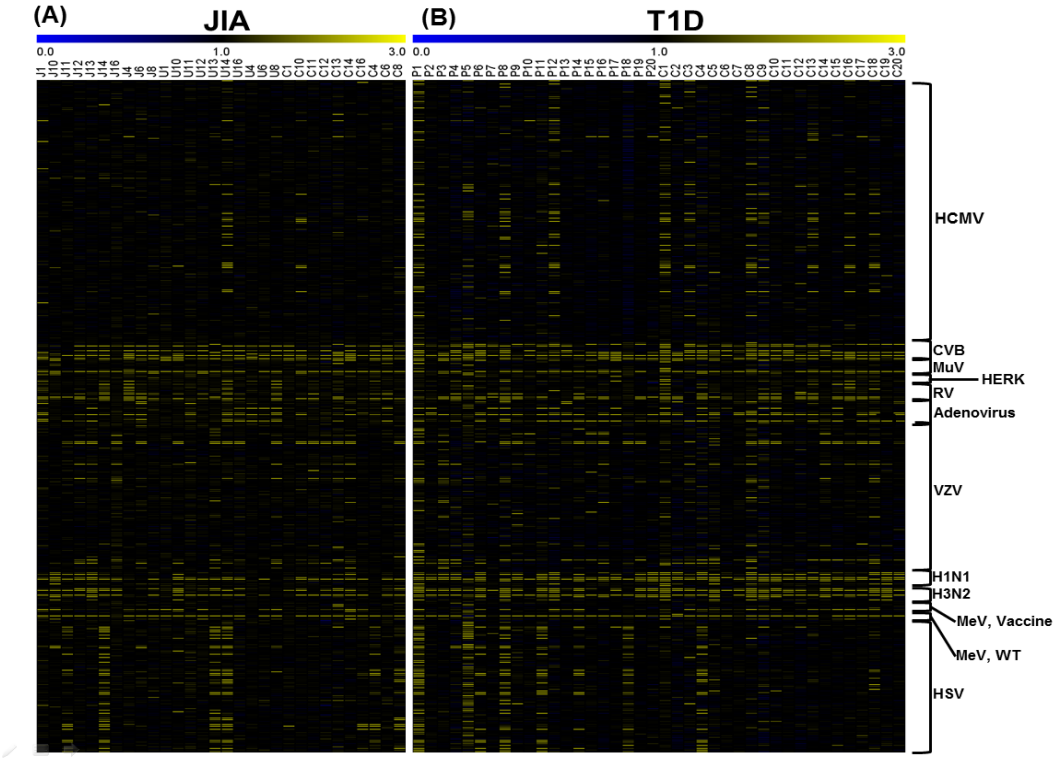


6 - 5 Anti-viral Antibodies to PB19, RUBA and EBV
(A) Heatmaps of S/B ratio of sero-reactivity to viral proteins from the three viruses on HD-NAPPA in T1D and JIA sample sets.
(B) Jitter plots of sero-reactivity to representative viral antigens (RUBA-capsid, EBV-BFRF3 and PB19-protein X) on HD-NAPPA in JIA sample set.

6.4.5 Profiles of Anti-viral Antibodies in JIA and T1D Samples

Profiles of anti-viral antibodies were generated on HD-viral-NAPPA and illustrated in the heatmaps (Figure 6-6). Overall, most samples (both cases and controls) were positive for CVB, RUBA, mumps virus (MuV), rotavirus (RV), adenovirus, influenza A virus and measles virus (MeV), while negative for Human endogenous retrovirus K (HERK) and varicella zoster virus (VZV). The JIA samples have less sero-

reactivity to human cytomegalovirus (HCMV) proteins compared with T1D samples which may result from the age and geographic difference between these two sample collections. Interestingly, antibody response to herpes simplex virus (HSV) was found to be more prevalent in both JIA and T1D patients than healthy controls.



6 - 6 Profiles of Anti-viral Antibodies in JIA and T1D Patients
(A) JIA sample set, J stands for JIA patients, U stands for JIA patients with uveitis, C stands for healthy controls.
(B) T1D sample set, P stands for T1D patients, C stands for healthy controls.

6.5 Discussion

Host innate and adaptive immunity works cooperatively to fight against viral infections (Anderson et al., 2011). Antibodies recognizing antigen proteins are produced during this process. Detection and quantification of these antibodies will aid sero-diagnosis of infections, design of preventive vaccines, discover innovative therapeutics and monitor of anti-viral treatments (Odumade et al., 2011). Traditional methods focusing on one-protein/one-virus at a time suffer greatly from the limitation of throughput. Protein arrays, as one of the key innovations in the era of functional proteomics, provide an ideal tool to profile antibody response to thousands of proteins on a microscopic slide in a multiplexed manner (Sutandy et al., 2013). NAPPA, as a robust *in situ* cell free protein array platform, prints full length cDNAs on the arrays instead of purified proteins (Ramachandran et al., 2008).

HD-NAPPA achieved higher density and less diffusion by expressing and capturing proteins in isolated sealed 'nanowells' (Takulapalli et al., 2012). It shares some common advantages with standard NAPPA. First, antigens are expressed by a HeLa cell lysate-based IVTT system, yielding high expression levels and functional conformations of displayed proteins. Second, the ability to reconfigure new arrays allows exploration of sero-reactivity to any antigens from new pathogens, new strains or new mutants in diseases as they emerge. This is especially important for microbial studies because conventional protein arrays for human and yeast, although expensive, are at least commercially available; but, similar arrays are not available for microorganisms. Third,

both assays are highly reproducible and the turnaround time for one assay for thousands of proteins is as short as one day.

In addition, HD-NAPPA introduces some unique advantages. First, based on its high density feature, as many as 24,000 proteins can be tested simultaneously compared to only 2,500 proteins by standard NAPPA on each array. Second, the high density nature reduces the sample amount needed for assaying each protein, thus preserving precious resources. Third, here HD-NAPPA was demonstrated to have higher sensitivity and better S/B in detecting antibody responses compared with standard NAPPA and ELISA. Fourth, HD-NAPPA protocols use much less DNA per printing batch when compared to standard NAPPA, which means that many more samples can be tested based on a single round of DNA preparation and reducing cost of array production. Fifth, HD-NAPPA has negligible diffusion between neighboring spots reducing false positives during serum screening.

Aside from the above advantages, there are some limitations of NAPPA technology. Although it is easier and more robust to prepare and print DNA than to express, purify and print proteins, it is nevertheless beyond the means for most general research labs to work with thousands of genes/proteins at the genome/proteome level. Collaboration or fee-for-service (such as http://nappaproteinarray.org/) might make more sense in this setting. Fast and accurate noncontact piezoelectric dispensing systems capable of targeting nanowells are required for printing HD-NAPPA but are inaccessible to most researchers. An affordable user-friendly non-contact printing instrument is currently in development to allow widespread adoption of HD-NAPPA. Finally, it should

be noted that this platform is well-suited for screening for possible interactions or responses at the proteome level. But, all candidates that emerge from such screens must be confirmed by orthogonal methods and, where relevant, with *in vivo* assays.

In this study, HD-NAPPA showed superior S/B ratio to standard NAPPA from a direct comparison of sero-reactivity to the same set of genes on these two platforms. On average, more than a seven fold increase in the S/B ratio of anti-EBNA1 sero-reactivity was obtained at various sample dilutions (Figure 6-4). The S/B ratio of HD-NAPPA also compares favorably to reported serum antibody studies using purified protein arrays (Robinson et al., 2002) or Luminex bead arrays (Wong et al., 2009). Thus, this tool detects serum antibodies at high sensitivity and can potentially improve the accuracy of clinical studies.

To demonstrate the great utility of HD-NAPPA, HD-viral-NAPPA was constructed containing 761 protein antigens from 25 viral strains. There are over 10,000 nanowells per SiNW array in the current configuration (Takulapalli et al., 2012). ORFs for all 761 viral antigens were printed in the nanowells, in duplicate, four times to produce four sub-arrays on one array. This allowed for the profiling of the sero-reactivity against all viral antigens of four serum samples on one array in parallel. The quality of protein display was evaluated by array-to-array reproducibility. 0.64%, 4.16 % and 3.81% of features with CVs higher than 20% was observed using the log transformed raw signal intensities when comparing intra-slide, inter-slide and inter-batch correlations, respectively. Preliminary studies suggest that this may be due to imperfect dispensing of the same amount of DNA into the nanowells, resulting in inconsistent protein display on

arrays from two different arrays. The piezoelectric printing protocol is currently under development to achieve higher dispensing reproducibility. Nonetheless, this affected a very small fraction of the features on the array and was mitigated somewhat by having duplicate spots for each gene on the array.

Anti-viral antibodies in the two most common juvenile autoimmune diseases, JIA and T1D were profiled, as a demonstration of HD-NAPPA in serological studies. It has long been suspected that certain types of viral infections are involved in the development of these two autoimmune diseases (Jun and Yoon, 2004; Ravelli and Martini, 2007). T1D controls were collected in the United States (US) as T1D patient samples, and JIA controls were collected in the United Kingdom (UK) as JIA patient samples. In the anti-viral antibody profile, the prevalence of sero-reactivity to HSV was higher in both JIA and T1D patients than healthy controls while the sero-reactivity to EBV was more prevalent only in T1D patients. The percentage of males is lower than females in JIA, it is the opposite in T1D. No obvious differences of anti-viral antibody profiles were seen between the genders. Although the sample size in this study is too small to draw any statistical conclusions, this may suggest a potential role of these viral infections in JIA or T1D development. Interestingly, the antibody responses to EBV proteins were higher in JIA patients and healthy controls than JIA patients with uveitis. It has been reported that JIA patients went into remission after EBV infection (Kawada et al., 2013). Therefore, the potential protection of EBV infection against uveitis in JIA patients warrants future investigation.

The protein array platform detects multiplexed antibody responses to viral antigens from the whole viral proteome. Many viruses undergo latency after active/lytic infection and may subsequently become reactivated after a latent stage (Odumade et al., 2011). Different viral antigens are preferentially expressed depending on these different states. Thus, both the antibody response and its magnitude to specific viral antigens may provide clues to the different stages of viral infection (Klutts et al., 2009). In cases where the lifecycle of a virus is well understood, a viral proteome array can be revealing. For example, EBV commonly enters latency in adults and in that state continues to produce the EBNA protein, which may explain the strong IgG response observed to that protein in adults (Klutts et al., 2009). The prevalence of strong anti-EBNA responses is much less common in children (unpublished observation). For less understood viruses, the inclusion of the whole viral proteome allows the investigation of every viral antigen that is linked to either active/lytic or latent stages of viral infection. Furthermore, IgM is often used to detect acute infection while IgG detects past infection. The array platform allows multiplexed detection of IgG and IgM using secondary antibodies labeled with two different fluorescent dyes. In summary, the inclusion of all viral antigens from the complete viral proteome and the ability of multiplexed detection of IgM and IgG may enable the discrimination between active/lytic or latent viral infection.

6.6 Conclusions

The utility of HD-viral-NAPPA in profiling anti-viral antibodies in diseases was successfully demonstrated. The small sample size used in this study may preclude reaching strong statistical conclusions. However, high quality serological profiles were generated in 70 subjects; observed reactivity differences among them and confirmed results with orthogonal ELISA data. HD-NAPPA may be applied to large scale anti-microbial antibody studies and help us better understand the role of microbial infections in various autoimmune diseases.

6.7 Acknowledgements

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CHAPTER 7

7 CONCLUSIONS AND FUTURE WORK

7.1 Conclusion

The major findings and contributions of my dissertation include:

(i) A proteome-scale, two-stage serological AAb screening against 6,000 human proteins was performed and identified 26 novel AAbs including a known T1D-associated AAb (ZnT8). An orthogonal immunoassay named Luciferase LIPS assay was developed and used to validate these candidate AAbs. AAb to a dual specificity typrosine-phosphorylation-regulated kinase 2 (DYRK2) was validated with 36% sensitivity at 98% specificity. The AUC for a combination of DYRK2A and the classical IA-2A was 0.90 compared to 0.72 for DYRK2A and 0.64 for IA-2A alone. This is the first systematic screening for novel AAbs against large number of human proteins by protein arrays in T1D.

(ii) A most comprehensive search for novel AAbs in T1D using a knowledge-based approach by ELISA and a screening-based approach by NAPPA was performed. Six AAbs were identified and validated with sensitivities ranged from 16% to 27% at 95% specificity. Their prevalence in T1D cases complement to the major known T1D-assocaited AAbs and varied in different age subgroups.

(iii) Seroreactivity to 646 viral antigens was assessed in 42 new-onset T1D patients and 42 age-gender matched healthy controls. Prevalence of anti-viral antibodies agreed well with the infection rates of the corresponding virus based on epidemiological studies. Antibody positive rate of EBV was significantly higher in cases than controls

(OR 6.6; 95% CI 2.0-25.7) while the other viruses did not differ between the two groups. The EBV and T1D association was significant in both genders and age subgroups (<=12 and >12). These results suggest a potential role of EBV in T1D development.

(iv) HD-NAPPA showed higher S/B ratio compared with standard NAPPA on planar glass slides. HD-NAPPA also showed high quality of protein display and serum profiling with average correlation coefficients within or between printing batches at 0.91 and 0.96, respectively. Common as well as unique antibody reactivity patterns were detected between patients and healthy controls in T1D on HD-NAPPA.

With these results, two papers were published, one manuscript is in revision and one manuscript is in preparation for publication. All of them are listed as below:

1. Miersch, S.*, **Bian, X.***, Wallstrom, G., Sibani, S., Logvinenko, T., Wasserfall, C. H., Schatz, D., Atkinson, M. A., Qiu, J., Labaer, J. (2013). Serological autoantibody profiling of type 1 diabetes by protein arrays. J Proteomics. 94, 486-96. PMID: 24148850. (*Co-first authorship)

2. **Bian, X.**, Wallstrom, G., Wasserfall, C. H., Wang, J., Barker, K., Wang, H., Atkinson, M. A., Schatz, D., Qiu, J., Labaer, J. Identification of novel autoantibodies in type 1 diabetes by protein arrays. *In preparation.*

3. **Bian, X.**, Wallstrom, G., Davis, A., Wang, J., Park, J., Throop, A., Steel, J., Yu, X., Wasserfall, C. H., Atkinson, M. A., Schatz, D., Qiu, J., Labaer, J. Immunoproteomic profiling of Virus Antibodies in New-Onset Type 1 Diabetes on Protein Arrays. *In revision.*

4. **Bian, X.**, Wiktor, P., Kahn, P., Brunner, A., Khela, A., Karthikeyan, K., Barker, K., Wasserfall, C. H., Gibson, D., Rooney, M. D., Qiu, J., LaBaer, J. Anti-Viral Antibody Profiling by High Density Protein Arrays. Proteomics. 2015 Mar 11. doi: 10.1002/pmic.201400612. [Epub ahead of print]. PMID: 25758251.

7.2 Future Work

The focus of this dissertation is tracking the humoral immune response in T1D including the AAbs and anti-viral antibodies by an innovative protein array platform. Novel AAbs provide value in identifying individuals at risk, stratifying patients with different clinical courses, improving our understanding of autoimmune destructions, identifying cellular immune response antigens and providing candidates for prevention trials. A complete picture of viral infections was drawn by profiling anti-viral antibody responses to individual viral proteins from the whole viral proteome which will give us a better understanding of the role of viral infections in T1D development. The work done in this dissertation successfully demonstrated the great use of innovative protein arrays in studying these two important questions in T1D, however, some future work can be continued from this study.

7.2.1 Enriching the Gene Collection to Cover the Full Human Proteome

The human genome project (HGP) has been successfully completed and revealed 20,500 human genes in human genome (Watson, 1990). More recently, the human proteome project (HPP) aims to identify all the proteins translated from human genome (Legrain et al., 2011). There is continuous effort to clone more human genes into a

protein array compatible expression vector that was used in this study. The additional genes need to be included in future studies.

7.2.2 Longitudinal Samples for AAb and Virus Study

In the AAb study, age-gender matched cases and controls were used to screen for novel AAbs. In the second study, cases were new-onset T1D patients and had a longer duration of disease than the first study. It is known AAbs may appear at different stages of disease progression including the prediabetic period. Because their levels might not persist, they may not have been detected in the blood samples taken at the time of diagnosis or in clinically active patients. AAbs might also be specific to certain individuals emphasizing the heterogeneous nature of the disease (e.g., different rates of progression, etiological factors, genetic backgrounds). In the future, a systematic study to profile AAbs using longitudinal samples collected before "seroconversion" to clinical T1D will enhance our understanding of the natural history and mechanisms leading to diabetes specific autoimmunity.

Viral infection or presence may act as a longitudinal factor during the process of the induction of single islet antibody, the simulation from single islet antibody to multiple islet antibodies, or the progression from β cell autoimmunity to clinical onset of T1D (Schneider and von Herrath, 2014b). Several studies reported that the initial development of autoantibodies and progression to multiple autoantibodies occurred at an early age. Subsequently, individuals progress to clinical T1D at different paces during which viral infections may act as an accelerator (Chmiel et al., 2015; Parikka et al., 2012). For example, enterovirus infection was shown to increase progression to clinical onset in the

Diabetes and Autoimmunity Study in the Young (DAISY) study (Stene et al., 2010). Profiling of anti-viral antibodies using longitudinal samples may discover viruses that induce “seroconversion” or trigger T1D.

7.2.3 Post-translational Modifications (PTMs) of Autoantigens

During T1D progression, β-cells suffer from ER and oxidative stress due to the inflammatory response from chemokines and cytokines. Proteins undergo missfolding or post-translational modifications (PTMs) in cells under these stresses. One hypothesis is that modified proteins generate new epitopes and are recognized as non-self by the immune system (Dunne et al., 2012). The types of antigen modifications include alternative splicing, overexpression, deamination, transglutamination, glycosylation and citrullination. Several studies reported autoimmunity against proteins with PTMs in T1D. The insulin A-chain epitope recognized by human T cells was modified by forming a vicinal disulfide bond in adjacent cysteine residues at position A6 and A7 (Mannering et al., 2005). WE14 peptide from chromogranin A can be modified by translgutaminase and release a highly reactive T-cell epitope in NOD mice (Delong et al., 2012). PTMs of peptide from insulin had higher T-cell autoreactivity than native peptides (van Lummel et al., 2014). Epitopes of citrullinated GAD65 were recognized in T1D patients (McGinty et al., 2014). Citrullinated glucose -regulated protein 78 is an autoantigen for both B-cell and T-cell response in NOD mice (Rondas D, 2015). Most of the above studies were focused on the detection of autoreactive T-cells against modified peptides in mice. There is no study that investigated the AAb response to antigens with PTMs in human. These modified autoantigens may be more relevant to T1D pathogenesis than their native form.

The protein array platform in this study will be much more powerful if it can display proteins with PTMs and assess their AAb responses in high-throughput manner.

7.2.4 Integration with T1D Microbiome and Virome

The human microbiome includes all the commensal, symbiotic and pathogenic microorganisms in the human body. The human virome is the collection of viruses in human body. They were suspected to be involved in T1D pathogenesis (Kondrashova and Hyoty, 2014). The role of bacteria in T1D onset was described in mouse models. Feeding probiotic bacteria can delay or prevent diabetes in NOD mice (Matsuzaki et al., 1997). Bacteria strains in diabetic mice and non-diabetic mice were different (Roesch et al., 2009). The T1D autoimmune microbiome was defined in human in multiple studies (Brown et al., 2011; Giongo et al., 2011; Mejia-Leon et al., 2014; Schneider and von Herrath, 2014b). The disease specific virome was investigated in inflammatory bowel disease (Norman et al., 2015). NGS was used to sequence the gut virome in children with rapid onset of T1D or islet autoimmunity (Lee et al., 2013). These DNA based approaches such as sequencing are clearly powerful to investigate the T1D mircobiome/virome asscoation. However, they may not tell the full story since viruses may be cleared by immune system. Anti-viral antibodies may persist longer after infection. So the profile of anti-mircobiome/viral antibodies can complement the genomic study. With the flexibility of the NAPPA platform, genes of newly discovered T1D-associated micobiome/virome can be easily cloned and their encoding proteins can be displayed on arrays to test the prevalence of anti-microbiome/viral antibodies.

In summary, T1D is a complicated chronic autoimmune disease that involves the interplay between genetic, immunological and environmental factors. There is an emerging need to understand T1D etiology from different perspectives of the humoral immune response. The use of protein arrays in this study provides a better understanding of T1

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APPENDIX A

IRB APPROVAL INFORMATION

ASU ARIZONA STATE UNIVERSITY
RESEARCH AND ECONOMIC AFFAIRS

Office of Research Integrity and Assurance

To:	Joshua Labaer Center for
From:	Debra Murphy, Director Office of Research Integrity and Assurance
Date:	08/04/2009
Committee Action:	**IRB Review Not Required**
IRB Action Date:	08/04/2009
IRB Protocol #:	0908004207
Study Title:	**The use of protein microarrays to study autoimmunity and diabetes**

The above-referenced protocol has been reviewed and it has been determined that IRB oversight is not required because the study does not meet the criteria under Federal Regulations, 45 CFR Part 46 for research involving human subject participation. This determination is based on OHRP Guidance on Research Involving Coded Private Information or Biological Samples.

The IRB understands that (1) this investigation involves only the use of specimens not collected specifically for the research proposed, (2) the samples are not individually identifiable and cannot be linked to specific individuals by the investigator either directly or indirectly, and (3) the investigator and the holder of any key to coded data agree that the key will not be released under any circumstances.

This determination applies to existing specimens, as well as to specimens to be collected in the future for purposes other than the currently proposed research.

You should retain a copy of this letter for your records.

APPENDIX B

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