IDENTIFICATION OF A NEW METHOD TO DETECT T CELL RESPONSE IN TYPE 1 DIABETES

Thesis leading to the title of
International PhD in Endocrinology and Metabolic Diseases

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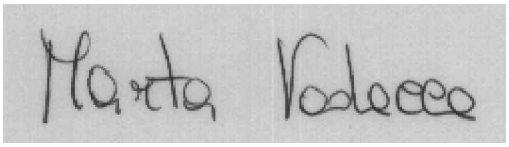

DECLARATION

I hereby certify that the work presented in this thesis is the result of my own investigation except where the reference has been made to published literature and where acknowledgement is made for unpublished data. During the course of this research I have not been registered or enrolled for another award from any academic or professional institutions.

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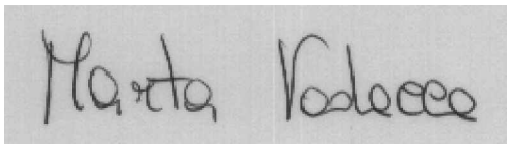

ACKNOWLEDGEMENTS

This project will be run with Antigen Express a biotech company located in Boston which provided the technical support needed by Dr. Marta Vadacca and the Ii-Key/MHC class II hybrids designed as requested in sufficient quantity to test diabetic subjects for the project length according to the research protocol.

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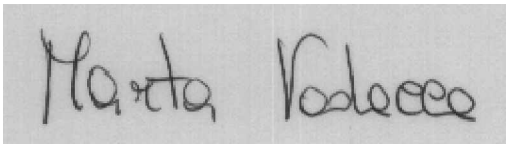
Thanks to Luciana Valente for teaching the required technical skills.

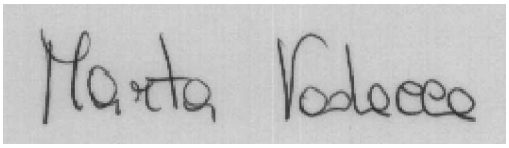


ABSTRACT

Il diabete tipo 1 (DT1) è una patologia autoimmune cronica geneticamente determinata. Al momento della diagnosi clinica circa l'80% delle isole pancreatiche non contengono più cellule deputate alla secrezione di insulina e il quadro istopatologico più frequentemente riscontrato è un infiltrato caratterizzato dalla presenza di macrofagi e linfociti T. Il danno immunologico è mediato sia da linfociti CD4 sia CD8: pertanto, la risposta immunitaria nel diabete di tipo 1, coinvolge sia la componente umorale che cellulo mediata ed entrambi i meccanismi rimangono attivi per numerosi anni dopo la diagnosi. Come in numerose altre patologie autoimmuni, lo specifico epitopo riconosciuto dagli autoanticorpi non è stato ancora chiaramente definito. Il profilo T cellulare coinvolto nella patogenesi della malattia e il meccanismo con cui le cellule T determinano il danno immuno-mediato rimangono tuttora un punto chiave da chiarire per meglio comprendere i meccanismi patogenetici alla base del DT1. Per tali motivi, uno dei più importanti obiettivi per lo studio del DT1 è rappresentato dallo sviluppo di nuove tecniche per identificare con alta specificità linfociti T reattivi verso epitopi antigenici coinvolti nella patogenesi della malattia.

Le citochine prodotte dai linfociti rappresentano un marker specifico del profilo T cellulare e la misura delle stesse è un ottimo indice della reattività immunitaria del soggetto esaminato. Tale evidenza è applicabile a tutte le patologie autoimmuni, compreso il DT1. L'utilizzo nella pratica clinica di questi marker rimane tuttavia limitato a causa della non ancora precisa caratterizzazione degli epitopi riconosciuti dalle stesse cellule e dello scarso numero di linfociti autoreattivi presenti nel sangue periferico dei soggetti affetti. In un processo autoimmune cronico, la continua stimolazione da parte dell'antigene endogeno determina un progressivo impoverimento del pool T cellulare autoreattivo. D'altra parte, in tali patologie, molto spesso cloni T cellulari autoreattivi sono presenti più nell'organo target (per esempio le isole pancreatiche) che nel sangue periferico. E' stato stimato che nel sangue periferico di soggetti affetti da DT1 meno di 1 linfocita ogni 30,000 riconosca e venga di conseguenza attivato da epitopi antigenici del GAD-65. Pertanto, le tecniche per studiare la risposta T cellulare nel DT1 devono assicurare un'altissima sensibilità. Recentemente, è stato dimostrato come la tecnica dell'ELISPOT (enzyme-linked immunosorbent spot) permetta l'identificazione di singole cellule T antigene-specifiche, sebbene esse siano presenti a bassissime concentrazioni nel sangue periferico. Infatti mediante ELISPOT è possibile misurare linfociti T autoreattivi anche ad una concentrazione di 1:1,000,000.

La presentazione di determinati peptidi da parte del MHC di classe II è un processo fondamentale per la risposta immunitaria e pertanto è altamente codificato e controllato. Gli epitopi presentati dal MHC di classe II vengono legati alle molecole MHC in un compartimento post-Golgi, presente in tutte le cellule presentanti l'antigene (cellule dendritiche, macrofagi e linfociti B) deputato esclusivamente a questo processo. A questo meccanismo partecipa attivamente una proteina denominata Ii, un cui peptide (Ii77-92; LRMKLPKPKPPKPVSQMR) aumenta *in vitro* la presentazione antigenica come dimostrato da studi su ibridomi T cellulari. Di questo peptide è stata isolata una breve sequenza che contiene solo quattro aminoacidi (LRKM) che è stata definita peptide Ii-key. Recentemente, è stato dimostrato che legando ad Ii-key un peptide antigenico mediante un semplice ponte

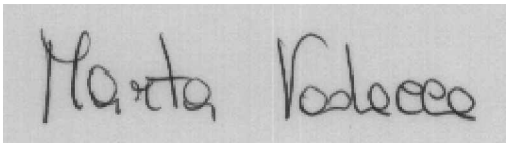


(-ava-) di polimetilene, è possibile aumentare la potenza della presentazione antigenica del peptide stesso. Il peptide derivante dall'unione dell'Ii-key con il peptide antigenico viene definita ibrido Ii-key. Il maggior vantaggio di quest'approccio è che esso permette di presentare il peptide antigenico sulla superficie delle molecole del MHC di classe II, bypassando il processo intracellulare. Questa strategia è completamente innovativa ed è stato dimostrato che utilizzando key-ibridi è possibile aumentare la stimolazione T cellulare sia *in vitro* che *in vivo*. La nuova tecnologia dei key-ibridi è stata recentemente applicata con successo a patologie infettive (HIV) e tumorali (melanoma, tumore al seno) e numerosi altri studi sono tuttora in corso.

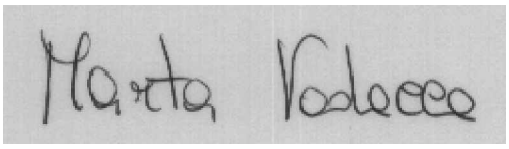
Scopo del presente studio è stato quello di valutare la risposta T cellulare in pazienti affetti da DT1 mediante la determinazione dell'INF-γ prodotto dai linfociti T in risposta alla stimolazione con peptidi di insulina e GAD, combinati con i peptidi "Ii-key" (Ii-key hybrids), utilizzando la tecnica dell'ELISPOT assay.

Gli epitopi dell'insulina e del GAD che sono noti essere riconosciuti dai linfociti T autoreattivi dei pazienti affetti da DT1 sono stati selezionati dalla letteratura. La sequenza dell'insulina e del GAD è stata ottenuta dalla Genbank. Applicando il programma Rammensee SYFPEITH (http://syfpeithi.bmiidelberg.com/Scripts/MHCServer.dll/EpPredict.htm) sono stati selezionati gli epitopi presumibilmente riconosciuti dal DRB1*0301 e DRB1*0401. E' stato pertanto possibile produrre peptidi ibridi Ii-key, costituiti da: a) una catena costante LRKM-ava- (ava=acido aminovalerico; 5 aminopentanoico) e b) la sequenza del peptide antigenico con una porzione C-terminale costante e una porzione N-terminale variabile in base alla delezione di un amino acido per ciascun ibrido della serie. La parte N-terminale è stata acetilata e la porzione N-terminale amidata per bloccare il catabolismo da parte di amino- e carbossi- peptidasi. Per ciascun antigene sono state isolate 5 serie di peptidi costituite ciascuna da un peptide di controllo (peptide antigenico insulinico e del GAD) e da un numero variabile (da 2 a 4) di key-ibridi. Sono quindi stati testati 15 ibridi per ciascun antigene e confrontati ciascuno con il rispettivo peptide di controllo. Sono stati effettuati prelievi di sangue periferico a digiuno. I linfociti sono stati separati utilizzando il metodo standard di separazione Ficoll Hypaque. Il test ELISPOT è stato effettuato utilizzando il kit BD Pharmingen per IFN-γ seguendo le istruzioni fornite dalla casa produttrice. Sono stati studiati in totale 38 pazienti affetti da T1D e 12 controlli con i peptidi/Ii-Key-hybrids dell'insulina e 8 pazienti e 7 controlli con i peptidi/Ii-Key-hybrids del GAD. Le immagini digitali degli spot sono state analizzate mediante il Software Eli.Analyse 4.0.

Il 20% dei pazienti testati con i Ii-Key hybrids dell'insulina mostravano una produzione di INF-γ maggiore rispetto alla produzione determinata con la stimolazione dei soli peptidi. Negli altri pazienti testati e nei controlli sani non era evidenziabile alcuna risposta né ai peptidi dell'insulina né ai Ii-Key-insulin-hybrids. Gli Ii-Key-insulin-hybrids testati sono stati riconosciuti solo dai soggetti DR3/DR4 0302+vi. Per quanto riguarda i peptidi del GAD, 2 su 8 pazienti (25%) ha mostrato una risposta positiva a uno o più Ii-Key-GAD-hybrids rispetto a nessuno dei controlli sani. Tali risultati supportano l'ipotesi che nei pazienti con DT1 i Key-ibridi riescono ad elicitare una risposta immunitaria anche nei soggetti che non presentano reattività T cellulare al peptide antigenico. Inoltre, il nostro studio dimostra che tale risposta è HLA dipendente. Questo è il primo studio che applica al DT1 la tecnica

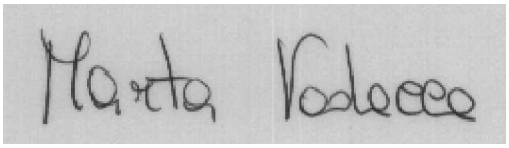


recentemente introdotta degli Ii-key hybrids. Gli Ii-Key hybrids possono rappresentare un nuovo strumento per la studio della risposta T cellulare nel T1D. La selezione di specifici Ii-Key hybrids e l'utilizzo combinato di Ii-Key-insulin-hybrids e Ii-Key-GAD-hybrids potranno portare ad incrementare la sensibilità del test.

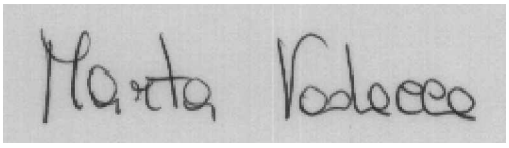


ABSTRACT

Type 1 diabetes (T1D) is the result of a T cell-mediated destruction of pancreatic β cells in the islets of Langerhans. In diabetic animal models and humans, a correlation between disease onset and cytokines, in particular interferon (INF)-γ has been observed and these studies support the promotion of inflammatory cellular immune responses by T lymphocytes. The development of T1D is also associated with the major histocompatibility complex (MHC) class II genes. An important role of the highly polymorphic HLA class II immune recognition molecules, DR and DQ, located on chromosome 6 has been consistently confirmed. In T1D, the specific epitopes recognized by pathogenic T cells remain poorly defined and the specific autoreactive T cells are difficult to detect due to the low number in the peripheral blood, the inhibition by populations of regulatory T cells and the inability to assay cells from the inflammatory lesion. Moreover, autoantigen reactive T cells are also detected in healthy individuals; therefore the development of new techniques to identify with high specificity β cells specific T cells in T1D subjects remains a major goal in the study of the disease. The normal process of MHC class II antigen charging and presentation is highly controlled, in order to assure the presentation of selected peptides. MHC class II epitopes are normally bound to MHC class II molecules in a post-Golgi, antigenic peptide-binding compartment of antigen presenting T cells (dendritic cells, macrophages and B cells). The Ii protein, which is coded by one gene in all humans, forms a trimer at synthesis in the endoplasmatic reticulum. The CLIP (cleaved leupetin-induced peptide) peptide of the Ii protein remains on some MHC class II molecules and might facilitate charging of MHC class II molecules with antigenic peptides. A second immunoregulatory peptide, called the Ii-key peptide, also regulates antigenic peptide binding and release of antigenic peptides, even on cell surface-expressed MHC class II molecules. A peptide of the Ii protein, Ii (77-92; LRMKLPKPKPPKPVSQMR), enhanced *in vitro* presentation of antigenic peptides. The shortest sequence with half-maximal activity of the most potent peptide LRMKLPK contains only four amino acids (LRMK). These Ii-key peptides appear to act at an allosteric site on MHC class II molecules to facilitate charging and presentation of vaccine peptides into the antigenic binding site. The covalent linkage of the Ii-Key core sequence with the antigenic peptide appears to lead to an increased level of binding and recognition of antigenic determinants. This could occur due to increased representation of the antigenic epitope on the surface of the APC, or to some favorable structure change in the MHC antigenic peptide T cell receptor complex. Hybrids consisting of antigenic peptide-flexible-linker-Ii-Key peptide have up to 250 times the potency of the respective antigenic peptide in a T hybridoma response assay. In type 1 diabetes is widely assumed that the pathogenetic process is orchestrated by autoreactive T cells, but to date the studies can usually make use of peripheral blood, in which islet autoreactive T cells are likely to be rare and the epitopes remain poorly characterized. For these reasons, assays must be highly sensitive. The cytokine enzyme-linked immunosorbent spot (ELISPOT) assay has many of the requisite qualities. Detection sensitives as low as one responder cell per million have been claimed. ELISPOT assay can be used to detect early antigen directed activation of lymphocytes subpopulation at the single cell level. The main objective of this study was to



investigate the cellular immune response in T1D by characterising the cytokines secreted by peripheral blood mononuclear cells (PBMCs) in response to the exposure to Ii-key/MHC class II insulin/GAD epitope hybrids. To date, this is the first study using the recently introduced novel technique of Ii-key/MHC class II epitope hybrids in diabetes. The first point of this study was to establish the validity of using Ii-Key/MHC class II insulin epitope hybrids to detect anti-insulin responses in patients with T1D. We focused first on MHC class-II presented epitopes in insulin because computer based analysis revealed a plethora of theoretical epitopes in the sequence of the insulin, which have been reported to contain active epitopes that stimulate responses by $CD4^+$ T cells of type 1 diabetics. We identified five epitopes series. Within a homologous series, the longest and shortest hybrids were taken for initial synthesis plus the shortest peptide of 9 amino acids being considered to be a control. Peptides of insulin shown to be recognized by $CD4^+$ T cells of T1D patients are selected from the literature. ELISPOT assay were performed with BD Pharmingen set for IFN-γ according to the manufacturer's instructions. The study has been carried out in 23 patients affected by type 1 diabetes and in 2 controls. When sufficient cells were available, all epitopes series were analyzed. Initial data showed that some hybrids, and not the epitope-only peptide, stimulate some reactions in some patients. It has been noted that the responder rate was lower in type 1 diabetes of long-standing than in newly diagnosed patients. There was a strong positive response to specific T cell antigen (PHA or PMA). To confirm these preliminary data, we successively performed the study in 15 patients affected by T1D and in 10 healthy Caucasian nondiabetic control subjects. In our tests we added IL-12, because it's a uniquely different cytokine that only acts on activated T cells. All cases and control subjects showed a detectable and significant INF-γ response to stimulation with the polyclonal T cell stimulus PMA/Ionomycin. Spontaneous production of INF-γ was present at similar, very low levels in both patients and healthy controls. Control subjects showed no detectable response to stimulation with Ii-key hybrids or peptides. Three out 15 patients (20%) demonstrated a positive response to one or more peptides compared with none of 10 nondiabetic control subjects tested. Tested peptides have been shown to be recognized in DR3/DR4 0302+ve diabetic patients, but not in other patients (DR3 or DR4+ve) or normal subjects. In the third part of the study we investigated the cellular immune response in T1D by characterising the cytokines secreted by peripheral blood mononuclear cells in response to exposure to Ii-key/MHC class II GAD epitope hybrids. We tested 8 patients affected by T1D and 7 control subjects. Two out 8 patients (25%) demonstrated a positive response to one or more Ii-Key/GAD65 hybrids compared to none of 7 nondiabetic control subjects. The Ii-key hybrid technique is a new strategy for augmenting the potency of MHC class-II restricted epitopes resulting in a profound increase in T cell stimulation. This study demonstrated in a first step that some Ii-key insulin hybrids and not the epitope-only peptide, stimulate strong reactivity in T1D subjects. As second step, we demonstrated a specific response to Ii-key/GAD65 hybrids only in T1D. The combined use of insulin and GAD key-hybrids might therefore assure a more selective and sensitive cytokine detection in response to antigen stimulation in T1D offering a new highly specific marker of cell mediated immunity in T1D.



ABSTRACTS

The following abstracts have been published and accepted for oral presentation in support of this thesis.

- **Vadacca M.,** *MG Valorani, L Valente, RE Humphreys, P Pozzilli*
  A novel approach to study T cell response to Ii-Key/MHC class II epitome hybrid peptides in type 1 diabetes.
  Diabetologia 2005;48 Suppl 1:A99.
  41st EASD Annual Meeting-Athens, 12-15 September 2005

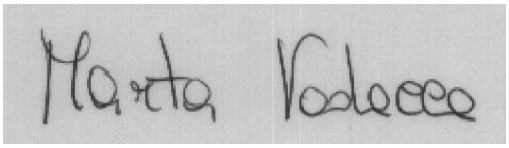
- **M. Vadacca,** *MG. Valorani, NL Kallinteris, L Valente, RE Humphreys, G D'Agostino, P Pozzilli, Gruppo IMDIAB*
  Peptidi antigenici Ii-Key/Insulina: un nuovo approccio per lo studio della risposta T cellulare in pazienti con diabete di tipo 1.
  Il Diabete 2006; Suppl. 1:C061.
  21° Congresso Nazionale SID-Milano, 17-20 Maggio 2006

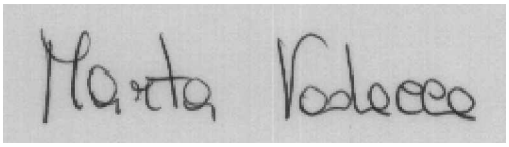
- **Vadacca M.,** *Robert E Humphreys, Nikoletta Kallinteris, Luciana Valente, Maria Giuditta Valorani, Paolo Pozzilli, IMDIAB Group*
  Ii-Key/Insulin MHC class-II antigenic epitope peptides in type 1 diabetes.
  Diabetes 2006; 55 Suppl 1: 378OR.
  66th ADA Scientific Session-Washington, 9-13 June 2006

- **M.Vadacca,** *M.G. Valorani, R.E. Humphreys, N. Kallinteris, L. Valente, P.Pozzilli, IMDIAB Group*
  Ii-key/MHC class II epitope hybrid technology and ELISPOT assay in type 1 diabetes.
  Diabetologia 2006; 49 Suppl.1:165-66.
  42nd EASD Annual Meeting Copenhagen, Denmark, 14-17 September 2006

- **M.Vadacca,** *M.G. Valorani, R.E. Humphreys, N. Kallinteris, L. Valente, P.Pozzilli, IMDIAB Group*
  Ii-key/MHC class II epitope hybrid technology to detect T cell response to insulin and GAD in type 1 diabetes.
  Diabetologia 2007; 50 Suppl 1:S30.
  43rd EASD Annual Meeting, Amsterdam, Netherlands, 16-21 September 2007

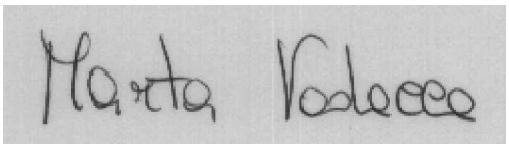
- **M.Vadacca,** *M.G. Valorani, R.E. Humphreys, N. Kallinteris, L. Valente, P.Pozzilli, IMDIAB Group*
  Ii-Key-Hybrid Technology To Detect T Cell Response To Insulin And GAD In Type 1 Diabetes.
  Diabetes 2008; 57 Suppl 1: page A18.
  68 th ADA Scientific Session-San Francisco, 6-10 June 2008





The following abstract has been published in support of this thesis.

- **Vadacca M,** *MG Valorani, RE Humphreys, N Kallinteris, L Valente, P Pozzilli, IMDIAB Group*
  Ii-Key/Insulin MHC class-II antigenic epitope peptides in type 1 diabetes.
  10th Annual CDA/CSEM Professional Conference and Annual Meetings
  Toronto, October 18-21, 2006



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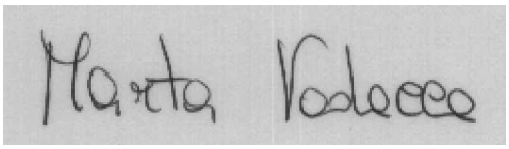
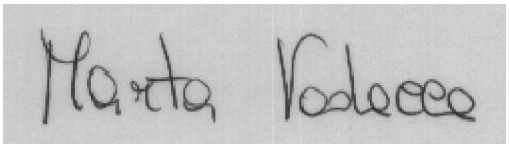
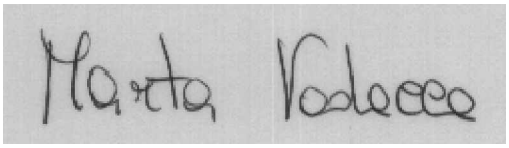
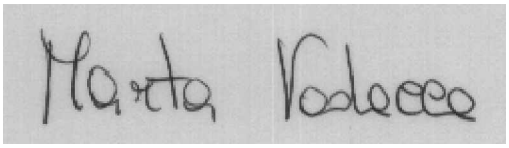


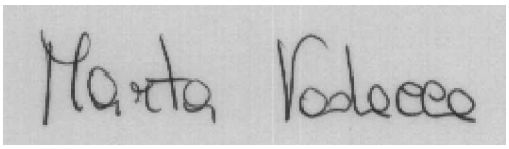
CHAPTER 1: GENERAL INTRODUCTION



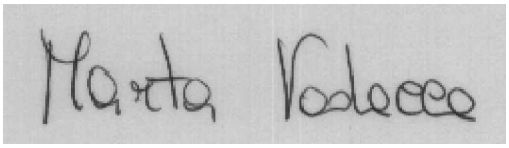


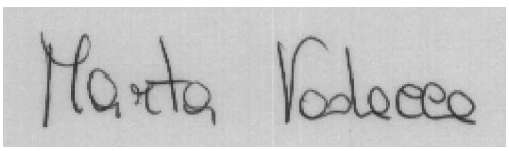


CHAPTER 2: INTRODUCTION TO THE STUDY

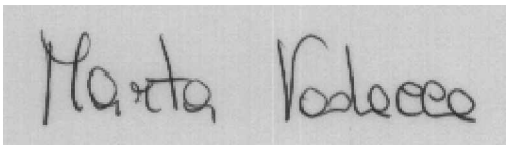


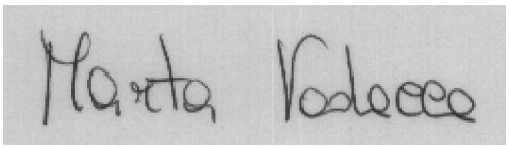
CHAPTER 3: MATERIALS AND METHODS



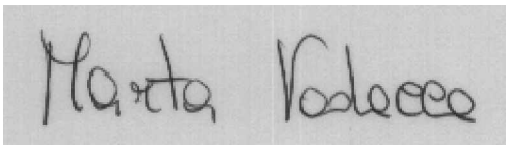


CHAPTER 4: SELECTION OF MHC CLASS II-PRESENTED EPITOPES IN PROINSULIN AND GLUTAMIC ACID DECARBOXYLASE (GAD) IN TYPE 1 DIABETIC PATIENTS

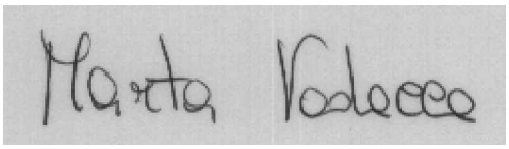


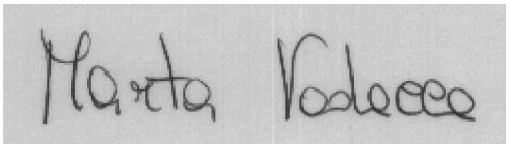


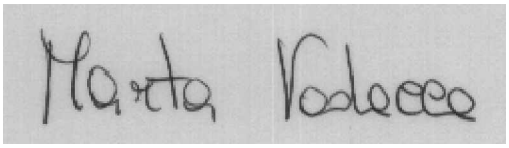
CHAPTER 5: Ii-KEY HYBRIDS PEPTIDES



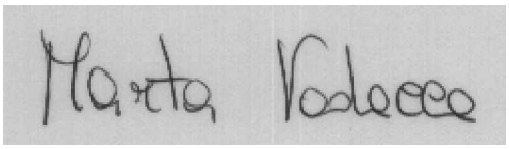
CHAPTER 6: II-KEY HYBRIDS TO DETECT T CELL RESPONSE IN TYPE 1 DIABETES

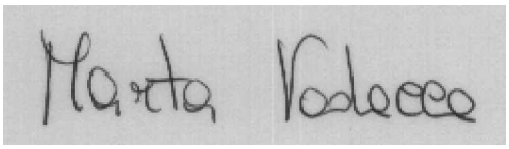


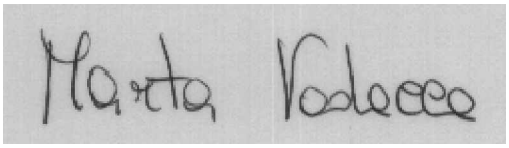




CHAPTER 7: DISCUSSION







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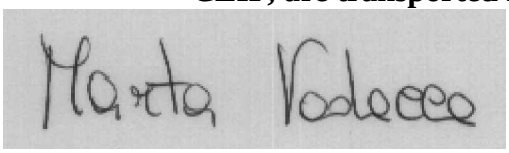

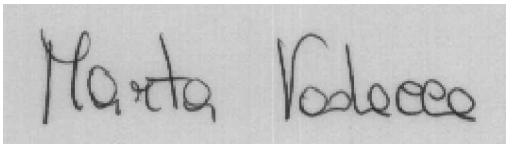

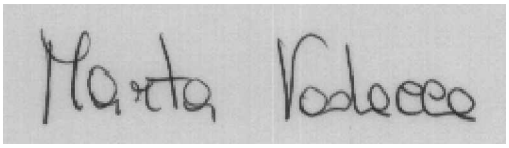

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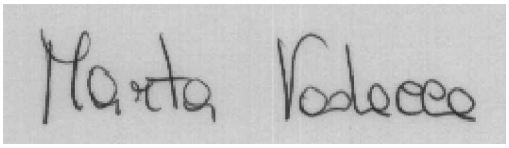

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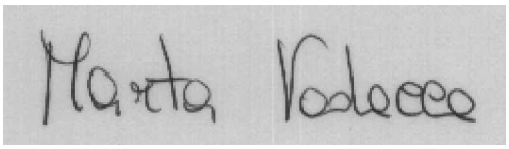
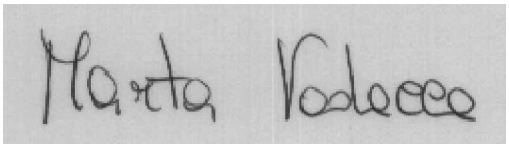
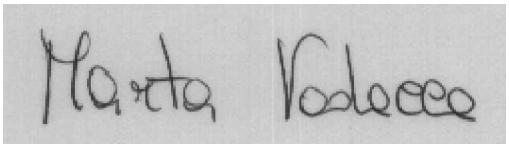


ABBREVIATIONS

APC	**antigen presenting cell**
AVA	**amino-valeric acid**
BSA	**bovine serum albumin**
CFA	**complete freunds adjuvant**
CK	**cytokine secreting cells**
CLIP	**class II-associated invariant chain peptide**
CMV	**cytomegalovirus**
CTLA4	**cytotoxic T lymphocyte antigen 4**
DMSO	**dimethyl sulphoxide**
DNA	**deoxyribonucleic acid**
DZ	**dyzigotic**
EAE	**experimental autoimmune encephalitis**
EGF-R	**epidermal growth factor receptor**
ELISA	**enzyme linked immunosorbent assay**
ELISPOT	**enzyme linked immunosorbent spot**
ER	**endoplasmic reticulum**
GAD	**glutamic acid decarboxylase**
GITR	**glucocorticoid induced tumour necrosis factor receptor**
HLA	**human leukocyte antigen**
IAA	**insulin auto antibodies**
IA-2	**insulinoma associated antigen**
ICA	**islet cell antigen**
Ii	**invariant chain**

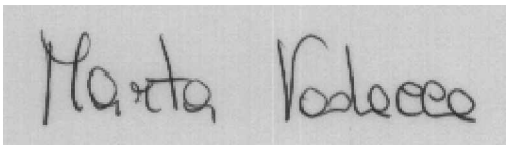


IL	**interleukin**
INF	**interferon**
LDA	**limiting dilution assay**
MHC	**major histocompatibility complex**
MS	**multiple sclerosis**
NK	**natural killer**
NO	**nitric oxid**
NOD	**non-obese diabetic**
PBMC	**peripheral blood mononuclear cell**
PE	**phycoerythrin**
PHA	**phytohemagglutinin**
PMA	**phorbol myristate acetate**
PTP	**protein tyrosine phosphatase**
SA	**streptavidin**
SD	**standard deviation**
SI	**stimulation index**
SLE	**systemic lupus erythematosus**
T1D	**type 1 diabetes**
TGF	**transforming growth factor**
Th	**T helper**
TLR	**toll like receptors**
TNF	**tumour necrosis factor**



UNITS

g	**gram**
h	**hour**
l	**litre**
M	**Molar**
m	**milli**
min	**minute**
n	**nano**
p	**pico**
s	**second**
v	**volume**
w	**weight**
%	**percentage**
°C	**degrees centigrade**
µ	**micro**



1.2 Diabetes Mellitus

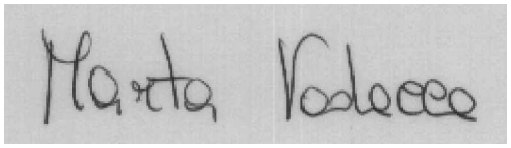
Diabetes mellitus represents a heterogeneous group of disorders. Some distinct diabetic phenotypes can be identified in terms of specific aetiology and/or pathogenesis, but in many cases overlapping phenotypes make etiological and pathogenic classification difficult (Pociot F, 2002).

1.2.1 Type 1 diabetes

Type 1 (insulin-dependent) diabetes mellitus (T1D) develops as a result of pancreatic beta-cell destruction and is characterised by absolute insulin deficiency, an abrupt onset of symptoms, propeness to ketosis and dependency on exogenous insulin to sustain life (Leslie RD, 2006).

1.2 Epidemiology of type 1 diabetes

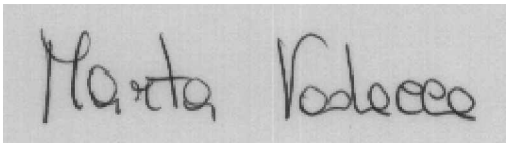
T1D is the most common form of diabetes among children and young adults in populations of Caucasoid origin, where the prevalence is approximately 0.4%. Large differences in incidence have been reported in Caucasoid populations living in relatively close proximity and among those who are genetically similar. Large interethnic differences in the incidence rates between Jewish and Arab populations have been reported in Israel (Laron-Kenet T, 2001). Geographical variation in incidence appears to reflect the global distribution of major ethnic populations, which demonstrates a different degree of genetic susceptibility to diabetes among populations (Holmberg H, 2006). The overall age-adjusted incidence of T1D varies



from 0.1/100.000 per year in China and Venezuela to 36/100.000 per year in Sardinia and Finland (Karvonen M, 2000; Carle F, 2004). Most of the information regarding T1D incidence thus far has come from regions with a high or intermediate incidence, mostly in Europe and North America where several registries have been established since the mid-1980s or earlier. The data from Asia, South America, and Africa are still sparse. Although the populations with very high incidence rates were europoid populations in Europe and other continents, populations with a relatively high incidence rate were also found in tropical or subtropical areas such as Kuwait and Puerto Rico. The incidence of T1D appears to be increasing in almost all populations worldwide and the increase is particularly high in populations with a low incidence. However, the global pattern of the incidence of T1D has not changed markedly since the reports published during the 1970s and 1980s (Karvonen M, 2000).

1.2.1 Cost to the community

It is generally recognized that the costs of treating chronic medical conditions are rising for health care systems because of an aging population and the increasing prevalence of such conditions. Estimating the progressive costs of diabetes is important for resource allocation, health policy, and perhaps even allocation of research funding. Accurate costing is important, given that a substantial portion of costs are not attributable directly to the condition itself but to accumulated complications and comorbidities (Johnson JA, 2006). Overall per capita health care costs for individuals with diabetes would increase over the 10 years after identification of diabetes, as a result of disease progression, accumulation of comorbidities, and deterioration of health status. After a large increase in costs in the



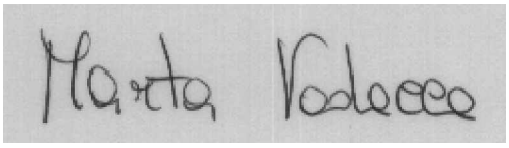
incident year, costs were relatively stable, despite increases in prescription, day surgery, and dialysis costs. Decreased hospital costs over the study period, which offset cost increases in the other categories, may reflect changes in hospitalization rates, length of stay, case mix, and service intensity. It is also possible that decreased hospital costs in the later years of follow-up reflect a survival bias in which healthier individuals survived to the end of the follow-up period. Prescription medications were the second largest cost category after hospitalizations during most years of follow-up. Medication costs increased considerably from 1991 to 2001 for both type 1 and type 2 diabetes. This trend could be the result of *1*) increased use of medications overall, *2*) increased use of more expensive disease treatment options for cardiovascular risk reduction, and *3*) use of newer, more expensive antidiabetic agents in the later years of follow-up (Johnson JA, 2006).

1.3 Causes of type 1 diabetes

T1D is due to the destruction of the insulin secreting cells in the islets of Langherans of the pancreas thought to be mediated by an abnormal immune response (Leslie RD, 2006). The risk of developing T1D in humans is determined by a complex interaction between inherited predisposing genetic factors and various environmental triggers (Beyan H, 2003). Evidence for genetic and non-genetic factors in causing T1D will be discussed in further detail.

1.3.1 Genetic influence in type 1 diabetes

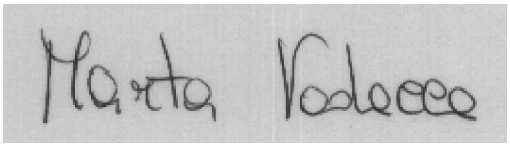
The simplest evidence that T1D has a genetic influence in man is shown by the observation that frequency of T1D is higher in relatives of diabetic patients than in the general population. In Caucasian populations, the overall lifetime risk of



developing T1D is approximately 0.4% (Knip M, 2002; Adeghate E, 2006). Family studies have shown that this risk increases among subjects with a positive family history of T1D. The average risk for siblings of a T1D patient is 5%-6%, therefore a sibling of a T1D patient is 15 times more likely to develop T1D than in general population. Children of diabetic patients also have a higher frequency of T1D, approximately 3%-6%. This increased risk may indicate a genetic contribution or shared environmental factors by them. To determine the degree genetic factors contribute to this increased risk, studies using twins have been performed. Twin studies are a powerful tool to assess genetic and non-genetic factors in multi-factorial, immune mediated diseases and to estimate the impact of genetic factors on a particular disease (Leslie RD, 2004). The difference in concordance rate between monozygotic twins (approximately 40%) and dyzigotic twins (approximately 6%) is consistent with a significant genetic contribution. The high discordance rate among monozygotic twins however indicates that the susceptibility genes have a low input to development of the disease. This is confirmed by the fact that many individuals who are at high risk of T1D do not develop the disease. This observation highlights the importance of environmental triggers, which may account for up to two-thirds of diabetes susceptibility.

1.3.1.1 HLA-encoded susceptibility to type 1 diabetes

Twin studies, family studies and animal models have helped to elucidate the genetics of T1D. At least 20 different chromosomal regions have been linked to susceptibility of T1D. The best evidence for a genetic susceptibility to T1D comes from studies of the HLA genes in both populations and families as well as from animal studies. It has



been estimated that HLA provides up to 40-50% of the familial clustering of T1D. The HLA region is a cluster of genes located within the major histocompatibility complex (MHC) on chromosome 6p21 (Pociot, 2002). Reports suggest that these genes located within the MHC account for at least 40% of the familial clustering of T1D (Petrone A, 2002). The genes encoding the MHC region encode MHC class I (Figure 1) and class II molecules (Figure 2), which are heterodimers made up of alfa- and beta-chains molecules.

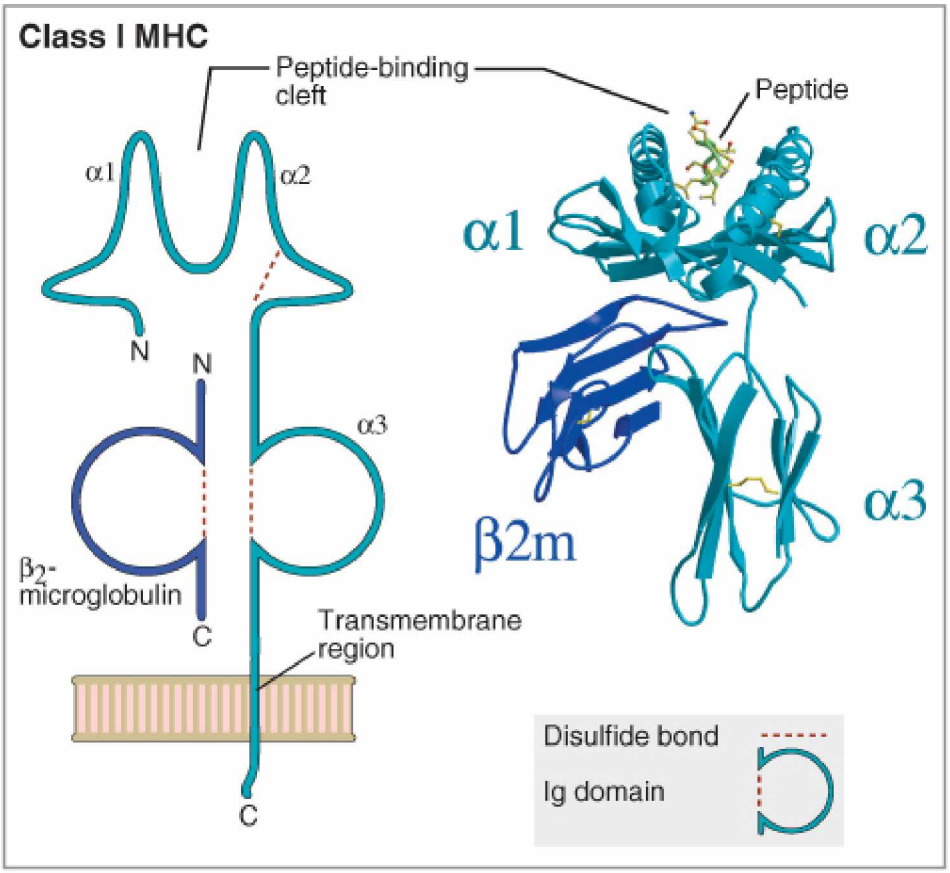
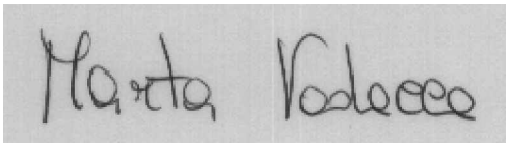


Figure 1.
The schematic diagram *(left)* illustrates the different regions of the MHC molecule (not drawn to scale). Class I molecules are composed of a polymorphic α chain no covalently attached to the non-polymorphic β_2-microglobulin (β2m). The α chain is glycosylated; carbohydrate residues are not shown. The ribbon diagram *(right)* shows the structure of the extracellular portion of the HLA-B27 molecule with a bound peptide, resolved by x-ray crystallography. (Courtesy of Dr. P. Bjorkman, California Institute of Technology, Pasadena.) Not copy-right issue.



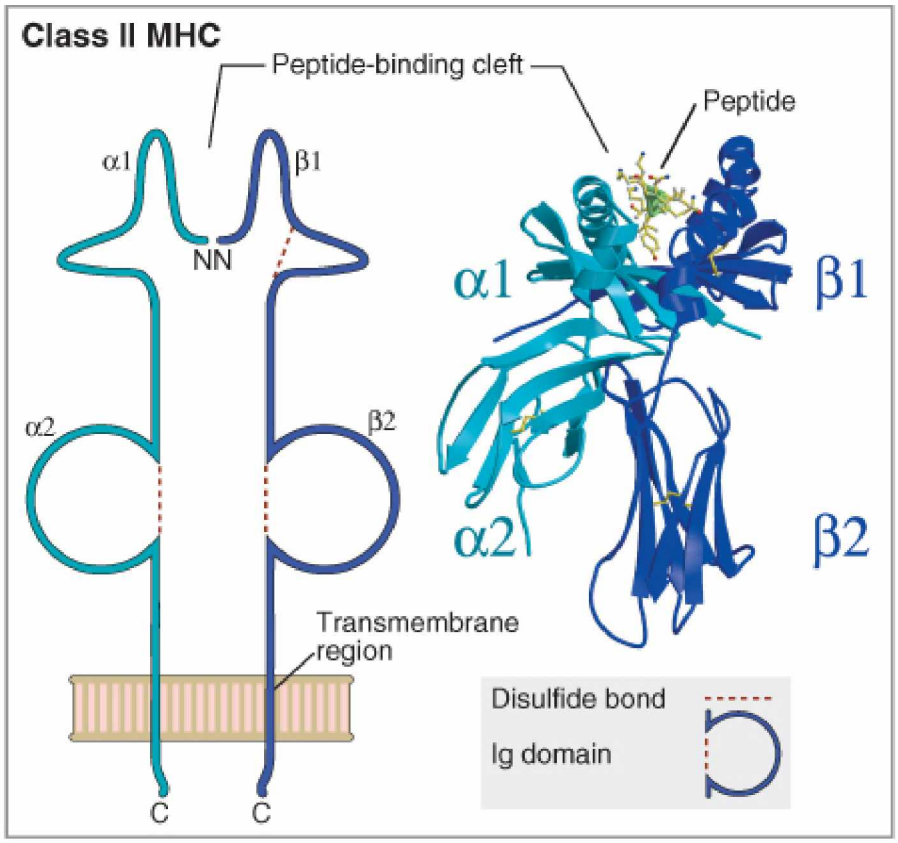
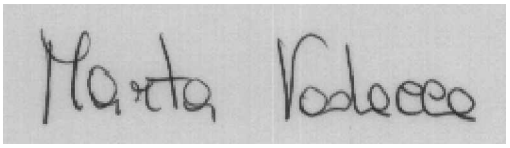


Figure 2.
The schematic diagram *(left)* illustrates the different regions of the MHC molecule (not drawn to scale). Class II molecules are composed of a polymorphic α chain no covalently attached to a polymorphic β chain. Both chains are glycosylated; carbohydrate residues are not shown. The ribbon diagram *(right)* shows the structure of the extracellular portion of the HLA-DR1 molecule with a bound peptide, resolved by x-ray crystallography. (Courtesy of Dr. P. Bjorkman, California Institute of Technology, Pasadena.) Not copy-right issue.

The main function of these molecules is to present antigens that have been processed into peptides to antigen specific receptors on CD4+ and CD8+ T lymphocytes (Figure 3).



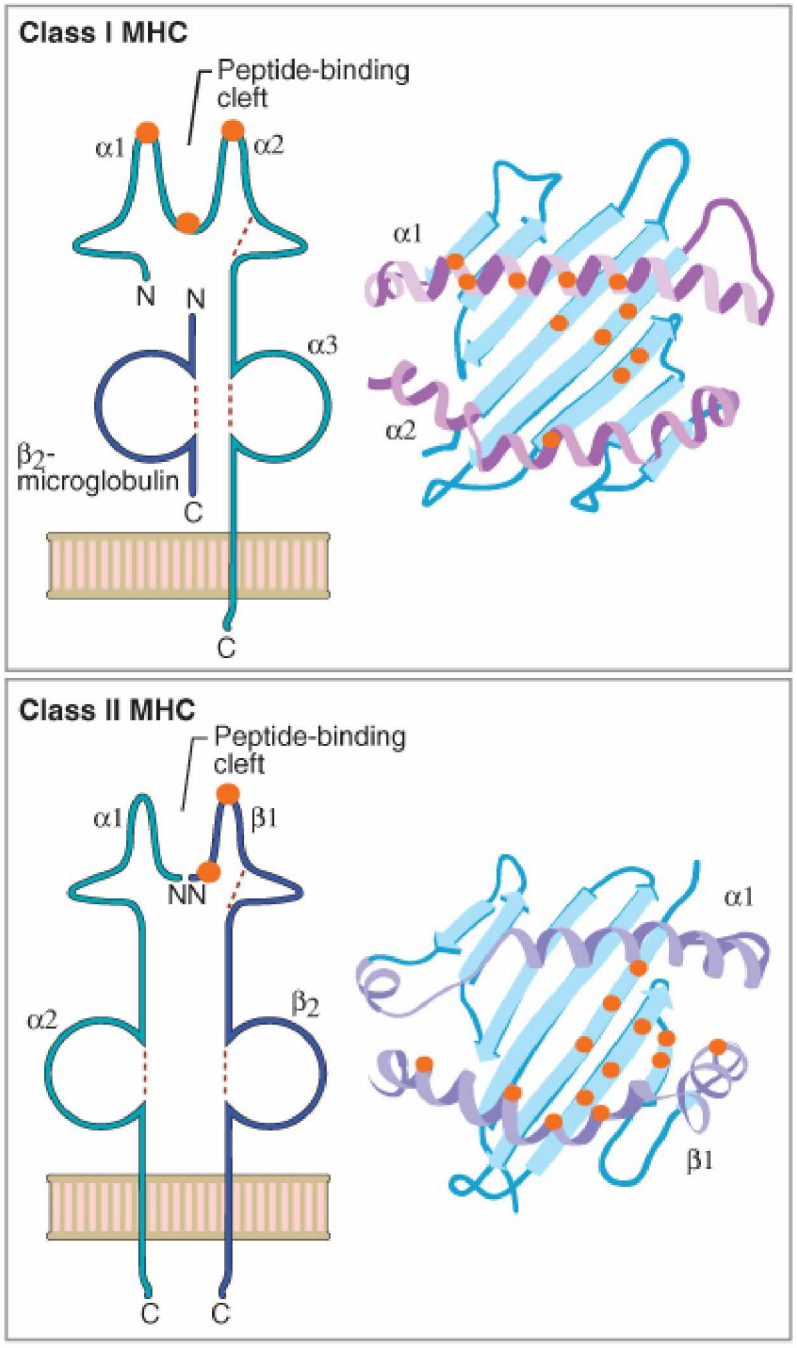
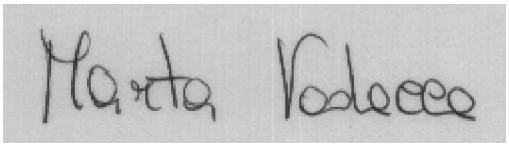


Figure 3.
The polymorphic residues of class I and class II MHC molecules (shown as red circles) are located in the peptide-binding clefts and the α-helices around the clefts. In the class II molecule shown (HLA-DR), essentially all the polymorphism is in the β chain. However, other class II molecules in humans and mice show varying degrees of polymorphism in the α chain and usually much more in the β chain. (Courtesy of Dr. J. McCluskey, University of Melbourne, Parkville, Australia.) Not copy-right issue.



Antigens bound to MHC class II molecules are recognised by CD4+ T lymphocytes. Nearly all nucleated cells express MHC class I molecules. MHC class II molecules are expressed by B lymphocytes, macrophages and other APCs, and, in humans by activated T lymphocytes. The MHC molecules are highly polymorphic which results from different genes encoding families of MHC molecules (Buzzetti R, 2004). The association of MHC alleles, also termed HLA alleles in humans and T1D was recognised more than 30 years ago by Nerup and colleagues (Nerup J, 1976). The statistically strongest genetic association with T1D is conferred by HLA class II gene alleles. The class II molecules, also known as immune responses genes, are termed DR, DP and DQ (Figure 4).

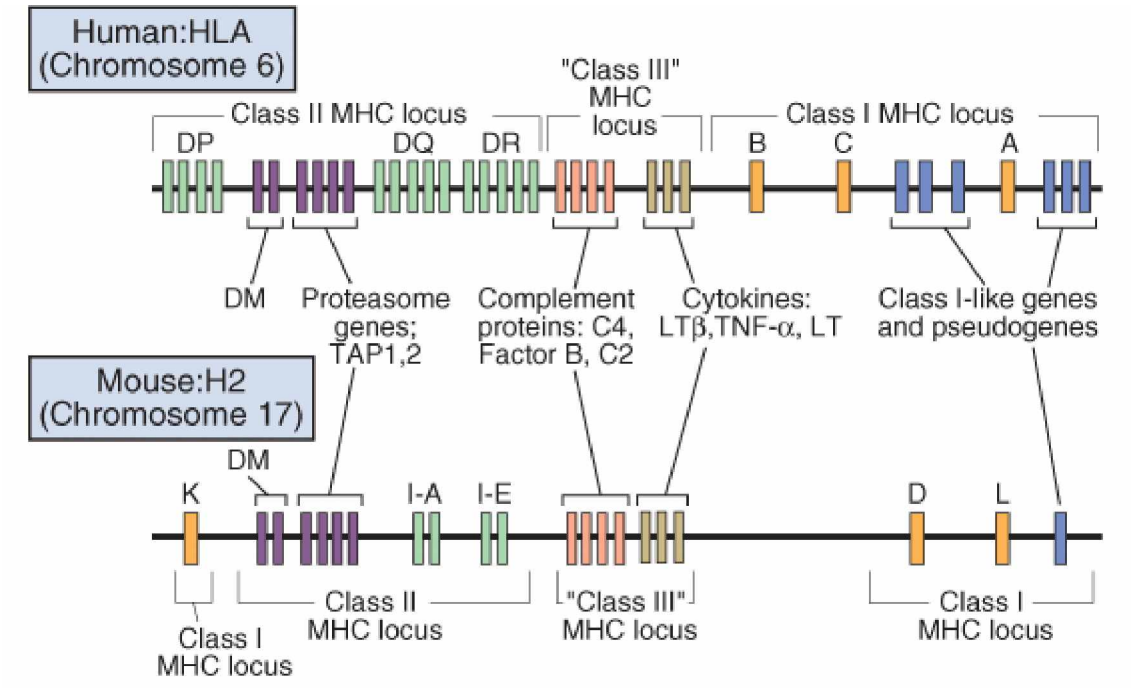
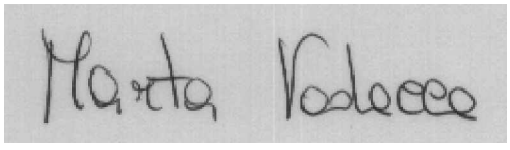


Figure 4.
Sizes of genes and intervening DNA segments are not shown to scale. Class III MHC locus refers to genes that encode molecules other than peptide-display molecules; this term is not used commonly. Not copy-right issue.



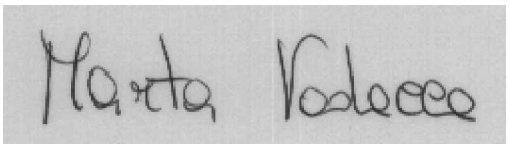
Extensive sequencing of MHC class II alleles has revealed a complex interplay between alleles of the two major isotypes of MHC class II molecules, HLA DR and DQ. HLA DR3 and DR4 have been shown to be strongly associated with T1D. DR3 and DR4 were found to be expressed in 55% and 75% of T1D children compared with 20% and 26% of controls, respectively. The combination of DR3 and DR4 were present in 37% of the diabetic children, compared with only 4% of the controls. Approximately 30% of T1D subjects are HLA-DQ2/DQ8 heterozigotes. By contrast, a particular DQ6 molecule is associated with strong protection from the disease, even in the presence of T1D-associated autoantibodies and/or of high risk HLA alleles, and has been reported in less than 1% of patients in most populations studied (Sanjeevi CB, 2002) (Table 1).

Table 1.

Type 1 diabetes-associated HLA class II alleles and haplotypes.

HLA-DQ alleles	**HLA-DR alleles**	**RR**
Positive associations (Susceptibility haplotypes)		
A1*0301-B1*0302	DRB1*04	2.5-9.5
A1*0501-B1*0201	DRB1*301	2.5-5.0
A1*0501-B1*0302	DRB1*301/ DRB1*04	12.0-32.0
A1*0301-B1*0201	DRB1*301/ DRB1*04	
A1*0301-B1*0402	DRB1*04/DRB1*801	4.0-15.0
A1*0301-B1*0201	DRB1*701	8.0-13.0
A1*0301-B1*0201	DRB1*901	5.5
A1*0301-B1*0401	DRB1*04	3.5-4.5
A1*0301-B1*0303	DRB1*901	2.0-4.5
Negative associations (Protective haplotypes)		
A1*0102-B1*0602	DRB1*1501	0.03-0.2
A1*0103-B1*0603	DRB1*1301	0.05-0.25
A1*0301-B1*0301	DRB1*04	0.2-0.5
A1*0501-B1*0301	DRB1*1101	0.05-0.5

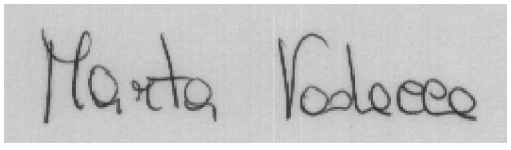
Relative risk (RR) is for the combined DQ-DR haplotype and refers to high risk populations. There is significant variation between different ethnic populations.



The fact that several different Class II MHC alleles and combinations of alleles may be associated with T1D implicates a hypothesis of a factor, which may be common to all these alleles and central to disease susceptibility e.g. the presence of specific amino acids. Certain amino acids of the DQ and DR chains correlate well with susceptibility and resistance to T1D. These residues are known to be critical for the peptide binding function of Class II molecule. In particular aspartic acid (Asp) at residue 57, which is pocket 9 of the HLA DQβ1 molecule, is encoded by a protective allele, whilst an alanine, valine or serine residue at the same position characterises predisposing alleles (Pociot F, 2002). The three dimensional structure of the class II antigen DQ showed that Asp-57 forms a salt bridge with a conserved arginine residue at position 76 of the DR-alpha chain. The beta chain residue at position 57 may affect antigen binding, by either being more effective in binding a diabetogenic antigen or by interacting with the T cell receptor, thus increasing diabetes susceptibility. However, this association does not always protect from T1D, as in Japanese patients Asp 57 predispose to T1D (Lee HG, 2005).

1.3.1.2 Non-HLA-encoded susceptibility to type 1 diabetes

There is a substantial role for non-HLA encoded susceptibility. The insulin gene represents a plausible candidate susceptibility locus since insulin or insulin precursor act as autoantigens. This susceptibility locus, *IDDM2* has been mapped to chromosome 11p15.5. Population studies of T1D patients and non-diabetic controls show a positive association between alleles of a variable number tandem repeat (VNTR) within the *IDDM2* and T1D in Caucasians (Pociot F, 2002). The fact that the autoimmune process leading to T1D is specific for beta-cells, the only cells that

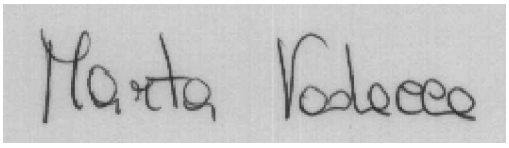


produce insulin suggests that this gene is a good susceptibility candidate. The 5'-VNTR is related to proinsulin expression in thymic epithelial cells. However IDDM2 is the only other generally accepted genetic contributor to T1D. At least 15 other genetic regions have been reported to be associated with T1D susceptibility; these have subsequently been termed IDDM3-IDDM18 (Mayans S, 2007; Ueda H, 2003).

1.3.2 Non-genetic influence in type 1 diabetes

The geographical variation in the incidence of T1D in childhood is substantial. For example a child in Finland is 36 times more likely to develop T1D than a child from Japan. Although some of this variation is due to differences in genetic susceptibility, observation from HLA studies make it unlikely that the 10 fold incidence difference in children in Europe could be exclusively due to genetic differences alone, supporting the role of environmental factors (Knip M, 2005). Studies on migrants moving from a low incidence area to a high incidence area also provide good evidence for environmental factors. There are several reports of a higher incidence of T1D in children after a low risk population has moved to a high-risk area (Knip M, 2005). For example, Bodansky and colleagues, studied the incidence of diabetes in 0-16 year old Asian children migrating from Pakistan (low incidence of diabetes) to Europe. The incidence of diabetes increased from 3.1/100,000 in the first 4 years of the study to 11.7/100,000 in the last 3 years of the study, which was comparable to the 12/100,000 cases in the control group (Cadario F, 2004).

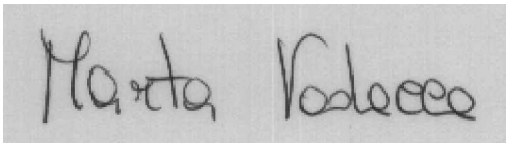
However, the most powerful evidence that T1D is due to non-genetically determined factors comes from the study of identical twins (Redondo MJ, 2001). Various group



in USA, Britain, Japan, have shown striking agreement, in that the majority of identical twins are discordant for T1D (Redondo MJ, 2001). In studies from USA and UK, concordance rates were remarkably similar and together showed only 39% of co-twins developed diabetes themselves, by 40 years of diagnosis of the index twin. Such discordance between identical twins for T1D indicates that non-genetically determined factors play an important role on causing diabetes.

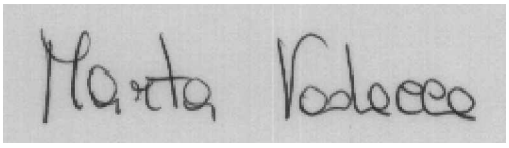
Viral infections have been associated with the development of T1D in humans for more than one hundred years (Filippi C, 2005). Viruses are thought to act against beta-cells by at least two possible mechanisms; either via a direct cytotoxic effect to the beta-cells or by triggering an autoimmune process, leading gradually to beta-cell destruction. The role of molecular mimicry in diabetes-associated responses has been indicated by the observation of structural and functional homology between viral structures and beta-cell antigens (Frisk G, 2004). In man, approximately eight viruses have been reported to be associated with the development of T1D. Candidate viruses that have been reported to be involved in the pathogenesis of diabetes include Coxsackie B virus (Yoon JW, 2006), Rubella virus (Yoon JW, 2006), Mumps virus (Bach JF, 2005), Cytomegalovirus (Bach JF, 2005; Roep BO, 2002), Epstein Barr virus (Filippi C, 2005), Varicella zoster virus (Yoon JW, 2006), Retrovirus (Bach JF, 2005), and Reovirus (Honeyman M, 2005).

Various dietary factors have been proposed as causing T1D. In this regard, it is interesting to note that there is a higher incidence in Northen Europe compared to Southern Europe (Cardwell CR, 2007). Diet has been implicated as one factor which



may explain the higher incidence of T1D found in Northern Europe, Sardinia, and in the large Mediterranean islands, that often have their own distinctive dietary patterns. One current theory is that there is a link between early cow's milk proteins, to which almost all Caucasian populations are exposed to in early life and development of T1D. This exposure early in life has been linked to the development of diabetes (Pozzilli P, 2003). It has been reported that a limited period of breast-feeding or early exposure to an artificial milk feed, increases the risk of T1D. Recent studies indicate that antibodies to cow's milk protein, in particular bovine albumine, are prevalent in children with T1D at diagnosis but rare in the general population (Pozzilli P, 2003). The cow's milk theory postulates that individuals genetically susceptible to T1D can generate antibodies against bovine albumin which cross-react with islet proteins such as protein p69 and hence through molecular mimicry lead to beta-cell destruction (Monetini L, 2003).

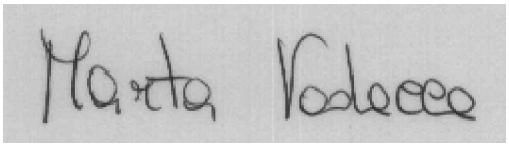
An association between the incidence of childhood T1D and the average yearly temperature in different countries has been reported, the incidence being higher in countries with a lower mean temperature (Levy-Marchal C, 2001). Data published in humans from the Eurodiab study demonstrated that in Europe reduction in Vitamin D supplementation is associated with a higher risk of the disease (Eurodiab Group, 1999). A study in Finland found that Vitamin D supplementation was associated with a decreased frequency of T1D when adjusted for neonatal, anthropometric, and social characteristics (Viskari H, 2006). Children who regularly took the recommended dose of Vitamin D (2000 IU daily) had a RR of 0.22 (0.05 - 0.89) compared with those who regularly received less than the recommended amount. Ensuring adequate



Vitamin D supplementation for infants could help to reverse the increasing trend in the incidence of T1D (Pitocco D, 2006). In NOD mice it was investigated whether 1,25-(OH)2D3 may protect human pancreatic islet cells from destruction induced by cytokines. Finally, it is of interest to mention that a primary prevention trial of T1D (Prevefin Trial) recently started in Italy uses Vitamin D supplementation at birth in genetically susceptible individuals to T1D (Lorini R, 2005; Pozzilli P, 2005).

1.4 Autoimmune diseases

The word « auto » is the Greek word for self. The immune system is a complicated network of cells and cell components that normally work to defend the body and eliminate infections caused by bacteria, viruses, and other invading microbes. A characteristic feature of autoimmune diseases is the selective targeting by the immune system (autoreactive T and B lymphocytes), which attacks specific self cells, tissues, and organs. There are many different autoimmune diseases, which can each affect the body in different ways. For example, the autoimmune reaction is directed against the brain in multiple sclerosis and the gut in Chron's disease. In other autoimmune disorders such as systemic lupus erythematosus, affected tissues and organs may vary among individuals with the same disease. One person with this disease may have affected skin, kidney and lungs (Figure 5).



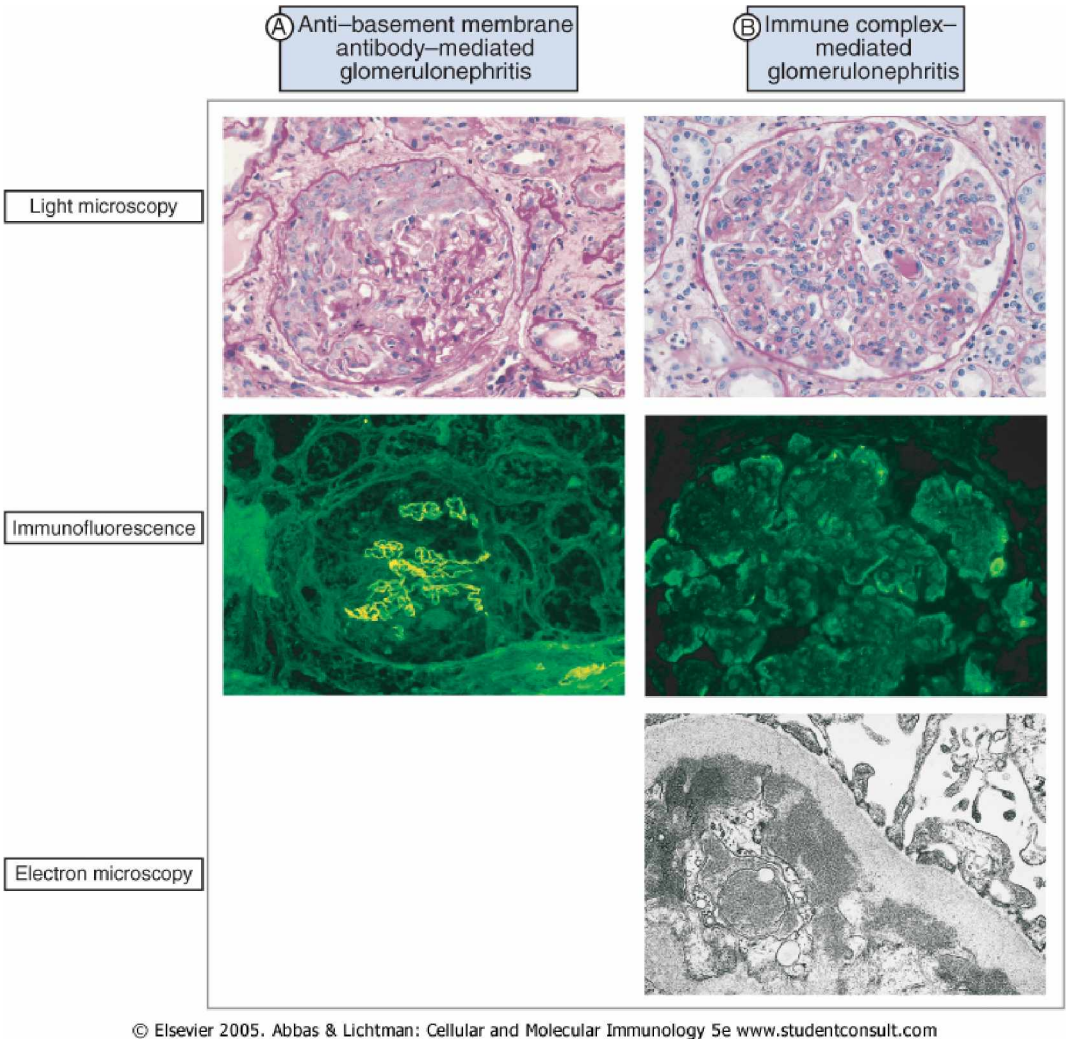
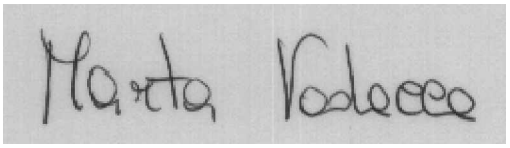


Figure 5.
Glomerulonephritis induced by the deposition of immune complexes (systemic lupus erythematosus): the light micrograph shows neutrophilic inflammation, and the immunofluorescence and electron micrograph show coarse (granular) deposits of antigen-antibody complexes along the basement membrane. (Courtesy of Dr. Helmut Rennke, Department of Pathology, Brigham and Women's Hospital, Boston.) Not copy-right issue.

Ultimately, damage to certain tissues by the immune system may be persistent, as with multiple sclerosis, rheumatoid arthritis and T1D. These are characterized by chronic inflammation, tissue destruction and malfunction of corresponding target organs (Hill NJ, 2007) (Figure 6).



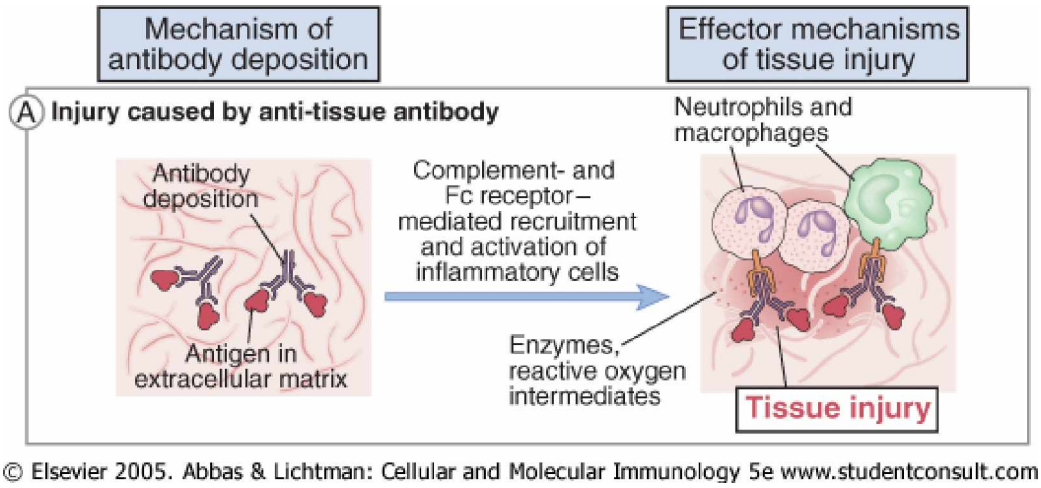


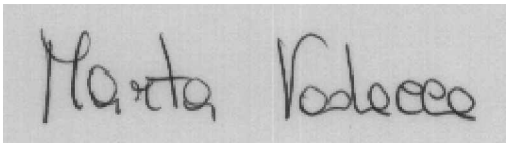
Figure 6.
Antibodies may bind specifically to tissue antigens (A), or they may be deposited as immune complexes that are formed in the circulation (B). In both cases, the deposited antibodies induce inflammation, leading to tissue injury. Not copy-right issue.

Autoimmune diseases, with the exception of rheumatoid arthritis are individually rare, but together affect approximately 5%-10% of the population of the Western countries (Molina V, 2005).

Disease	Organ	Examples of known autoantigens	Mechanism of damage	Prevalence (%)
Organ-specific autoimmune diseases				
Thyroiditis (autoimmune)	thyroid	thyroglobulin, thyroid peroxidase	T cells/antibody	1.0–2.0
Gastritis	stomach	H^+/K^+ ATPase, intrinsic factor	T cells/antibody	1–2% in > 60-y-old
Celiac disease	small bowel	transglutaminase	T cells/antibody	0.2–1.1
Graves disease	thyroid	thyroid-stimulating hormone receptor	antibody	0.2–1.1
Vitiligo	melanocytes	tyrosinase, tyrosinase-related protein-2	T cells/antibody	0.4
Type 1 diabetes	pancreas β cells	insulin, glutamic acid decarboxylase	T cells	0.2–0.4
Multiple sclerosis	brain/spinal cord	myelin basic protein, proteolipid protein	T cells	0.01–0.15
Pemphigus	skin	desmogleins (for example, desmoglein 1)	antibody	< 0.01 – > 3.0
Hepatitis (autoimmune)	liver	hepatocyte antigens (cytochrome P450)	T cells/antibody	< 0.01
Myasthenia gravis	muscle	acetylcholine receptor	antibody	< 0.01
Primary biliary cirrhosis	liver bile ducts	2-oxoacid dehydrogenase complexes	T cells/antibody	< 0.01
Systemic autoimmune diseases				
Rheumatoid arthritis	joints, lungs, heart etc.	IgG, filaggrin, fibrin etc.	T cells in joint?/antibody	0.8
Systemic lupus (SLE)	skin, joints, kidneys brain, lungs, heart, others	nuclear antigens (DNA, histones, ribonucleoproteins), others	antibody	0.1
Polymyositis/dermato-myositis	skeletal muscle (predominant) lungs, heart, joints, others	muscle antigens, aminoacyl-tRNA synthetases, other nuclear antigens	T cells/antibody	< 0.01

Diseases are listed by category (organ-specific versus systemic) and then by prevalence. Unless referred to in this review, only diseases with a prevalence of greater than 0.1% (> than 1 in 1000) are included in this table. Diseases without a known antigenic target such as inflammatory bowel disease (ulcerative colitis and Crohns disease) or spondyloarthropathies, are also not included. Many of the papers on the prevalence of these diseases have been reviewed[1]. Other sources were also used[96–98].

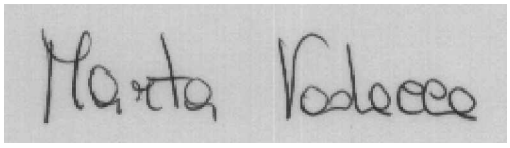
Table 2.
Examples of organ-specific and systemic autoimmune diseases with known autoantigen targets



In 1957, rational steps were drawn up to establish the autoimmune aetiology of human diseases (Milgrom F, 1962). These were modelled on Koch's postulates and termed Witebsky's postulates. These postulates required that an autoimmune response be recognised in the form of autoantibody or cell-mediated immunity; that the corresponding antigen be identified, and that an analogous autoimmune response be induced in an experimental animal model. Finally, the immunized animal must also develop a similar disease. These are stringent criteria and form a good basis for defining autoimmune diseases. The criteria were reviewed in 1993 (Rose NR, 1993) and a new set of criteria developed, which were as follow:

1. Defined autoantigens and autoantibodies must be present.
2. Passive transfer of T-lymphocytes (non-specific or specific) must lead to disease development.
3. Immunomodulation of subjects with disease must ameliorate symptoms.

Each of these postulates will be considered with respect to T1D, and evidence presented for the argument that T1D is an autoimmune disease.



1.4.1 Type 1 diabetes as an autoimmune disease

Over the last few years, evidence from man and mouse models has accumulated that T1D is an autoimmune disease or at least has an autoimmune component. The evidence comes from three major sources: the presence of inflammatory infiltrate (insulitis) in the islets (Figure 7); a strong linkage between T1D and certain alleles of the MHC and autoantibodies that react with islet antigens (Atkinson MA, 2002) (Figure 8).

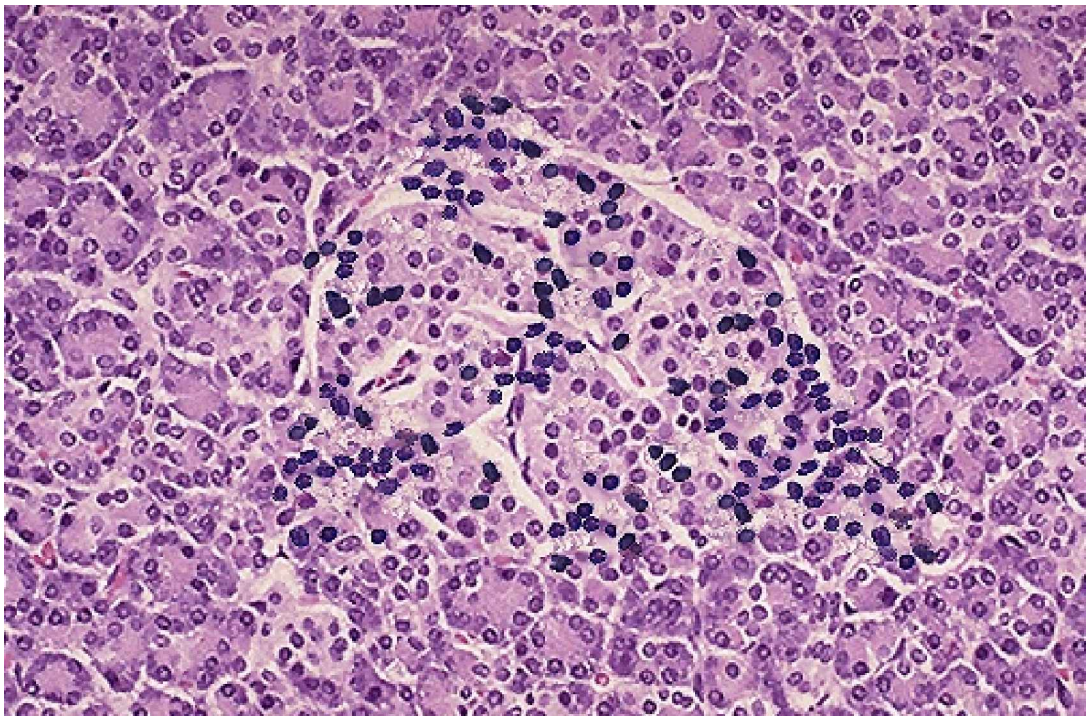
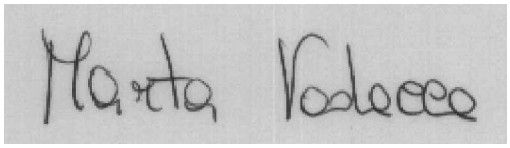


Figure 7.
An islet of Langerhans demonstrates insulitis with lymphocytic infiltrates in a patient developing type 1 diabetes mellitus. This lesion precedes clinical onset of diabetes mellitus.



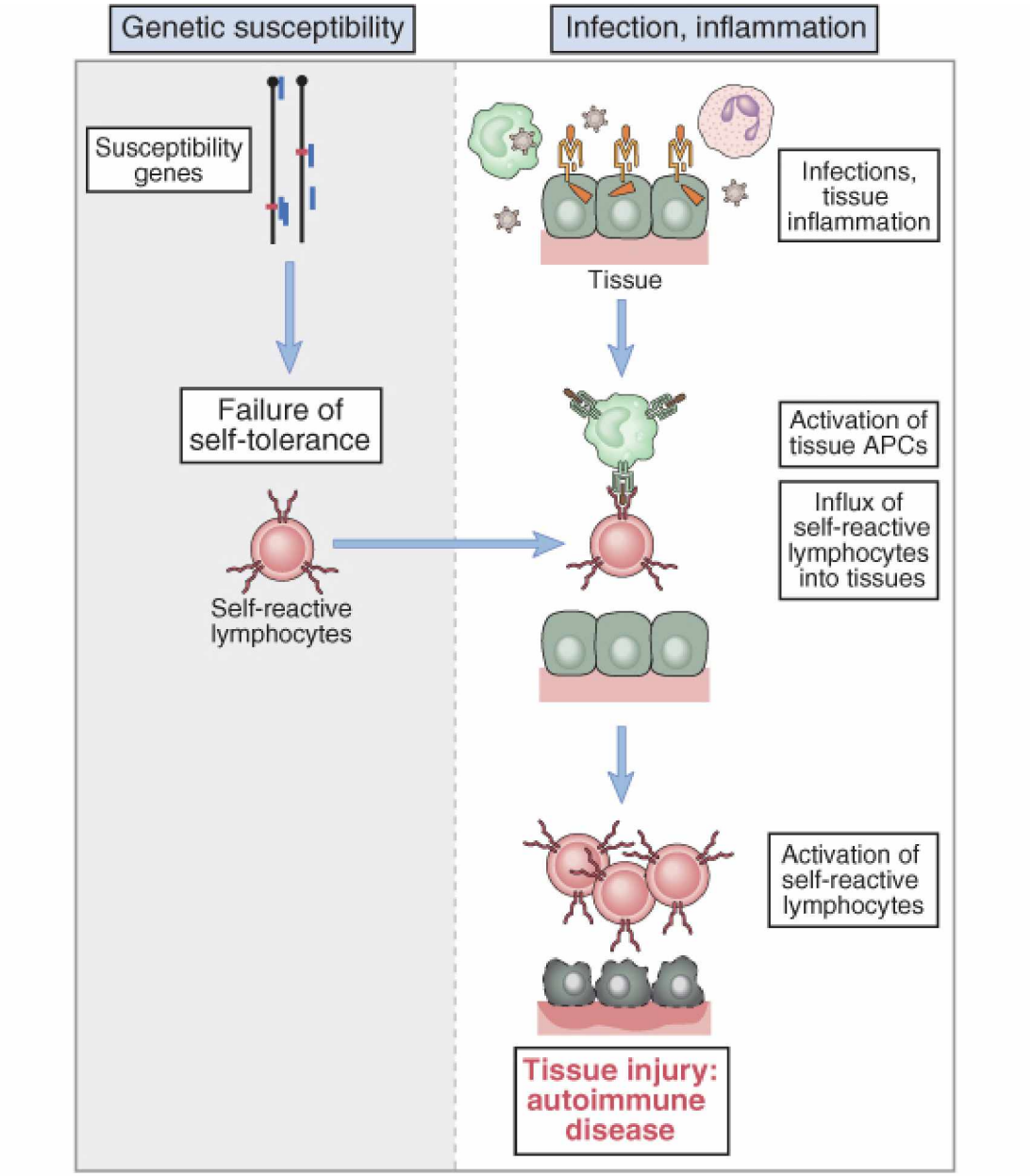
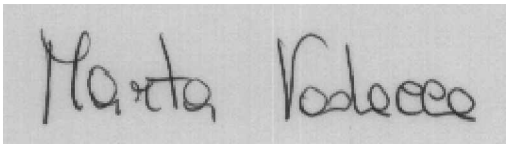


Figure 8.
In this proposed model of an organ-specific T cell-mediated autoimmune disease, various genetic loci may confer susceptibility to autoimmunity, in part by influencing the maintenance of self-tolerance. Environmental triggers, such as infections and other inflammatory stimuli, promote the influx of lymphocytes into tissues and the activation of self-reactive T cells, resulting in tissue injury. Not copy-right issue.



1.4.1.1 Autoantigens and Autoantibodies

The initial evidence for autoimmunity in patients with T1D came from immunofluorescence studies, which showed that a high percentage of sera from newly diagnosed T1D patients, reacted with pancreatic islets (Bottazzo GF, 1974). These lead to an extensive search for the actual autoantigens with which these ICA cells reacted. Three major autoantigens have now been identified.

The first is an isoform of Glutamic Acid Decarboxylase (GAD) (GAD65), which was identified in 1980s. GAD is an enzyme localized in the cytoplasm and microsecretory vesicles of γ-amino butyric acid (GABA)-secreting neurons and pancreatic β cells that catalyzes the conversion of glutamic acid to the inhibitory neurotransmitter GABA (Figure 9).

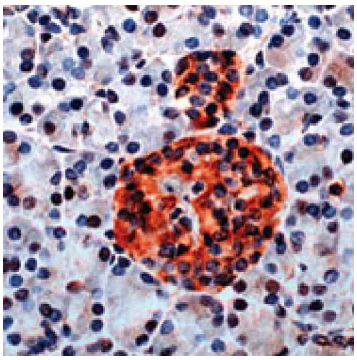
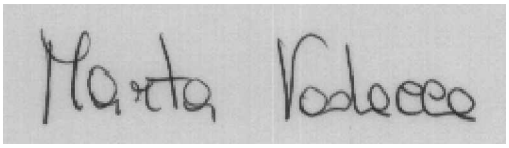


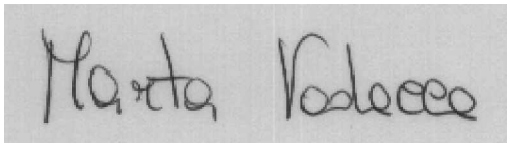
Figure 9. Formalin-fixed, paraffin-embedded human pancreas stained with GAD65 using peroxidase-conjugate. Note cytoplasmic staining of islet cells. Not copy-right issue.

It exists as two protein isoforms of 65 kDa (585 AA) and 67 kDa (594 AA) which are encoded by two distinct genes (Chessler SD, 2000), that may have developed from a common ancestral gene during vertebrate phylogeny. GAD 67 and GAD 65



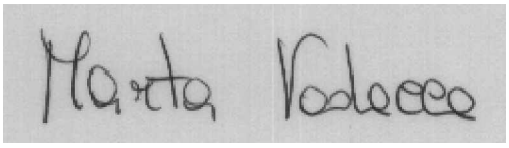
are highly diverse in the first 95 amino acids but share significant homology in the rest of the molecule (Richter et al, 1993). In type 1 diabetes, dominant epitopes are reported to be predominantly conformational and have been mapped within the central (*amino acids 243-444*) and COOH terminal (*amino acids 445-585*) portions (Wei J, 2005). This gene encodes one of several forms of glutamic acid decarboxylase, identified as a major autoantigen in insulin-dependent diabetes. The enzyme encoded is responsible for catalyzing the production of gamma-aminobutyric acid from L-glutamic acid (Wei J, 2003). A pathogenic role for this enzyme has been identified in the human pancreas since it has been identified as an autoantibody and an autoreactive T cell target in insulin-dependent diabetes. This gene may also play a role in the Stiff person syndrome (Costa M, 2002).

The second major antigen, IA-2, was identified in the early 1990s (Christie MR, 1992). IA- 2 is a member of the transmembrane protein-tyrosine phosphatase (PTP) family. It is 979 amino acids in length, has a molecular weight of 106,000 kDa and is encoded by a gene on chromosome 2q35. Because of a critical aminoacid replacement at position 911 (Asp for Ala), which is required for enzymatic activity, IA-2 is catalytically inactive (Westerlund A, 2005). IA-2 is a transmembrane protein found in the secretory vescicles of both endocrine and neuronal cells (Pietropaolo M, 2005). Recent evidences have shown that IA-2 may play a role in insulin secretion (Saeki K, 2002). Approximately 37%-57% of newly diagnosed patients with T1D have autoantibodies against IA-2 and GAD (Leslie D, 2001; Hoppu S, 2005).



The third major autoantigen is insulin, which was also identified in the 1980s (Palmer JP, 1983). This protein is only 51 amino acids in length, and its gene is on chromosome 11p15. As with GAD65 and IA-2, the majority of autoantibodies recognize conformational epitopes, mainly on the B chain of insulin (Al-Bukhari TA, 2002). Autoantibodies against insulin are the first autoantibodies to appear in the prediabetic state and are usually found in very small children (Borg H, 2004). Between 30% and 50% of young children with T1D have autoantibodies to insulin (Barker JM, 2004; Dretzke J, 2004). The frequency of IAA is substantially lower in individuals who develop T1D at an older age (Borg H, 2003).

Although autoantibodies have turned out to be an excellent diagnostic and predictive marker for T1D, it is generally thought that they play a minor role, if any in the pathogenesis of the disease (Ronkainen MS, 2006). Instead the cell-mediated immune response is believed to be responsible for the beta-cell destruction. Inflammatory cells are found in and around the pancreatic islets, but in some individuals these inflammatory cells can be present for years without clinical symptoms (Jaume JC, 2002). In fact, some individuals with autoantibodies and insulitis do not go on to develop diabetes. It has been estimated that in animal models approximately 90% of the islets need to be destroyed before diabetes is clinically manifested. This has not been yet confirmed in humans. Autoantibodies alone do not appear to be sufficient to induce beta-cell destruction (Wang J, 2006). Transplacentally transferred antibodies related to T1D are usually eliminated from the peripheral circulation of infants before 9 months of age and in a German follow-up study no difference was found in autoantibody frequencies between the offspring



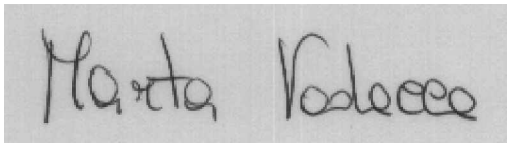
from mothers versus fathers with T1D up to the age of 5 years (Eisenbarth GS, 2004; Barker JM, 2004).

1.5 Immunology of diabetes

The immune system is designed to protect individuals from foreign antigens, whilst maintaining homeostasis, thus advoing the induction of autoimmune disease. The immune system has traditionally been divided into two components: innate, which is specific for invading (infectious) non-self molecules and the adaptative, potentially specific for all non-self as well self molecules. The innate and adaptive immune system differ both in their mode of immune recognition of triggering factors and in their ability to respond to further signals. The innate immune components generate an immediate immune response in response to foreign antigens; the adaptive immune response follows and is delayed, as it was in evolutionary terms. Activation of the adaptive immune system can be harmful to the host when antigens are self or environmental, since immune responses to such antigens can lead to autoimmune diseases (destruction of self, due to a breakdown in mechanisms meant to preserve tolerance itself and severe enough to cause a pathological condition). The adaptive response is generated in such a way, to retain memory, the basis for vaccine design and immunity. The innate response on the other hand does not possess immune memory (Abbas AK, 2005).

1.5.1 Innate immunity

Recently, the innate immune response has been shown to be important in determining how the immune system mounts an immune response. The receptors of



the innate immune system are expressed on many effector cells, most importantly on monocytes/macrophages, dendritic cells, natural killer cells (NK). Recognition of these receptors by the innate immune system induces these cells to produce soluble components to influence the immune response including complement, cytokines i.e. TNF, IL-1, IL-12 and IL-18 and chemokines, which recruit and activate antigen-specific lymphocytes and initiate adaptive immune responses (Figure 10).

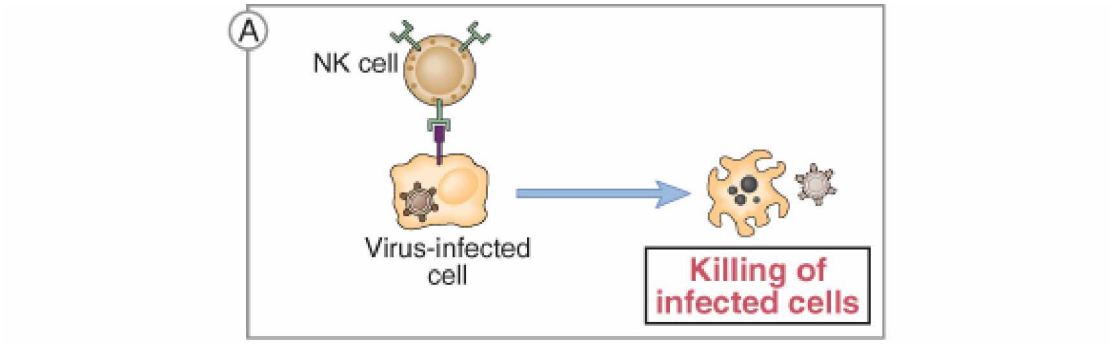
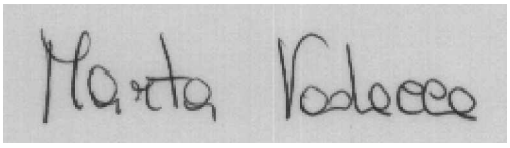


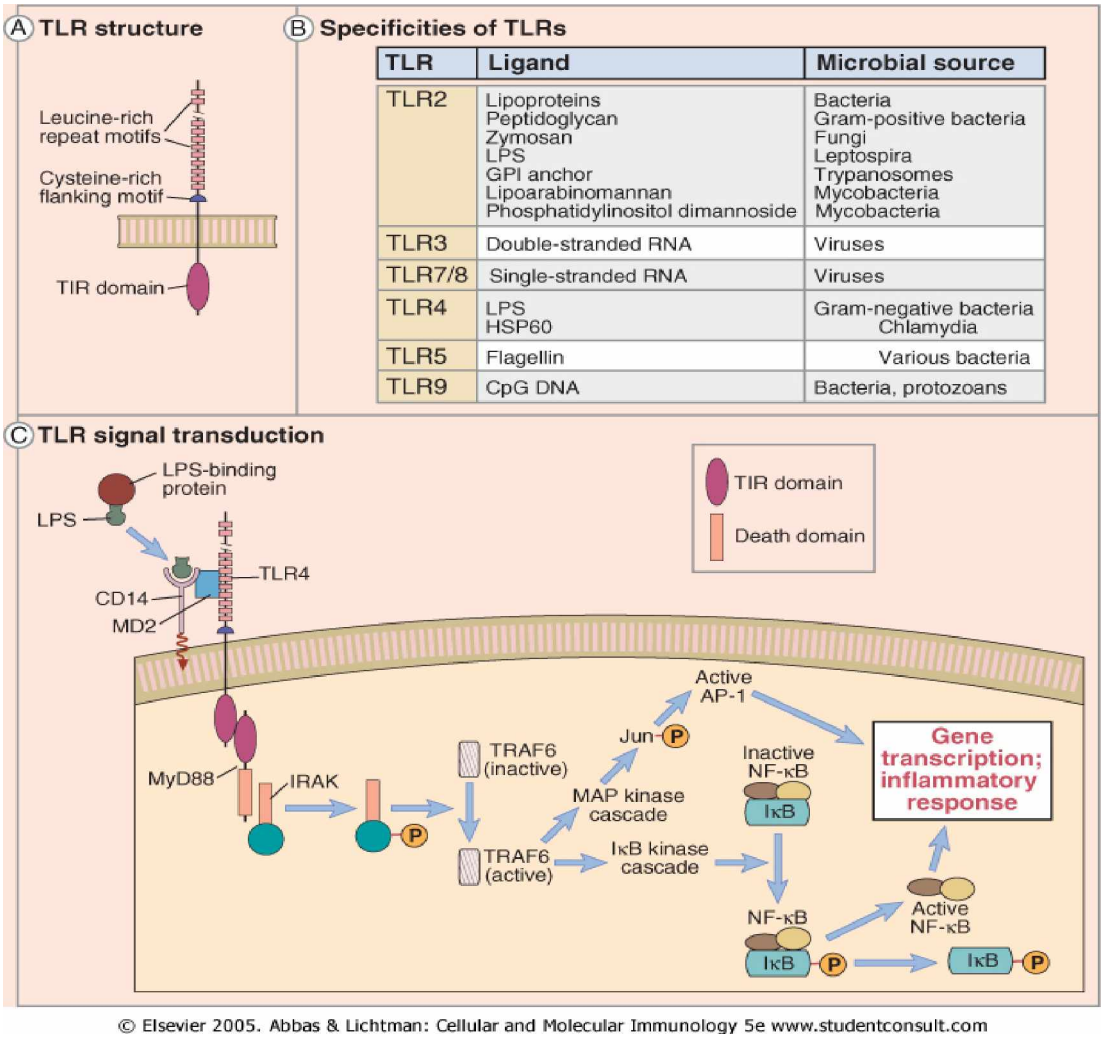
Figure 10.
NK cells respond to IL-12 produced by macrophages and secrete IFN-γ, which activates the macrophages to kill phagocytized microbes. Not copy-right issue.

The innate immune response is immediate, but lacks flexibility. The innate immune recognition is mediated by germ-line encoded receptors. The strategy of the innate immune system response may not be to recognise every possible antigen, but rather to focus on a few, highly conserved structures, associated with microbial infection, including conserved carbohydrate, lipid, protein and DNA structures, respectively. These structures are referred to as pathogen associated molecular patterns (PAMS) and hence the receptors of the immune system are referred to as pattern-recognition receptors. Probably the best described cell surface receptor system for recognition by the innate immune system is the LPS recognition complex composed of CD14 and toll like receptor (TLR) family. Both CD 14 and TLR family of receptors function



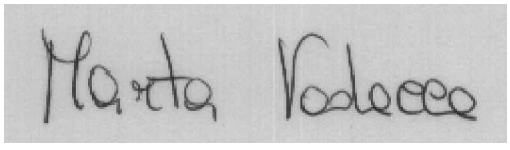
together as a receptor complex for LPS and other microbial products (Abbas AK, 2005).

Recent studies suggest that CD14 is a multifunctional receptor serving not only as an immune receptor for non-self components such as LPS, but also interacting with self components (apoptotic cells).



TLR	Ligand	Microbial source
TLR2	Lipoproteins Peptidoglycan Zymosan LPS GPI anchor Lipoarabinomannan Phosphatidylinositol dimannoside	Bacteria Gram-positive bacteria Fungi Leptospira Trypanosomes Mycobacteria Mycobacteria
TLR3	Double-stranded RNA	Viruses
TLR7/8	Single-stranded RNA	Viruses
TLR4	LPS HSP60	Gram-negative bacteria Chlamydia
TLR5	Flagellin	Various bacteria
TLR9	CpG DNA	Bacteria, protozoans

Figure 11.
The signalling pathway triggered by TLRs that results in the generation of the NF-κB transcription factor is shown. Intracellular adapter proteins other than MyD88 may also be involved in some TLR signalling pathways. In addition to NF-κB activation, TLRs are also linked to AP-1 activation. Not copy-right issue.



1.5.1.1 Antigen presenting cell populations

Monocytes, macrophages, dendritic cells and B lymphocytes belong to the APC group, with some diversity between the cell types in terms of antigen presentation capacities. These APCs play a pivotal role in the immune response, because they bridge the local innate immune response, which occurs in specific tissues, to the subsequent adaptative immune responses which taken place in more specialised lymphoid organs (Abbas AK, 2005) (Figure 12).
Various reports have provided that APCs including macrophages, dendritic cells and

B lymphocytes contribute to the initiation and progression of beta-cell autoimmunity

(Chen YG, 2007).

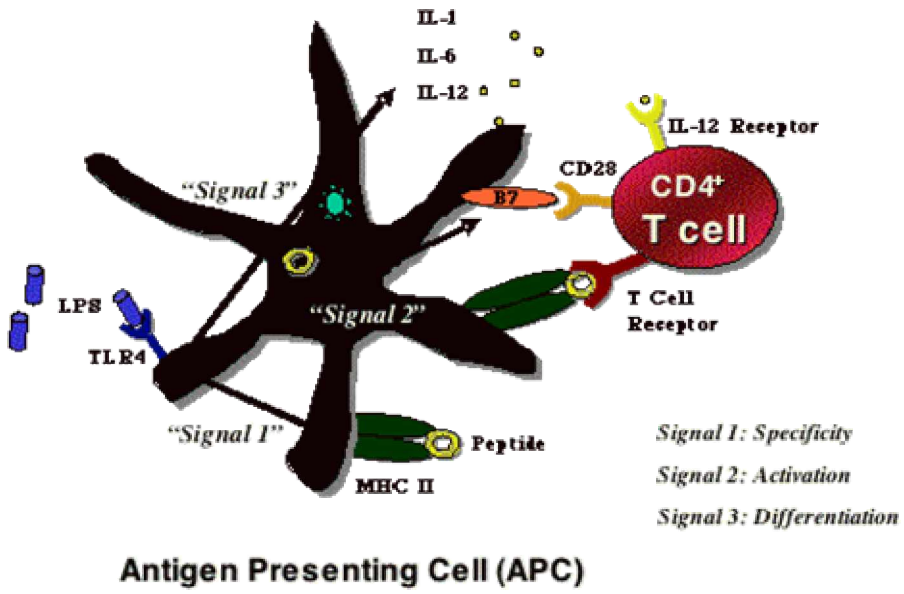
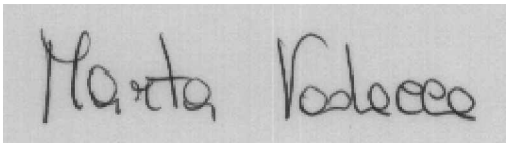


Figure 12.
T cell Activation by an Activated APC. Innate immune system dependent adaptive immune activation: A series of pattern recognition receptors (e.g., Toll-like receptors or TLRs) are present on antigen presenting cells. They recognize common molecules of infectious agents (pathogen associated molecular patterns or PAMPs) and stimulate the antigen-presenting cell. Subsequently, MHC and B7 molecules are unregulated on the surface of the APC and interact with the T cell receptor and CD28, respectively. Furthermore, interleukins are produced, such as IL-12, which help determine T cell differentiation ("signal 3"). The T cell requires multiple signals for activation; in the absence of both signal 1 and 2, TCR legation results in anergy rather than activation.

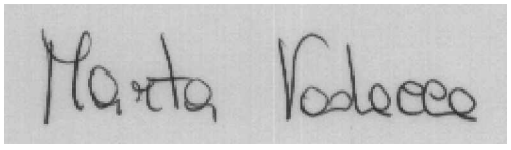


1.5.1.2 Monocytes and macrophages in type 1 diabetes

The function of the innate system is to confer rapid self protection by generating an immediate immune response to clear invading environmental pathogens or by reducing their spread. Monocytes and macrophages secrete cytokines in response to intracellular infection. These cytokines are involved in directing the innate immune response to intracellular infection. These cytokines are involved in directing the innate immune response by activating other cells including NK cells. Several studies support a role for macrophages in human T1D. Hanninen et al, identified the presence of monocytes/macrophages in islet infiltrates and demonstrated that they are involved in pancreatic beta-cell destruction at the onset of T1D (Hanninen A, 1992).

1.5.1.3 Dendritic cells in type 1 diabetes

Dendritic cells are widely distributed in tissues and in lymphoid organs. Immature dendritic cells readily take up large quantities of antigens. From tissue sites, dendritic cells migrate to the lymph nodes where they mature losing their capacity to present antigens, but markedly increased their capacity to activate T lymphocytes. Mature dendritic cells are 10-100 times more efficient in activating naive and primed T lymphocytes than are B lymphocytes or macrophages. The efficiency of dendritic cells as APCs is in their capacity to express high constitutive levels of MHC class I and II molecules and CD86, the ligand for the T cell costimulatory molecule CD28. Dendritic cells respond to many exogenous factors including bacterial (endotoxin) and viral (nucleic acid) signals. These signals initiate dendritic cell maturation, change dendritic cell migratory characteristics, increase their APC activity with



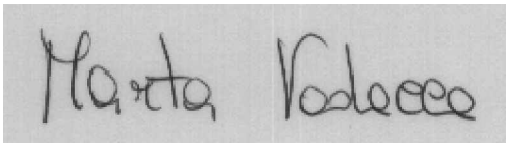
release of pro-inflammatory cytokines and initiate the immune response (Abbas AK, 2005).

Dendritic cells in human T1D have shown a reduced ability to stimulate autologous or allogenic T lymphocytes and a reduced dendritic cell function has been detected in relatives of patients with T1D, estimated to be a high disease risk because they had diabetes associated autoantibodies (Perone MJ, 2006). Since dendritic cells instruct the adaptive immune system and processing antigens, abnormalities in these cells could dictate the masking and unmasking of cryptic epitopes relevant to autoimmunity (Lo J, 2006).

1.5.1.4 NK cells in type 1 diabetes

NK cells are an important component of the immune response against several pathogens and some tumours. In autoimmune diseases, NK cells are frequently present in many of the target organs e.g. muscle, rheumatoid synovial tissue, pancreas and brain (Miyake S, 2007). However, direct lysis of target organs by NK cells has, in general, not been demonstrated, suggesting that NK cells might be involved in the regulation of immune responses. NK cell function is regulated by NK receptors that interact with MHC class I molecules on target cells (Abbas AK, 2005).

A decreased NK cell function has been demonstrated in patients with several autoimmune diseases, including MS and myasthenia gravis (Rinaldi L, 2007; Linsen L, 2007; Bielekova B, 2006). Though it remains unresolved as to whether the changes are primary, that is involved in causing the disease, or secondary, that is resulting from the consequences of the disease or its treatment (Novak J, 2007; Rodacki M, 2007).



1.5.2 Adaptive immunity

Of the many defence systems described, from the simplest single cell bacteria to the most complicated plants and animals, none has been more intensively studied than the mammalian adaptive system. The primary effector molecules of the adaptive immune system are the antigen binding receptors, Ig and TCR expressed on B and T lymphocytes respectively, each members of the Ig superfamily (Figure 13).

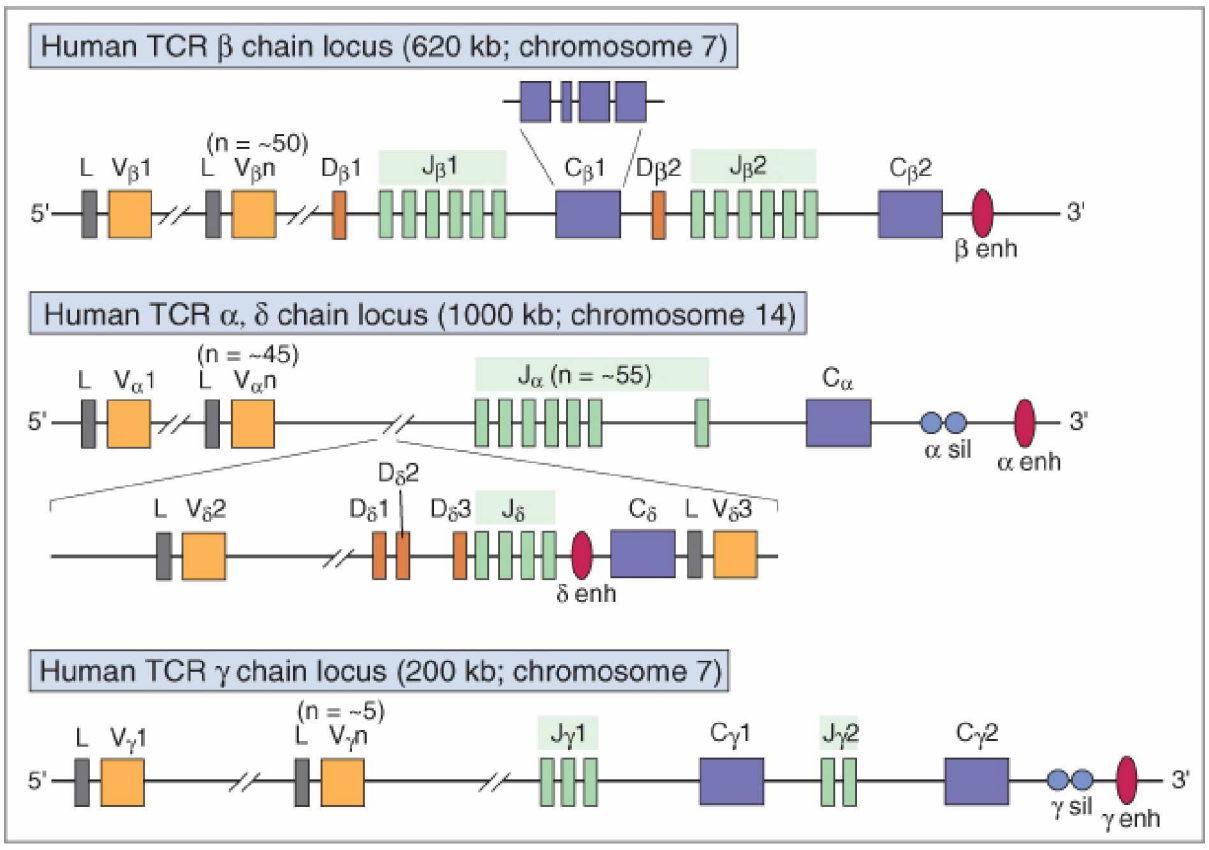
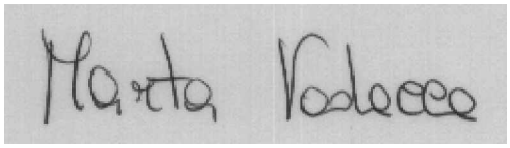


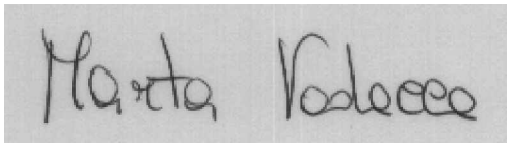
Figure 13.
The human TCR β, α, γ, and δ chain loci are shown, as indicated. Each constant (C) gene is shown as a single box but is composed of several exons, as illustrated for C_β. Gene segments are indicated as follows: L, leader (usually called signal sequence); V, variable; D, diversity; J, joining; C, constant; enh, enhancer; sil, silencer. Not copy-right issue.



The somatic diversification of the antigen binding regions of Ig and TCR is typically associated with a unique form of genetic rearrangement. T and B cells have a unique combination of gene segments that specify the variable region (the part of the receptor that binds the antigen). In the developing B lymphocyte, random recombination of heavy-chain genes and light chain genes shuffling the numerous variable region genes deals each B lymphocytes a structurally distinct unique receptor. A similar principle underlains the formation of the TCR. This allows these cells to generate a response to different fragment of different pathogens (Abbas AK, 2005).

1.5.2.1 B lymphocytes in type 1 diabetes

The possible role of B lymphocytes and autoantibodies in T1D in man has not been fully resolved, however it has been demonstrated that T1D can be transmitted in humans via bone marrow transplantation, between HLA identical siblings (Lampeter EF, 1998). Another study reported the development of T1D in a patient with X-linked agammaglobulinemia (Martin A, 2001). X-linked agammaglobulinemia is a human immunodeficiency disease characterised by a blocking of B lymphocyte differentiation and hence a marked decrease in the number of B lymphocytes in peripheral blood. Development of T1D in a patient with this condition implies that neither, autoantibodies or B lymphocytes functions are critically involved in the pathogenesis of T1D. B lymphocytes in T1D are driven by T cells. The central role of T cells in autoimmune diabetes was demonstrated in the NOD model by the ability of T cells to transfer disease and the protection afforded by immunotherapies targeting T cells. The precise function of autoreactive B cells has been more difficult to establish. Islet-specific autoantibodies targeting major autoantigens such as glutamic acid decarboxylase and insulin can be detected in the serum in NOD mice and in patients and have been an excellent indicator of autoimmunity and predictor of disease in diabetic patients and relatives. However, a direct pathogenic role for autoantibodies is controversial. In NOD mice, the importance of B cells was clearly demonstrated by the dramatic reduction in insulitis and diabetes incidence following B cell depletion using anti IgM antibodies at birth or in NOD mice genetically deficient in B cells. Evidence from many different studies suggests that B cells function as islet antigen–presenting cells for autoreactive T cells in NOD mice and that autoantibodies expressed on the cell surface improve the capture and presentation of autoantigens. Importantly, early loss of B cells does not eliminate insulitis in NOD mice, suggesting that autoreactive B cells may be essential at late



stages of disease to enhance autoreactivity and promote epitope spreading (Bour-Jordan, 2007).

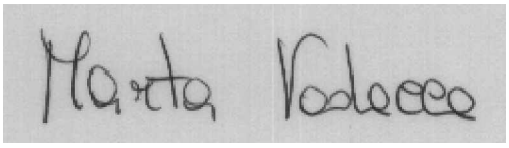
1.5.2.2 T lymphocytes in type 1 diabetes

Diabetes transfer studies have demonstrated that bone marrow derived cells from hosts with autoimmune diabetes can transfer beta-cells destructive insulitis to non-diabetes prone human, mouse or rat pancreas, thereby indicting that an undergoing abnormality in T1D resides in immune system. In man, an adoptive transfer study using bone marrow that was not depleted for T cell demonstrated a transfer of diabetes from a diabetic donor to a non-diabetic immuno-compromised recipient relative providing evidence for an important role for T lymphocytes in the pathogenesis of T1D in human (Lampeter EF, 1998).

The importance of T lymphocytes in the pathogenesis of T1D is well established and T1D is classified as an example of a T lymphocyte mediated autoimmune disease.

T lymphocytes recognize antigen when it is in combination with MHC molecules on the surface of APCs. Phagocytic cells i.e. dendritic cells and macrophages, engulf antigens, process them into peptides and display the fragments on their surface by means of MHC molecules to T lymphocytes. This recognition is facilitated by the membrane bound TCR specific for an antigen (Abbas AK, 2005).

The role of T lymphocytes in the development of beta cells destruction is further supported by the efficacy of immunosuppressive agents, which have been shown to slow the progression of beta-cell damage in patients with newly diagnosed T1D. These immunosuppressive agents include azatiopirine and cyclosporine and act mainly at the T lymphocyte level (Bresson D, 2007). Further, peripheral blood lymphocytes from patients with T1D proliferate in vitro on exposure to islet antigens, indicating T lymphocyte mediated autoimmunity in T1D. Furthermore, the inflammatory lesion in the islets (insulitis) consists mostly of mononuclear cells, mainly T lymphocytes and macrophages or monocytes.



1.5.2.3 CD4+CD25+ Regulatory T cells in type 1 diabetes

CD4+CD25+ regulatory cells have been described as important mediators of peripheral tolerance in a variety of experimental models including inflammatory bowel disease, EAE, and type 1 diabetes (Bach JF, 2003; Homann D, 2004) (Figure 14).

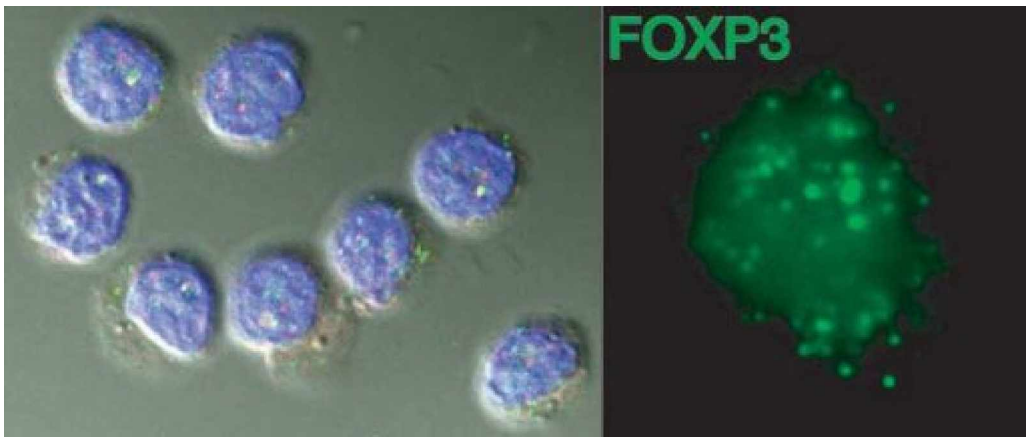
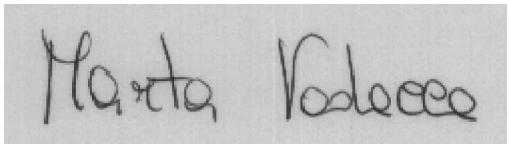


Figure 14.
Human regulatory T cells viewed using fluorescence microscopy after immunostaining (left). FOXP3 protein stained by anti-FOXP3 antibody within one regulatory T cell (right).
Image Credit: Kathryn T. Iacono, University of Pennsylvania School of Medicine; Proceedings of the National Academy of Sciences. Not copy-right issue.

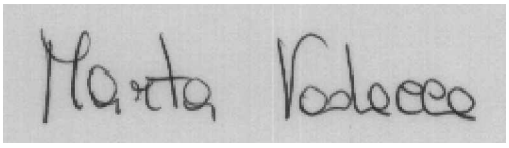
CD4+CD25+ cells recently have been described as very efficient in the protection and cure of NOD mice from diabetes development (Salomon B, 2000; Tang Q, 2004). This class of T regulatory cells has been identified as being of thymic origin, anergic, dependent on IL-2 stimulation for their survival (Sakaguchi S, 2001) as well as constitutively expressing CTLA-4, OX-40 , L-selectin (McHugh RS, 2002), and the glucocorticoid induced tumour necrosis factor receptor (GITR) (Shimizu J, 2002). In vitro studies have shown that CD4+CD25+ regulatory T cells act cell contact dependent while cytokines such as IL-10 and TGF- appear not to be necessary for the suppression of CD4+CD25+ cells (Shevach EM, 2002; Piccirillo



CA, 2002). However IL-10, IL-4, and TGF were required in vivo for the suppressive effect of CD4+CD25+ cells (Read S, 2000). It has also been postulated that a different class of CD4+CD25+ cells, derived from CD4+CD25+ cells in the periphery, is cell contact-independent and exerts its function through suppressive cytokines such as IL-10 and TNF (Jonuleit H, 2003). Priming of naïve autoreactive T cells to a memory/effector state does not inevitably result in autoimmune disease. Several gate-keeping functions prevent autoreactive effector T cells from mediating immune pathology; control by CD4+CD25+ regulatory cells is one of these mechanisms. Therefore, it is conceivable that NOR mice do not develop T1D because, in contrast to NOD mice, their primed ICA (islet cell antigen)-reactive T cells are more effectively controlled by CD4+CD25+ regulatory cells (Otto PA, 2005).

1.5.3 Cytokine profile in type 1 diabetes

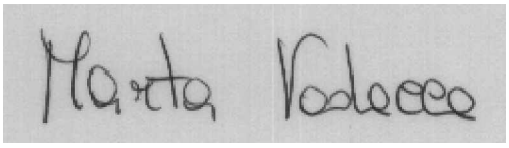
Destructive insulitis in NOD mouse and bio-breeding rat has been connected to the expression of proinflammatory cytokines IL-1, TNF-alfa and INF-alfa and Th1 cytokines (IL-2, TNF-beta and INF-γ) which contribute to cell-mediated immunity, e.g., cytotoxic and inflammatory responses mediated by T cells, NK cells and macrophages (Karlsson Faresjo MGE, 2004). On the contrary, the expression of Th2 cytokines (IL-4, IL-5, IL-6, IL-9, and IL-13) connected to antibody production and enhanced eosinophil proliferation, and anti-inflammatory IL-10 tends to correlate with benign insulitis in these animals. Type 1 diabetes has been shown to be transferable by CD4 T cells expressing a Th-1 like cytokine profile in neonatal NOD mice. On the other hand, treatment with IL-10 and IL-4 or expression of IL-4 in



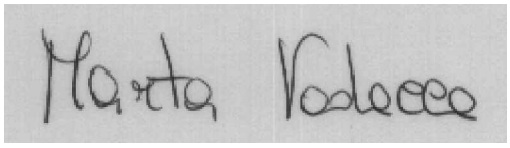
pancreatic beta-cell of transgenic mouse was shown to protect from insulitis and type 1 diabetes in NOD mice (Karlsson Faresjo MGE, 2004). IL-13 produced mainly by activated Th-2 like lymphocytes and NK cells, shows sequences similarity with IL-4. The level of IL-13 was significantly decreased in high risk first-degree relatives of diabetic children in comparison with healthy controls (Kretowski A, 2000), and treatment with IL-13 seemed to prevent the development of insulitis and diabetes in NOD mice. Insulin is shown to induce cellular response in both pre-onset and newly diagnosed diabetic patients. GAD-reactive T cells of a T like phenotype have been obtained after in vitro stimulation of PBMC from a leukocyte human antigen DR34 heterozygous type 1 diabetic patient. During the first month after diagnosis, INF-γ mRNA expression increased, whereas expression of IL-4 mRNA decreased during the same interval, showing a Th1 deviated cytokine profile (Karlsson Faresjo MGE, 2004).

1.5.4 Dysregulation of immunity in type 1 diabetes

In T1D it is likely that non-genetic, probably environmental factors play a major role in causing T1D. It is likely that these factors operate trough the induction of an autoimmune response (Larsson K, 2004), however the exact mechanism by which the immune response provides a rapid anti-microbial host defence that precedes the adaptive immune system. The innate immune system is mediated by a few, relatively inflexible cell populations and soluble components. The innate immune defence also has an additional role in determining the nature of downstream adaptive immune response. Hence, the induction of autoimmunity probably involves the adaptive immune system and innate effector cells which could be important in priming or



promoting these responses (Neighbors M, 2006). The innate system plays a major role in the induction on a response to a foreign antigen and normally it will only respond to these antigens. Infectious antigens can produce autoimmunity through molecular mimicry, in which an immune response to one fragment of a molecule (epitope) induces an immune response to the same epitope within a different molecule as well as bystander and polyclonal activation (Abbas AK, 2005). In the former, released self-antigens from damaged tissue can be processed by macrophages and dendritic cells which in turn can be activated by cell debris or factors secreted by activated T lymphocytes. Macrophages and dendritic cells which in turn can be activated by cell debris or factors secreted by activated T lymphocytes. Since macrophages and dendritic cells can act as APCs it follows that these events might lead to autoimmunity. Polyclonal activation by inflamed tissue could also activated anergic cells either by inflammatory mediators or by naive cells in an inflammatory setting to induce autoimmunity (Abbas AK, 2005). Destructive autoimmunity is the final consequence of a complex multi-step process, and is strongly supported by inflammation. Antigens can induce or promote inflammatory events, and can intervene at any step of the pathogenic sequence of events. Each step in the inflammatory process is controlled by a multitude of mechanisms, including those of the innate immunity, either supporting or suppressing the development of autoimmunity (Figure 15).



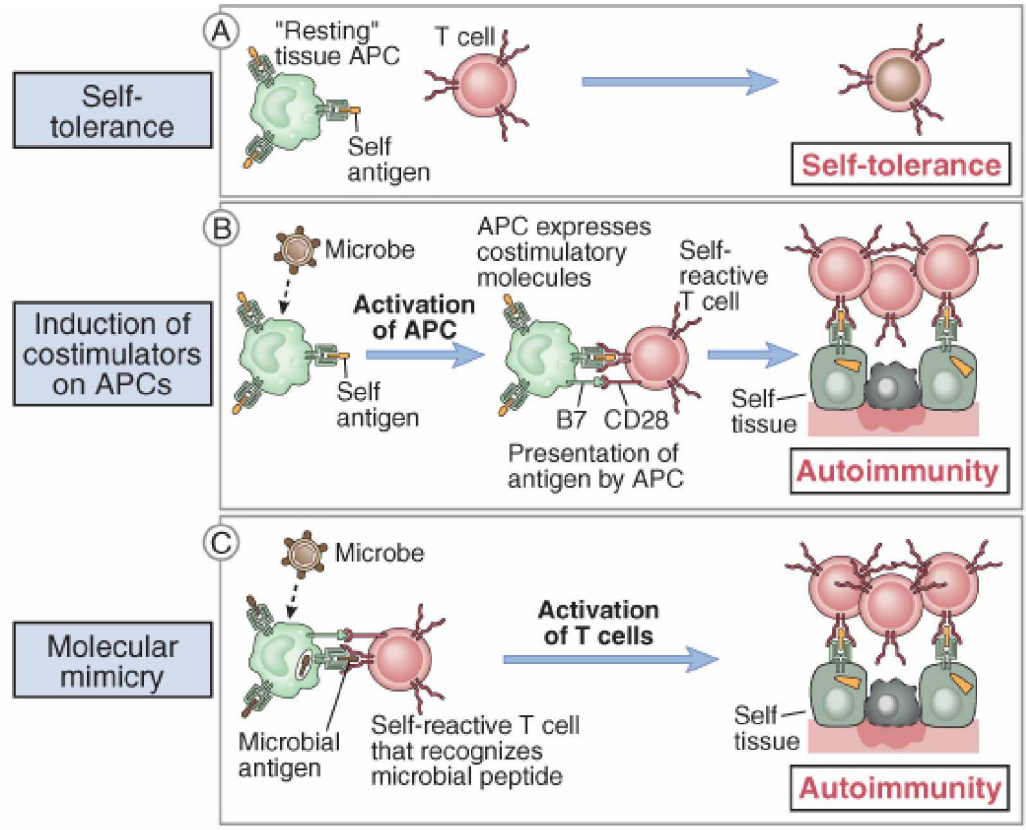
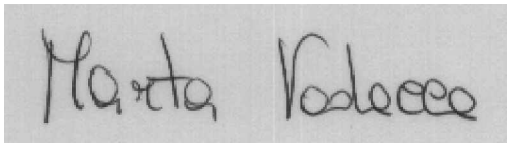


Figure 15.
Some microbial antigens may cross-react with self antigens (molecular mimicry). Therefore, immune responses initiated by the microbes may activate T cells specific for self antigens. Not copy-right issue.

During the inflammatory process before the development of destructive autoimmunity, both infected and non-infected host cells can succumb to cell death induced by either direct infection or by killing (i.e. by NK cells).

The function of the innate immune system is to promote self protection by clearing pathogens or reducing their spread. In this process several molecules can be released, such as antigens derived from destroyed pathogens and/or normal components of host cells. These products can trigger the release of anti-inflammatory mediators including IL-1, IL-6, IL-12, IL-18, TNF and NO. These can on one hand amplify T



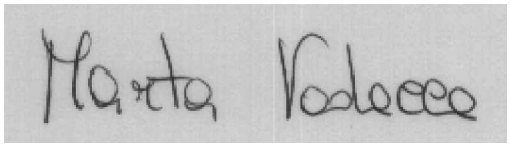
lymphocytes to enhance self protection against infected cells, or on the other hand can also effect the development of destructive autoimmunity. An intracellular bacterial or viral infection can induces monocytes/macrophages promptly to produce innate cytokines such as IL-1, IL-12, IL-18, and TNF which, in turn, independently or synergistically direct autoreactive Th1 cell development.

Combination of the innate response might result in a concerted effect that promotes downstream adaptive responses (i.e. T lymphocyte and or B lymphocyte mediated autoimmunity). There is a close relationship between innate and adaptive immunity and changes in the former could provoke inappropriate responses in the latter (Abbas AK, 2005).

1.6 Detection of autoreactive T cells in type 1 diabetes

1.6.1 Autoreactive T cells in Type 1 diabetes

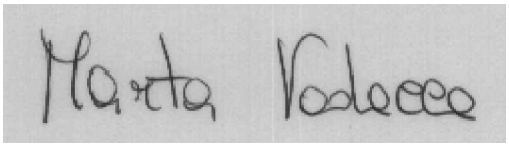
Type 1 diabetes is the result of a T cell-mediated destruction of pancreatic beta-cells in the islets of Langerhans (Otto PA, 2005). In diabetic animal models and humans, a correlation between disease onset and cytokines, in particular interferon INF-γ, has been observed and these studies support the promotion of inflammatory cellular immune responses by T lymphocytes (Karlsson MGE, 2005). The development of T1D is also associated with the major histocompatibility complex (MHC) class II genes. An important role of the highly polymorphic HLA class II immune recognition molecules, DR and DQ, located on chromosome 6 has been consistently confirmed (Galgani A, 2004; Barrat BJ, 2004). Both these molecules are essential for the presentation of specific peptides to autoreactive T cells (Ilonen J, 2002). In T1D, the specific epitopes recognized by pathogenic T cells remain poorly defined and the



specific autoreactive T cells are difficult to detect due to the low number in the peripheral blood, the inhibition by populations of regulatory T cells and the inability to assay cells from the inflammatory lesion (Schloot NC, 2003; Meierhoff G, 2002). Moreover, autoantigen-reactive T cells are also detected in healthy individuals; therefore the development of new techniques to identify with high specificity beta-cells-specific T cells in T1D subjects remains a major goal in the study of the disease.

1.6.2 T cell receptor

Antigen-specific immune recognition, the hallmark of the adaptive immunologic response, is achieved through clonally distributed arrays of variable receptors (i.e., immunoglobulins on B cells and αβ T-cell receptor [TCR] on CD4 and CD8 T cells) capable of recognizing thousands of disparate ligands. A central role in initiating immune responses and providing subsequent helper functions for antibody and cytolytic function is played by the CD4+ T cell, wherein the antigen-specific TCR recognizes a molecular complex consisting of specific antigenic peptides bound by particular HLA class II molecules. Because presentation of specific HLA-peptide complexes to a cognate TCR is a crucial event leading to immune activation, this same structural interaction can be exploited experimentally to identify and probe the CD4+ T-cell response. The technology that has been developed to accomplish this task, referred to as HLA class II multimers or tetramers, promises to provide sensitive and specific tools for detecting, evaluating, and manipulating the antigen-specific immune response (Buckner JH, 2002).



1.6.3 Tetramers

New techniques, such as the use of multimeric peptide-MHC complexes and enzyme-linked immunospot (ELISpot) assay, have enabled the detection of autoreactive T-cells at a very low frequency. ELISpot techniques have been used in the identification of low-frequency autoreactive T-cells in different autoimmune diseases. Although ELISpot analysis allows detection of individual T-cells directly from peripheral blood and determination of their antigen-specific cytokine release, the advantage of tetramer staining is that it provides the ability to isolate the autoreactive T-cells for further characterization. MHC class I and class II multimers (or tetramers) have been successfully used in enumeration of $CD8^+$ and $CD4^+$ T-cells, respectively, that are specific for viral, but it has been more challenging to apply MHC class II tetramers in identifying of $CD4^+$ T-cells in autoimmune diseases where the precursor frequency of antigen-specific T-cells is very low.

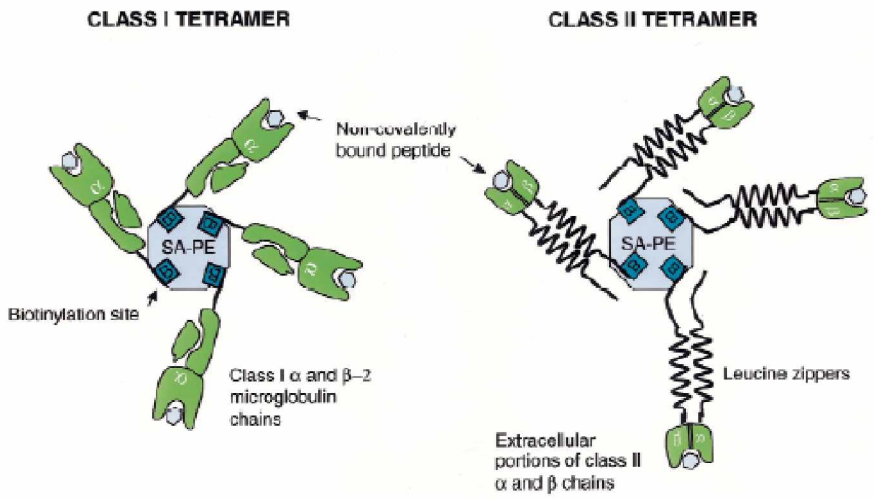
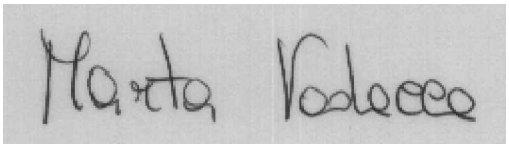


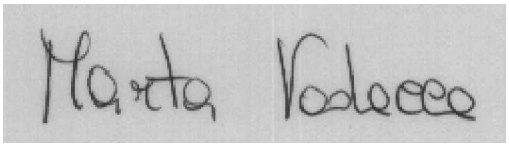
Figure 16.
Schematic illustration of multimeric MHC-peptide molecules suitable for detection of antigen-specific T cells. SA, streptavidin; PE, phycoerythrin.



In contrast to high-affinity T-cell responses to foreign antigens, circulating autoreactive T-cells may display low-to-moderate affinity for self antigens, which makes the staining of these T-cells by class II MHC tetramers cumbersome (Reijonen H, 2002).

1.6.3.1 Class I tetramers

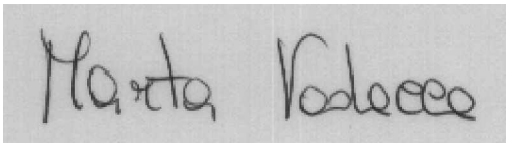
Tetramer analysis was developed to bypass this need by providing a direct structural basis for detection of T cells expressing particular TCR molecules. MHC tetramers were originally developed for analysis of class I restricted CD8+ T cells, in which the TCR recognizes an MHC class I antigen complex. Class I heavy chains are expressed in *Escherichia coli*, purified from inclusion bodies, denatured, and denatured in the presence of specific antigen to form class I peptide complexes, which are then multimerized with a streptavidin- biotin interaction to form tetramers. (Burrows SR, 2000) Alternatively, single-chain forms of the class I peptide complex have been engineered, (Denkberg G, 2000) and dimeric forms of MHC class I–peptide complexes have been described using an immunoglobulin scaffold as the basis for multimerization. When coupled to fluorescent tags, these class I– peptide multimers readily bind to $CD8^+$ T cells carrying the appropriate specific TCR. $CD8^+$ T lymphocytes are principle effector cells in antiviral and antitumor immunity, and the corresponding class I tetramers are frequently used in clinical studies to monitor specific immunity or after vaccine administration. The growing use of class I tetramers in these clinical applications is facilitated by 2 key features: first, the structural features of the denatured class I heavy chain are conducive to standardized production methods, and second, the frequency of particular antigen-specific $CD8^+$ T



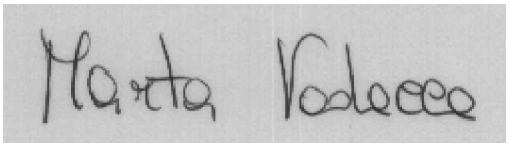
cells in peripheral blood is high, ranging from 1 in a few thousand to greater than 1 in 100. Recently, class I tetramers have also been used in immunohistology to directly visualize antigen specific CD8+ T cells within tissue sections (Skinner PJ, 2000). With respect to class II tetramers, in contrast, several barriers need to be overcome to fully exploit the technology for clinical application (Buckner JH, 2002).

1.6.3.2 Class II tetramers in type 1 diabetes

MHC class II tetramers are a useful tool for detection of autoreactive $CD4^+$ T-cells in the peripheral blood. Tetramer MHC class II-peptide complexes bind antigen-specific $CD4^+$ T cells with a high degree of specificity and enable isolation of the T-cells for further characterization (Reijonen H, 2002; Buckner JH, 2002). A study demonstrates that GAD65 and proinsulin tetramer binding $CD4^+$ T cells are detectable more often in peripheral blood of type 1 diabetes patients and at-risk subjects than healthy individuals. HLA-DR4+ type 1 diabetes patients and at-risk subjects had T-cells specific for both GAD65 peptides tested (GAD65 555–567 557I and GAD65 274–286), while the majority of the $DR3^+$ type 1 diabetes patients displayed response to a proinsulin peptide, B24–C36. Autoantibody positive children responded frequently to the GAD65 555–567 557I peptide, while none of the control subjects recognized it (Oling V, 2005). Autoantibodies specific for different islet cell proteins such as insulin, GAD65 and IA-2, are the most informative biological markers for the prediction of type 1 diabetes. In contrast, T-cell profiling in type 1 diabetes, and in human autoimmunity in general, is in its early stages. The development of new techniques such as MHC class II tetramers now makes it possible to probe T cell compartments for analysis of specificity for several

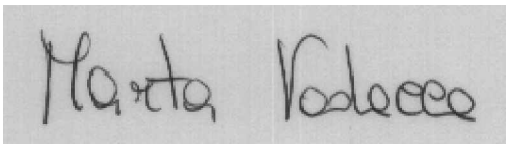


autoantigens and epitopes. Precise definition of the MHC/peptide specificity and functional properties of $CD4^+$ T cell populations is required for mechanistic understanding of the disease process of type 1 diabetes. In order to design prevention therapies and for proper immunomonitoring we need to learn the specificity and phenotype of the T-cells that are prerequisite for the disease outcome and sufficient for the clinical disease. In both human and NOD mice a small number of autoantigens including insulin (or proinsulin), GAD65 and IA-2, have been shown to be targets of the autoimmune attack (Lieberman SM, 2003). Insulin is the only beta-cell specific autoantigen. In humans the strongest susceptibility conferred by a locus outside of MHC class II region is the insulin gene (Ins) variable number of tandem repeats (VNTR) regulatory region. The disease associated polymorphism (class I repeats) correlates with reduced thymic expression of the Ins gene, which has been suggested to lead to defective negative selection and impaired induction of central tolerance to insulin and proinsulin. In a recent study less than half of the type 1 diabetes patients were $DR3^+$ but five out of eight of this group displayed T-cell response to the proinsulin epitope. Two very recent studies have highlighted the key role of proinsulin in NOD and human diabetes (Nakaiama M, 2005; Kent SCD, 2005). However, the role of GAD65 as a primary antigen cannot be excluded, at least in human type 1 diabetes. Even if insulin turns out to be a primary autoantigen involved in the early events of beta cell destruction, GAD65 or another autoantigen could still play a crucial role in the amplification of the autoimmune process and the intermolecular epitope spreading. It is likely that several autoantigens and epitopes contribute to the disease process although the hierarchy of epitopes may vary along the progression of autoimmunity. The highest risk to progress to the clinical disease



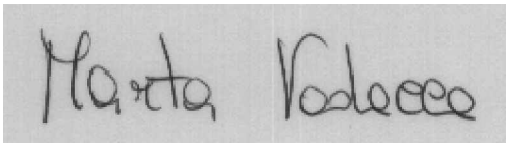
might correlate with the number of the T-cell epitopes recognized, which would be similar to the predictive value of having multiple autoantibodies. In a recent study type 1 diabetes patients displayed T-cell response to both GAD65 and proinsulin, but only one patient had reactivity to both autoantigens. However, only 11 patients were analyzed with multiple tetramers due to the limited sample size. T-cell response to the multiple autoantigens and potential epitope spreading during the preclinical phase of the disease process needs to be investigated in an extended study in a larger cohort of type 1 diabetes patients and at-risk subjects.

There are several technical constraints in the development of T-cell assays to identify autoreactive T-cells in the peripheral blood of type 1 diabetes patients. The difficulty to discriminate patients from normal subjects has been addressed in several individual studies and T cells cell workshops (Peakman M, 2001). Reliable detection of rare autoantigen specific T-cells in the peripheral blood have been challenging. It has been estimated that only one in 30,000 or less $CD4^+$ T cells in peripheral blood are GAD65 reactive. This low number of cells cannot be readily detected by flow cytometry and therefore assay relies on in vitro expansion of antigen specific T cells prior to the tetramer staining. Disadvantages of in vitro expansion include potential activation-induced apoptosis of the high avidity cells during the culture. The fact that T-cells with the highest avidity have been shown to be most readily detectable by tetramers suggests that the frequencies of T-cells determined in this study are conservative estimates (Reichstetter S, 2000). It is also technically challenging to design the optimal culture conditions to facilitate the detection of antigen specific cells without confounding parameters such as unspecific by-stander proliferation and activation of regulatory cells.



In the reported study the presence of GAD65 specific T-cells were followed on two occasions in four at-risk subjects. Two subjects displayed a consistent pattern of strong staining with a tetramer, and one individual became tetramer positive in the second sampling. However, in one at-risk child who had displayed the highest number of GAD65 557I tetramer binding cells in the study (53.60% of the $CD4^{high}/CD25^{+}$ activated cells), disappearance of these GAD65 557I specific T-cells in the sample drawn 3 months later was observed. This kind of fluctuation in the level of circulating islet antigen specific T-cells is reminiscent of observations by Trudeau and colleagues (Trudeau JD, 2003) who demonstrated in NOD mice that murine $CD8^{+}$ tetramer binding T-cells specific for the mimetic NRP epitope appear in the peripheral blood in cycles prior to the onset of hyperglycaemia. Even mice that developed diabetes had undetectable levels of autoreactive T-cells in some blood samples. The authors suggest that these cycles possibly reflect clonal proliferation of antigen specific T-cells and target organ homing during the inflammatory process. This possibility is much more difficult to test in humans, but we can speculate that active beta-cell targeted immunity possibly leads to fluctuation and even periodic disappearance of circulating autoantigen specific T cells in peripheral blood. Whether these kinds of changes in the detectable levels of tetramer binding T cells are a general phenomenon preceding the clinical onset of the disease remains to be investigated in a longitudinal study of a larger cohort of at-risk subjects with frequent sampling intervals.

Autoreactive T-cell responses in type 1 diabetes are very complex and much is still unclear about the target of the autoimmune attack. Epitope spreading may occur and there may be individual differences in the processing of these epitopes that contribute

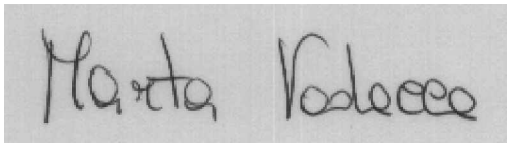


to the progression of the type 1 diabetes. Oling et al., demonstrates that MHC class II tetramers are a promising tool for monitoring disease progression in the individuals at risk to develop type 1 diabetes and in the identification of autoreactive T cells in type 1 diabetes patients (Oling V, 2005).

1.7 Ii-key hybrids

1.7.1 MHC class II molecules

The normal process of MHC class II antigen charging and presentation is highly controlled, in order to assure the presentation of selected peptides. MHC class II epitopes are normally bound to MHC class II molecules in a post-Golgi, antigenic peptide-binding compartment of antigen presenting T cells (dendritic cells, macrophages and B cells). At synthesis in the endoplasmic reticulum, MHC class II molecules are bound by the invariantly chain (Ii) which blocks the antigenic peptide binding site until the complex has been transported into a post-Golgi, antigenic peptide binding compartment. There the Ii chain is cleaved and released, by proteases which could also cleave antigenic proteins (Adams S, 1977; Xu M, 1999) (Figure 17).



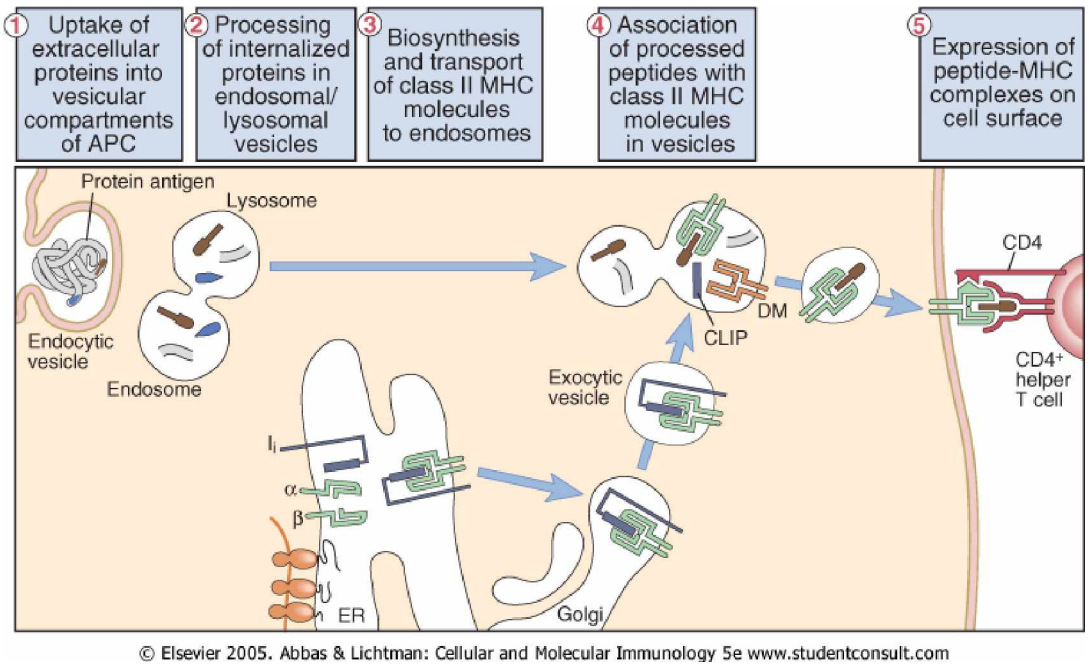
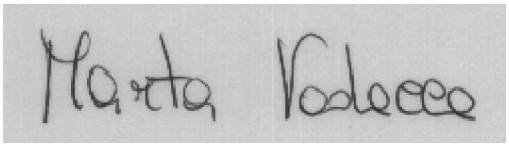


Figure 17.
The numbered stages in processing of extracellular antigens correspond to the stages described in the text. APC, antigen-presenting cell; CLIP, class II-associated invariant chain peptide; ER, endoplasmic reticulum; I_i, invariant chain.

1.7.1.1 Antigenic peptide charging

Antigenic peptide charging might proceed through two, not mutually exclusive, mechanisms. The first is by antigenic peptide exchange for a residual, proteolysis fragment of Ii, CLIP, hIi81-104. CLIP must be removed to permit antigenic peptide binding to MHC class II molecules. HLA-DM (DM) is required for efficient peptide loading of MHC class II molecules. Purified DM catalyzes release of CLIP peptides from, and binding of antigenic peptides to HLA-DR molecules. DM has editing and chaperon effect in the selection of antigenic peptides for presentation by MHC class II molecules. A second mechanism appears to be the binding of antigenic peptides to MHC class II molecules as a concerted process during the cleavage and release of Ii (Adams S, 1977; Xu M, 1999) (Figure 18).



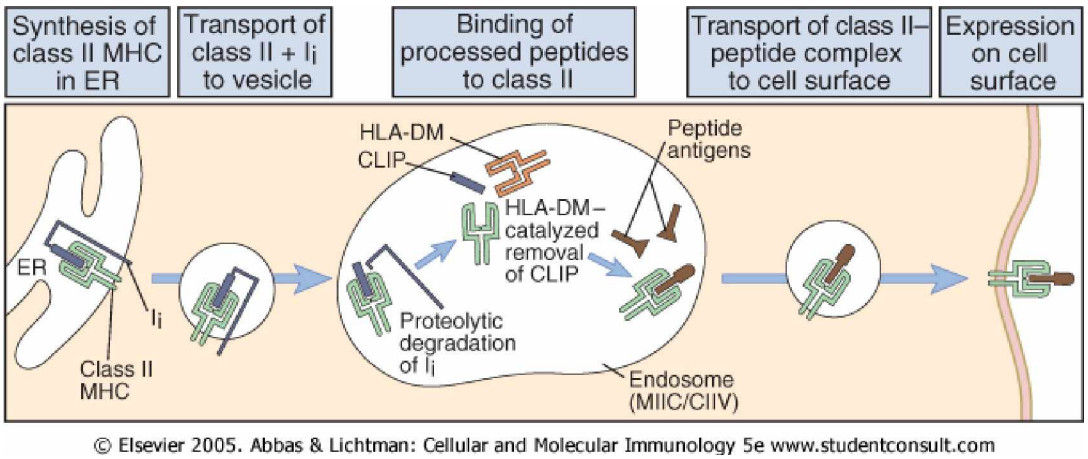
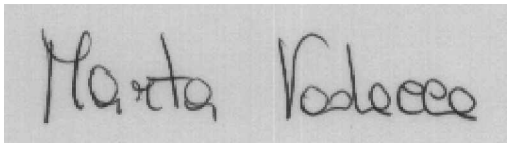


Figure 18.
Class II molecules with bound invariant chain, or CLIP, are transported into vesicles (the MIIC/CIIV), where the CLIP is removed by the action of DM. Antigenic peptides generated in the vesicles are then able to bind to the class II molecules. Another class II-like protein, called HLA-DO, may regulate the DM-catalyzed removal of CLIP. CIIV, class II vesicle; CLIP, class II-associated invariant chain peptide; ER, endoplasmic reticulum; I_i, invariant chain; MIIC, MHC class II compartment.

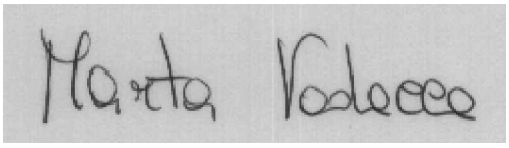
1.7.2 Ii-key peptides

1.7.2.1 Identification of the active Ii-key core sequence (LRMK)

The aminoacid sequence Ii81-89 within CLIP itself has an activity to release both longer and short forms of CLIP and some antigenic peptides from purified HLA-DR molecules (Kropshofer H, 1995). Toward a better understanding of the functional role of that region, a peptide of the Ii protein, Ii (77-92; YRMKLPKPKPPKPVSKMR), the hIi77-92 homolog, has been synthesized (Xu M, 1999). This first Ii-key peptide was synthesized to test for biological activity related to regulation of MHC class II antigenic peptide binding, because its primary sequence suggested a regulatory structure signal (six positive amino acids, no negative amino acids, four spaced prolines and recurrent cationic-hydrophobic doublets reminiscent of protease-cleavage sites) (Xu M, 1999). The activities of 160



homologs of Ii77-92 peptide (Ii-key peptide) were characterized in a murine T hybridoma activation assay as well as in an antigenic peptide binding and release assay, using immuno-purified exomembranal forms of HLA-DR1 (Xu M, 1999). In studying the molecular mechanism of immunoregulatory Ii peptides, Adams et al. found that coincubation of the Ii-key peptide greatly enhanced the presentation of certain antigenic peptides to their respective murine T hybridomas (Adams S, 1995; Adams S, 1997; Xu M, 2001). The mechanistic hypothesis has been that the Ii-key moiety binds initially to an allosteric site just outside the MHC class II binding groove inducing a conformational change in the trough for more accessible antigenic epitope charging (Sotiriadou NN, 2006). The response of T hybridomas recognizing the pigeon cytochrome C (PGCC) 81-104 increased up to 40 times baseline with limiting doses of antigenic peptide presented on a syngeneic APC-cell line. Structure activity relationship studies of 160 homologs revealed a shorter core sequence ii77-83 (LRMKLPK) with significantly greater activity than the original 16-amino acid peptide. A minimal fragment was also found, Ii77-80 (LRMK), which had half-maximal activity relative to the Ii-key core segment. The linear distance in the Ii protein sequence from this fragment to the region of Ii protein binding in the antigenic peptide-binding groove indicates that the Ii-key core interacts at an allosteric site away from the antigenic peptide binding groove (Xu M, 1999; Adams S, 1997) (Figure 19).



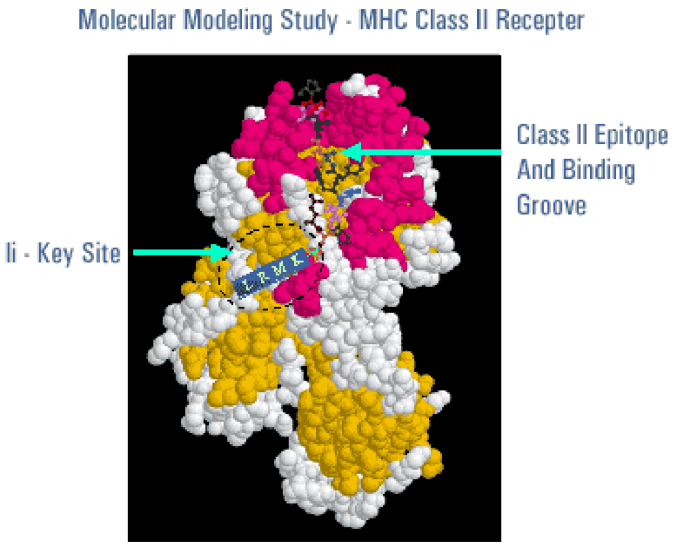


Figure 19.
Ii-Key allosteric site

1.7.2.2 Demonstration of the active Ii-key/ PGCC hybrid peptides

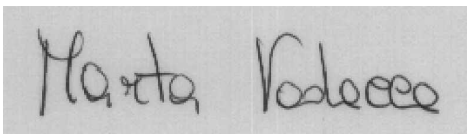
The design rationale for Ii-key/antigen hybrids was drawn from data presented in the previous section and X-ray crystallographic data indicating that the Ii-key binding site lies outside of the antigenic peptide-binding trough of MHC class II molecules. A series of hybrids has been synthesized containing Ii-key derivatives (beginning with LRMK, the shortest active derivative) coupled to the PGCC test antigen using a (δ-amino-*n*-valeric acid; ava) linker (Table 3).

Table 3. Design of hybrids with variable spacers between the Ii-key core motif and antigenic epitope

.

HYBRID #	SEQUENCE			SYMBOL
	Ii-Key	***SPACER***	**ANTIGEN**	
	Ac-		IAYLKQATAK-NH_2	r
1	Ac-LRMK-	ava*-	IAYLKQATAK-NH_2	i
2	Ac-LRMK-	ava- ava-	IAYLKQATAK-NH_2	o
3	Ac-LRMK-	LPKS-	IAYLKQATAK-NH_2	l
4	Ac-LRMK-	LPKS-AKP-	IAYLKQATAK-NH_2	n
5	Ac-LRMK-	LPKS-AKP-VSK-	IAYLKQATAK-NH_2	u

**ava* = 5-aminopentanoic acid [δ-amino-*n*-valeric acid].



Both the length and composition of the linker sequence between Ii-key and the PGCC MHC class II epitope peptide was varied within the series. Activity was measured by induction of proliferation and interleukin release in the murine T-cell hybridoma cell line Tpc9.1, which is specific for the PGCC peptide test antigen (Ghosh P, 1995). The hybrid of initial interest (No. 5) was one in which the PGCC antigenic epitope was linked with an *–ava-* spacer to the Ii protein residues LPKSAKPVSK (amino acids 81-90). In this homologs, the amino acids of the Ii peptide are in exact registry with the CLIP fragment of Ii, as residues of that Ii protein derived peptide overlay residues of the HA peptide, when respective X-ray crystallographic images of CLIL and HA are double-imposed (Stern LJ, 1994; Xu M, 2000). As shown in Figure 20, that hybrid was significantly more active than the antigenic peptide alone.

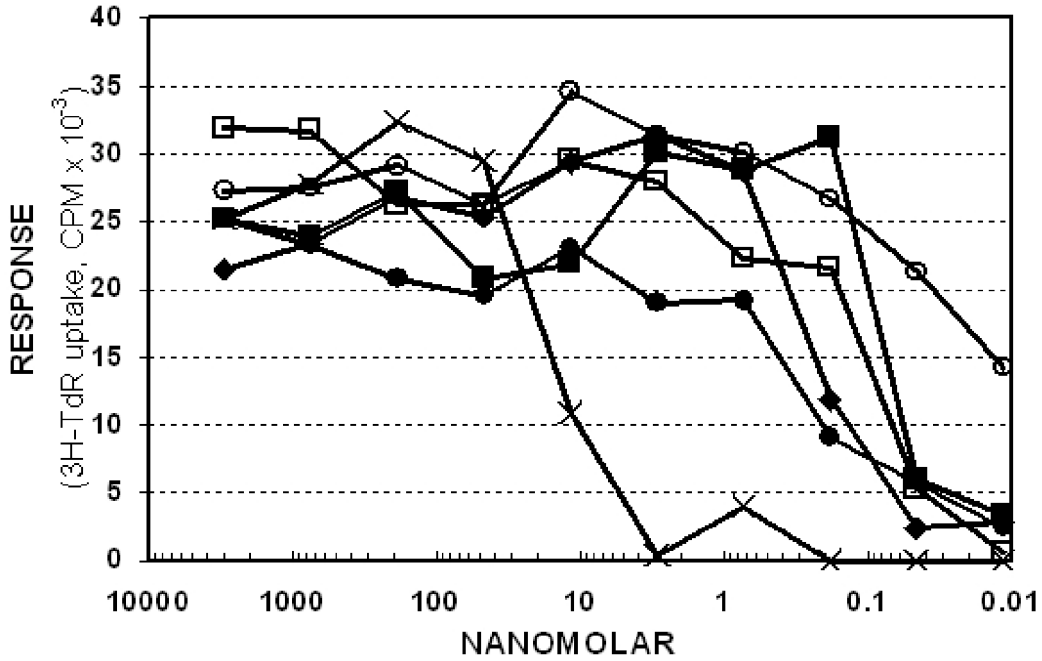
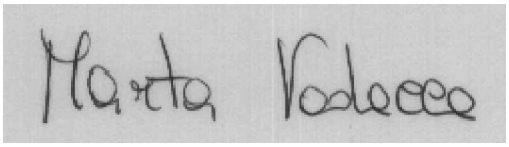


Figure 20.

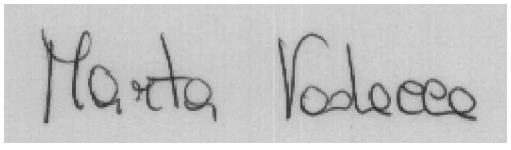
Potency of Ii-Key Hybrids. The immunological response to the antigenic epitope measured by tritiated thymidine uptake (y axis in thousands of counts/min), is presented as a function of the dilution factor of the hybrid (1:4 serial dilution from a 3 μM stock solution). The symbols for the respective hybrids and their structures are presented in Table 3. These results show that a simple 4-amino-acid Ii-Key motif with one of the shortest spacers has potent activity in this assay.

Of greater interest was the finding that the Ii-key core (LRMK) linked to the antigen by a simple methylene bridge using amino-n-valeric acid yielding a shorter linker sequence relative to peptide 5 had similar or greater potency. All Ii-key hybrid derivatives induced greater responses than naked epitope. Such Ii-key hybrid peptides use an immunomodulatory segment of the Ii protein (the Ii-key peptide) to loosen the epitope-binding groove of MHC class II molecules to permit insertion of a tethered MHC class II epitope (Xu M, 1999). In model in vitro systems, the enhancement in stimulation over free epitope peptide is >250 times when the N-terminus of the epitope is covalently linked through a chemical spacer to the C-terminus of the Ii-Key peptide, forming an Ii-Key/antigenic epitope hybrid (Humphreys RE, 2000; Kallinteris, NL 2006). This result fits an allosteric site model theory for epitope charging of MHC class II molecules. A number of studies have provided evidence that MHC class II molecules possess an allosteric site that profoundly alters epitope charging (Xu M, 1999; Xu M, 2001). Using purified soluble exomembranal HLA-DR1, Xu *et al* confirmed the existence of an allosteric site by competitive binding experiments with biotinylated Ii-key peptides or antigenic peptides (Xu M, 1999). Predictions from these data, in the context of X-ray crystallographic studies on the association of CLIP, hemaglutinin antigen and



hMBP with HLA-DR1 (Adams S, 1997; Ghosh P, 1995; Stern LJ, 1994), indicate that the active site lies just outside of the antigenic peptide binding groove. This is further suggested by NMR and computer modelling studies on the interaction between Ii-Key derivatives and HLA-DR1. Binding of a ligand to the allosteric site induces a conformational change in the antigenic peptide-binding groove such that it adopts a more accessible conformation. The allosteric site ligand, with a lesser binding affinity than the antigenic peptide for the antigenic peptide-binding groove (Xu M, 1999), dissociates to allow stabilization of the MHC class II/epitope complex (the 'clamshell' closes). Hammerling's group (Kropshofer H, 1995; Kropshofer H, 1995) has demonstrated that the N-terminal segment of CLIP (81-91) (which overlaps Ii-key) loosens the binding groove and releases the core CLIP (81-105) and other self-epitopes from the groove. Without CLIP (81-91), the core CLIP (91-105) cannot be released from the groove even at acidic conditions. The interaction of the Ii-key moiety with the allosteric site allows for direct charging of an associated epitope to any MHC class II molecule present on the surface of any cell, entirely circumventing the normal process of antigen phagocytosis, processing and intracellular charging onto MHC class II molecules. This offers us an efficient way to augment the binding of epitopes to the antigenic peptide-binding groove and thus enhances the immunogenecity of epitopes.

Linkage of MHC class II therapeutic peptides to the Ii-key (LRMK) moiety through a simple polymethylene (carbon hydrogen) spacer *(-ava-)* significantly enhances potency of presentation of the tethered peptide. One advantage of this approach is that it allows direct charging of cell surface MHC class II molecules, bypassing the need for intracellular antigen processing (Figure 21). This type of charging is



difficult if not impossible for free antigenic peptides because MHC class II molecules on the surface of cells have their antigen-binding groove occupied with endogenously charged antigenic peptides. Displacing these ambient peptides requires saturating concentrations of the therapeutic peptide.

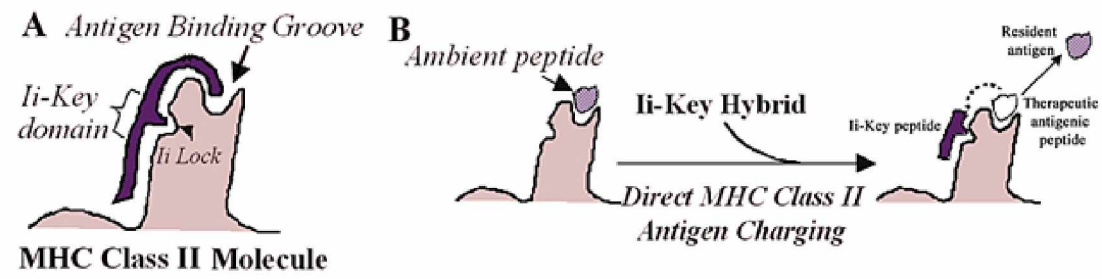
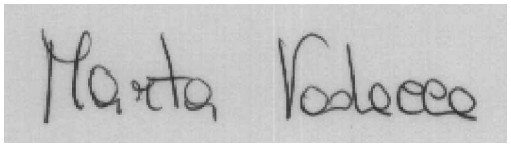


Figure 21.

Direct charging of MHC class II molecules with Ii-Key hybrid vaccine. A: Ii-Key, a small domain on the Ii protein, interacts with Ii lock, an allosteric site distal to the antigen binding groove of MHC class II molecules. B: Ii-Key hybrids consist of an Ii-Key peptide linked to a therapeutic antigenic peptide; when they bind to previously charged MHC class II molecules, the resident antigen is charged and the antigenic end of the hybrid occupies the antigen binding groove.

1.7.2.3 Ii-key hybrids in cancer, infection and autoimmune diseases

A novel technique to boost the potency of MHC class II-presented epitope peptides has become available in the form of Ii-key/MHC class II hybrid peptides (Xu M, 2001; Humphreys RE, 2000; Kallinteris NL, 2006). This is an entirely new strategy for augmenting the potency of MHC class-II restricted epitopes and results in a profound increase in both *in vitro and in vivo* T cell stimulation (Figure 22). It is also a powerful tool to efficiently expand CD4+ T-cells *ex vivo*.



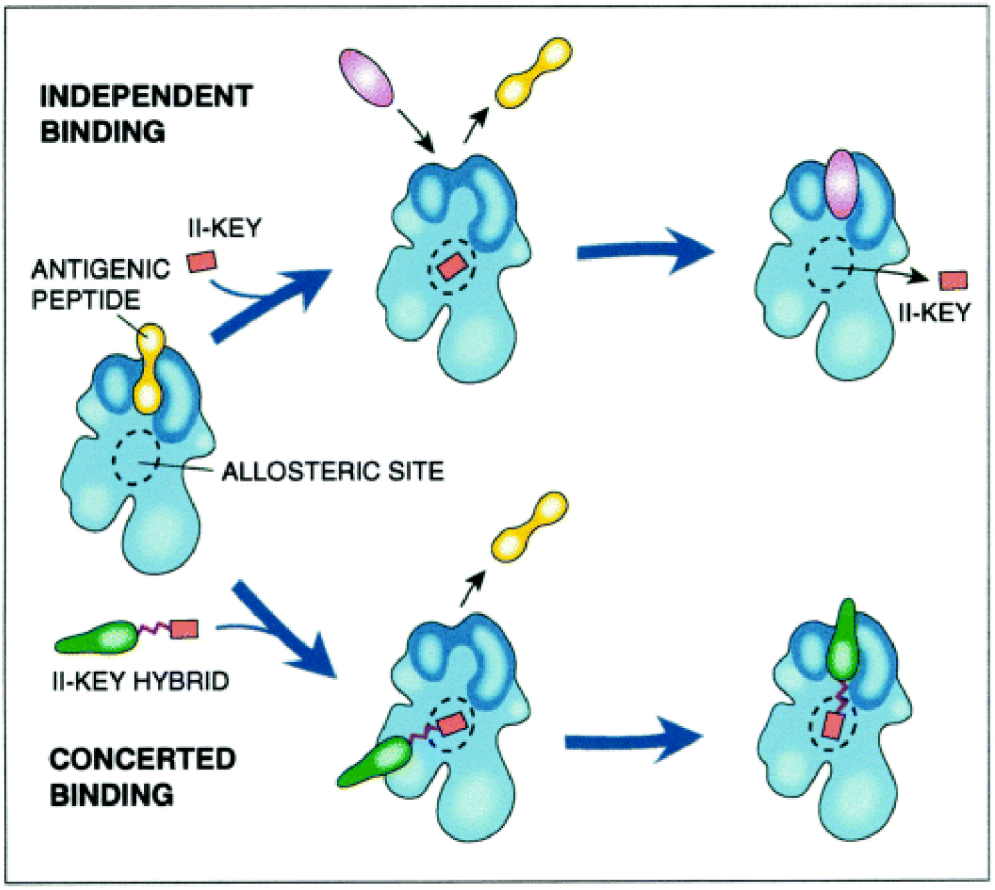
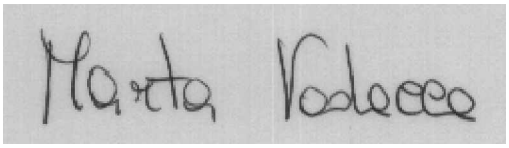


Figure 22.

The Ii-Key allosteric site ligand has different immunological activities depending upon whether it binds independently of major histocompatibility (MHC) class II restricted peptide epitopes or whether it is covalently attached to the epitope. When it binds alone, it causes 'spilling' of any antigen occupying the antigenic peptide binding groove (e.g. auto-antigens) and subsequent re-charging with ambient peptides. When hybridized to a therapeutic antigen (e.g. from an infectious agent or a tumor-associated antigen) the Ii-Key/antigenic peptide hybrid replaces the endogenously bound antigen with the therapeutic antigen directly. This diagram illustrates the allosteric site lying alongside the end of the antigenic binding site holding the N-terminal end of the antigenic peptide.

These observations have been supported by studies in cancer and infectious diseases (Kallinteris NL, 2006; Voutsas IF, 2007). In an initial preliminary study, HIV gag-hybrid peptides consistently elicited IFN-γ responses**.** In breast cancer, the Ii-key modified HER-2/*neu* hybrid peptide greatly augmented proliferative responses in



human PBMC and lymphocytes from metastatic lymph nodes (Gillohgly ME, 2005). Recent studies with DR4 transgenic mice demonstrated that Ii-key/ gp100 (46-58) MHC class II melanoma hybrid mediated a significantly greater magnitude of IFN-γ memory recall T-cell responder cells comparable to those cells generated specific to the native peptide sequence (Kallinteris NL, 2005). Overall, Ii-key/MHC class II hybrid peptides induce strong Th1 responses to various MHC class II epitopes from HER-2/*neu*, NY-ESO and tyrosinase melanoma, and influenza H5 hemagglutinin proteins (unpublished observations). Such hybrids can be applied to many diagnostic and therapeutic uses for cancer and infectious diseases, and to modify allergy and autoimmune diseases (Kallinteris NL, 2006).

1.7.2.4 Ii-key hybrids and HIV

In an initial preliminary study, HIV gag-hybrid peptides consistently elicited IFN-γ responses**.** Immunogenecity of HIV gp160 hybrids and HIVgp160 epitope-only peptide was tested in C3H/HeJ (H-2k), C57BL/6 (H-2b), and B10.A(5R) (H-2Ek-d) mice (Table 4 and 5).

Table 4.
Ii-Key/HIV gp160(843–852)

Peptide	Amino acid position	Sequence
Gp160-A	843–852	Ac-LRMK-AYRAIRHIPR-NH_2
Gp160-B	844–852	Ac-LRMK-*ava*-YRAIRHIPR-NH_2
Gp160-C	843–852	Ac-LRMK-*ava*-*ava*-AYRAIRHIPR-NH_2
Gp160-D	843–852	Ac-AYRAIRHIPR-NH_2

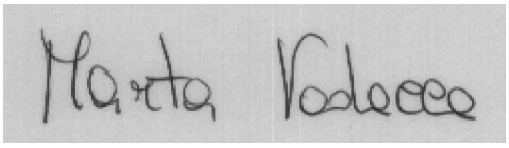
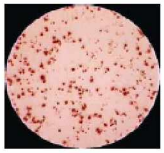
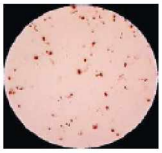
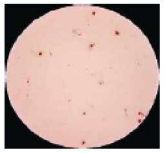


Table 5.
HIV gp160 MHC class II epitope hybrid immunization

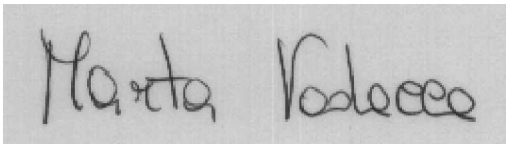
	In vivo immunogen		
	gp160-B[a]	gp160-D[b]	Naive[c]
			
Spots: in vitro stimulator			
gp160-D	208 ± 78	52 6 ± 6 14	12 ± 10
None	6 ± 6	17 6 ± 6 23	4 ± 1

[a] Injection of immunogen in IFA subcutaneously at the base of the tail on days 0, 14, and 36. On day 40, single splenocyte cell suspensions were restimulated in cultures (10^6 cells per well) containing peptide (5 μg per well) and ELISPOT assayed for IL-4 or IFN-γ cytokine recall responses. Data are expressed as the mean and S.D. spot forming cells from three mice tested individually in one experiment representative of three performed. Deviation in S.D. reflects mouse to mouse variability.
[b] The activity of gp160-D was the positive control for epitope-only peptide immunogenicity. Activity of hybrids was compared with epitope-only peptide.
[c] Positive controls for the ELISPOT included naive splenocytes (3×10^5 cells per well) stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) generated a mean number greater than 200 spots per well.

In the case of viral infections, the enhanced sensitivity afforded by Ii-Key hybrids in measuring T cell responses could be invaluable in early diagnosis of viral infections, such as the SARS agent or respiratory syncitial virus. It has been demonstrated the sensitivity of Ii-Key/HIV MHC class II epitope hybrids. Such hybrids can also be used to vaccinate individuals, either as a stand-alone vaccine, or to prime for a DNA vaccine, recombinant protein, or before an attenuated virus vaccine. (Kallinteris NL, 2003).

1.7.2.5 Ii-key hybrids and breast cancer

HER-2/*neu* is a proto-oncogene encoding a 185-kDa transmembrane protein with tyrosinase kinase activity and extensive homology to the epidermal growth factor receptor (EGF-R). HER-2 is compelling vaccine candidate for a broad spectrum of cancers due its over expression in a variety of tumours (Baxevanis CN, 2004). Pre-existent immunity has been evident at the humoral and cellular level in both normal



donors and cancer patients (Perez SA, 2002; Disis ML, 2000). HER-2 is present in normal tissues as a single copy and amplified in numerous malignant cell types. HER-2 protein contains both an intracellular and extra cellular domain, and thus both MHC class II and class I peptides have been identified (Knutson KL, 2002; Sotiriadou R, 2001). In particular, HER-2/*neu*(776-790) is a well established MHC class II epitope used commonly in clinical trials. A series of homologous Ii-Key/HER-2/*neu*(776-790) hybrid peptides, varying systematically in the length of spacer between the epitope(s)-containing segment and Ii-key moiety (LRMK) were significantly more potent than the native peptide [HER-2/*neu*(776-790)] in assays using T-cells from patients with various types of tumours over-expressing HER-2 (Voutsas IF, 2007 and Sotiriadou NN, 2007). In particular, priming patients' PBMCs with Ii-key hybrid peptides enhances recognition of the native peptide either pulsed onto autologous dendritic cells (DCs) or naturally presented by IFN-γ-treated autologous tumour cell targets. Cancer patient PBMCs were stimulated *in vitro* with either hybrid peptides –B, -C, -D, -E, and –F or native peptide (Figure 23).

PEPTIDE	SEQUENCE
Native peptide	Ac- GVGSPYVSRLLGICL-NH_2
B	Ac- LRMK-*ava* GVGSPYVSRLLGICL-NH_2
C	Ac- LRMK-*ava*- VGSPYVSRLLGICL-NH_2
D	Ac- LRMK-*ava*- GSPYVSRLLGICL-NH_2
E	Ac- LRMK-*ava*- SPYVSRLLGICL-NH_2
F	Ac- LRMK- GVGSPYVSRLLGICL-NH_2

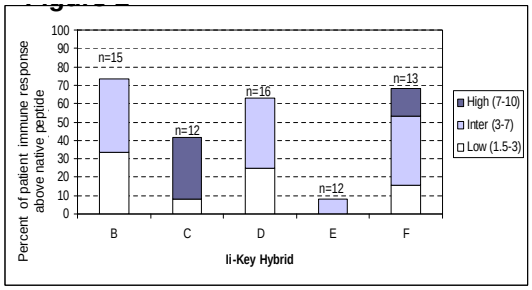


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Figure 23.
PBMC stimulation with Ii-key HER-2/neu hybrid peptides

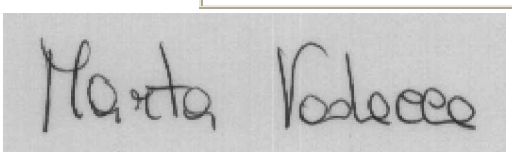
A week later, $CD4^+$ T cells recovered from growing micocultures were assessed for IFN-γ cytokine recall response against unpulsed or pulsed DC with the native peptide. Figure 23 displays the percentage of patients' immune response above the native peptide (immune response is defined as specific IFN-γ spots = native peptide-pulsed DC minus unpulsed DC). All Ii-key/HER-2/neu hybrid peptides except hybrid peptide –E augmented potency of response comparable to the native peptide. Overall, the majority of patients tested indicated strongest responses to either one of hybrid peptides –B, -D, or –F than the native peptide with a low (1.5- 3), intermediate (3-7), or high (7-10) stimulation index (Figure 23). Furthermore, such patients' CD4+ T-cells primed with the hybrid peptides provide a significantly stronger helper effect to autologous CD8+ T-cells specific for the HER-2(9_{435}) CTL epitope as illustrated by either IFN-γ ELISPOT assays or specific tumour cell lysis (Sotiriadou NN, 2007).

1.7.2.6 Ii-key hybrids and melanoma

A series of melanoma Ii-Key/gp100 (46–58) hybrids were synthesized (Table 6).

Table 6. Design of Ii-Key/gp100 (46–58) hybrids

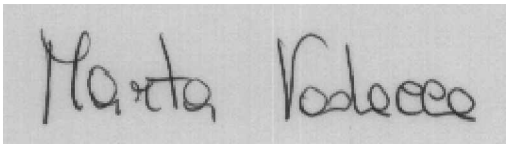
Sequence	Abbreviation
Ac- RQLYPEWTEAQRL -NH_2	A
Ac-LRMK- *ava*- NRQLYPEWTEAQRL -NH_2	B
Ac-LRMK- *ava*- RQLYPEWTEAQRL -NH_2	C
Ac-LRMK- *ava*- QLYPEWTEAQRL -NH_2	D



Sequence	Abbreviation
Ac-LRMK- *ava*- LYPEWTEAQRL -NH_2	E

Ii-Key was linked to different amino acid of the N-terminus of gp100 (46–58) by one *ava* (5-aminopentanoic acid) spacer. The flexible polymethylene spacer (δ-aminovaleric acid, -*ava*-) was a non-natural amino acid incorporated during peptide synthesis. All peptides were terminally acetylated and amidated to inhibit exopeptidases.

It has been shown that gp100 (46–58)-specific antibody titers are significantly enhanced by Ii-Key in a spacer length independent manner. In those studies, the enhancement of $CD4^+$ Th cell activation is spacer length-dependent, with the most potent hybrid being the one in which Ii-Key links to the P1 residue of the epitope through one *ava*. A mechanism for the phenomenon of space length-dependent enhancement of Tcell activation and spacer length-independent enhancement of antibody production by Ii-Key is proposed below. B cell activation needs help from antigen-specific Th cells through both T-B cell contact and cytokine release. For the activation of Th cells, hybrids are picked up by dendritic cells (DC) through cell surface MHC class II molecules. The direct charging of hybrids to MHC class II molecules on DC may be spacer length-dependent and thus the activation of epitope-specific Th cells exhibits a spacer length-dependency (Humphreys RE, 2000; Gillogly ME, 2004). In order to produce epitope specific antibodies, epitopes or hybrids must first be picked up by B cells through surface IgD and internalized for binding to MHC class II molecules, which then travel to the surface for Th cell recognition. The Ii-Key group may greatly facilitate the binding of a hybrid to MHC class II molecules inside of the B cell under acidic conditions in a spacer length



independent fashion. In this study the authors detected only gp100 (46–58)-specific IgG1 antibody, but not IgG2a when IFA of CFA were used as adjuvants.

In these studies, gp100 (46–58)-specific IgG2a titers were evident only when vaccinations were performed using CpG (Figure 24).

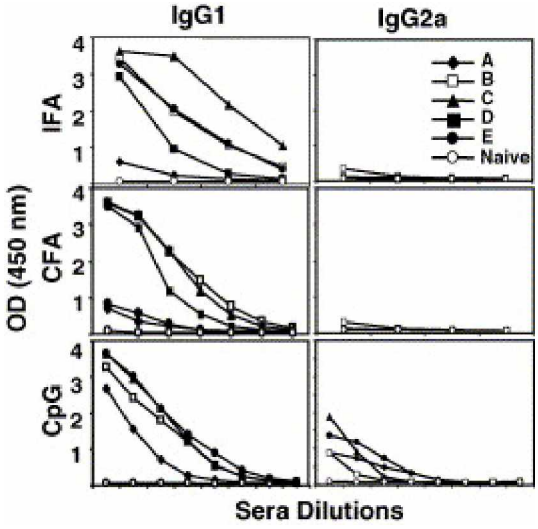
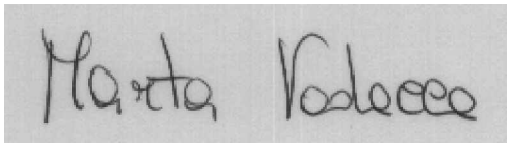


Figure 24.

Epitope-specific isotypes profile induced by Ii-Key/gp100 (46–58) hybrids and epitope-only vaccinations in IFA, CFA, and CpG. Vaccinations performed in IFA and CFA demonstrated stronger IgG1 response by hybrids B–D than the epitope alone. Hybrid E did enhance the IgG1 antibody production in IFA but not in CFA. IgG2a was negative in all samples. Vaccinations administered along with the CpG motif elicited a stronger IgG1 response for all hybrids. IgG2a was induced for hybrids in 4 out of 10 mice when compared to the gp100 (46–58) epitope alone. Data are averaged from 5 (IFA), 25 (CFA), and 10 (CpG) mice per group. The starting dilution is 1:20 and each point is a consecutive three-fold dilution.

The fact that 4 out of 10 mice in hybrid E group and 2 out of 10 mice in peptide A group induced higher IgG2a titre indicates that a vaccine boost might be necessary to induce IgG2a in all mice. These studies indicate that IL-4 production in CFA immunization might inhibit an isotypes switch to IgG2a while CpG enhances IgG2a



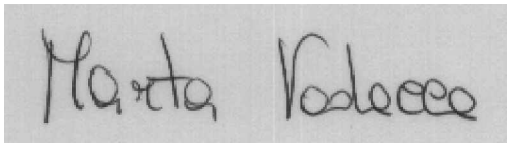
production through down-regulation of IL-4 (Kallinteris NL, 2005; Kallinteris NL, 1997).

1.8 Cytokine detection in type 1 diabetes

The measure of the clonal sizes of the islet reactive T cells provides direct information on the magnitude of the autoimmune response, and the cytokine signature of the autoreactive T cells provides information of the effector T cell class. In T1D it is widely assumed that the pathogenetic process is orchestrated by autoreactive T cells, however current studies in this respect are limited by the fact they make use of peripheral blood in which islet autoreactive T cells are likely to be rare and the epitopes remain poorly characterized. In T1D, exhaustion of the reactive T cell pool by the chronic stimulation with the endogenous antigen and the possible retention of the reactive T cells within the islets might contribute to the low frequency of autoreactive T cells in peripheral blood (Oling V, 2005). Several techniques have been used to identify autoreactive T cells in the peripheral blood of T1D. The difficulty to discriminate affected from control subjects has been reported in T cell workshops (Peakman M, 2001).

1.8.1 T cell proliferation assay

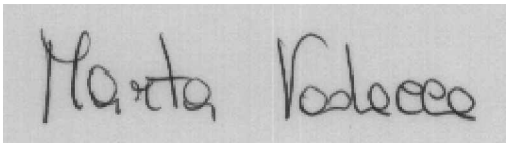
T cell proliferation assays have been applied to studies in T1D since the 1980s (Miller GG, 1987). Antigen specific T cells serve a read out for T cell reactivity and are used to monitor tolerance induction, reoccurrence of autoimmunity or immunization with antigens. The advantage of applying these bulk cultures consisting of T lymphocytes, B lymphocytes and monocytes/macrophages is several



folds: first, they are relatively easy to perform; second, they can be used to screen considerable numbers of subjects; third, the cells are tested immediately after isolation from in vivo source and have not undergone extensive in vitro cell culture (Table 7).

Table 7. Overview of different methods to be used to investigate antigen-reactive T cells

	Proliferation test	**Cytokine ELISA from supernatant**	**ELISPOT**	**Flow cytometry**
Red out	Proliferation	Amount of cytokines secreted by cells into supernatant	Spots, representing cytokine release of single cells over time	Cell surface markers, intracellular cytokines
Cells detected	CD-4 IL-2 producing T cells Antigen reactive precursor frequency	Cytokine producing cells reactive for the test antigen	Cytokine producing cells reactive to test antigen Precursor frequencies of antigen reactive T cells	Immune cells Precursor frequencies of antigen-reactive cells
Cells for analysis	Freshly isolated cells preferred	Whole blood or isolated cells	Freshly isolated cells or frozen cells	Whole blood or isolated cells, fresh cells recommended
Definition of positive response	SI > 2 or 3, mean cpm	Stimulated response minus background response	Stimulated response minus background response	Depending on threshold, gate definition
Cell survival	By split well assay	Cells survive test	Cells survive test	No cell survival
+	Relatively easy, fast	Relatively easy, fast Ex vivo production of multiple cytokines can be analyzed from same sample Supernatant can be stored	Highly sensitive Ex vivo frequencies of antigen precursor reactive T cells Evaluation of multiple cytokines	Multiparameter analysis for phenotype and cytokines
-	One parameter analyzed Bystander proliferation Bystander	Less sensitive Dilution effect might level out low or high concentration Requires standardization	Spot analysis requires special instruments	Less sensitive Cytokine content but not secretion is determined Cost intensive



	suppression/regulation			

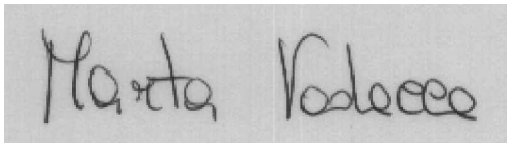
The results are expressed as stimulation index (SI), which is calculated by the cmp without stimulation (background) (Meierhoff G, 2002).

1.8.1.1 Technical limitations

CD4 Th1 cell producing IL-2 are the cell population preferentially detected in proliferation assays, because via autocrine production of Il-2, T cells are capable of generating their own growth factors. CD4 or CD8 positive cells that do not produce IL-2 cannot be detected by proliferation assays and they are thereby missed. Therefore, effector memory types of T cells that produce INF-gamma or IL-4 will remain undetected by proliferation (Gezinat G, 2001).

Another limitation is that proliferation assay tends to have a strong component of bystander proliferation. The IL-2 released by antigen specific cells can induce irrelevant, third party antigen specific T cells to also engage in proliferation. Furthermore, it renders the readout sensitive to regulatory effect by cytokines, such as IL-10 or TGF-beta, accumulating at a later stage of response. Therefore, it can recall antigen specific memory cells secreting such cytokines are present in the test sample; bystander suppression can result, thereby masking memory cell populations capable of proliferation (Meierhoff G, 2002).

Different international T cells workshops (International Immunology of Diabetes Society Workshop for Standardization of T cell assay in T1D) were performed in order to find an appropriate assay for the identification, quantification and



characterization of T cell reactive with islet antigens (Atkinson MA, 2000; Roep BO, 1999).

1.8.1.2 Selective detection of antigen-specific cells

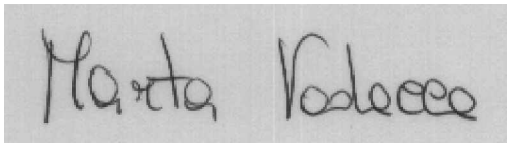
T cell proliferation based on cultures with freshly isolated PBMC represents the outcome of an exponential cell growth. Because of the bulk culture effect of coincubation and cell-cell interaction, analysis of single T cells with regard to cytokine release, epitope specificity or cross-reactivity is impossible. In an attempt to investigate single T cells, limiting dilution assay (LDA) were developed. Past a certain dilution, an all-or-none response is seen, depending on the presence or absence of one type of cell, critical for reactivity. However, these experiments are much elaborated and are not suited for a large scale use. Overall, side-by-side comparison of LDA and ELISPOT analysis showed that LDA tends to underestimate the true frequencies of antigen specific T cells (Lalvani A, 1997).

Cytokine analysis of supernatants derived from T cell clones is a way to circumvent the problem of bulk culture, since it allows the investigation of cytokine-producing single antigen specific T cells. However, the generation of T cell clones by itself is a long, artificial process and can only be used to address very specific question (Meierhoff G, 2002).

1.8.1.3 Stimulation kinetics

The majority of T cell assays investigate the proliferative response after a fixed incubation time depending on the experience of the laboratory and ranging from 72 to 144 h. Although the applied protocols are usually the outcome of an optimized incubation time, the kinetics of proliferative profiles or cytokine secretion profiles are not taken into account. Thereby, much information is lost. The amount of cells available and experimental conditions usually limited the extension of such assay (Meierhoff G, 2002).

1.8.2 ELISA



Enzyme Linked Immuno Sorbent Assay (ELISA) is at present the gold standard for determining cytokine concentrations in blood fluid such as serum or plasma. It is also used to determine cytokine concentrations in supernatants of stimulated immune cells. Immunoassays are highly specific and relatively easy to perform on a large number of samples, but often reach their detection limit with certain cytokines from ex vivo stimulated cells.

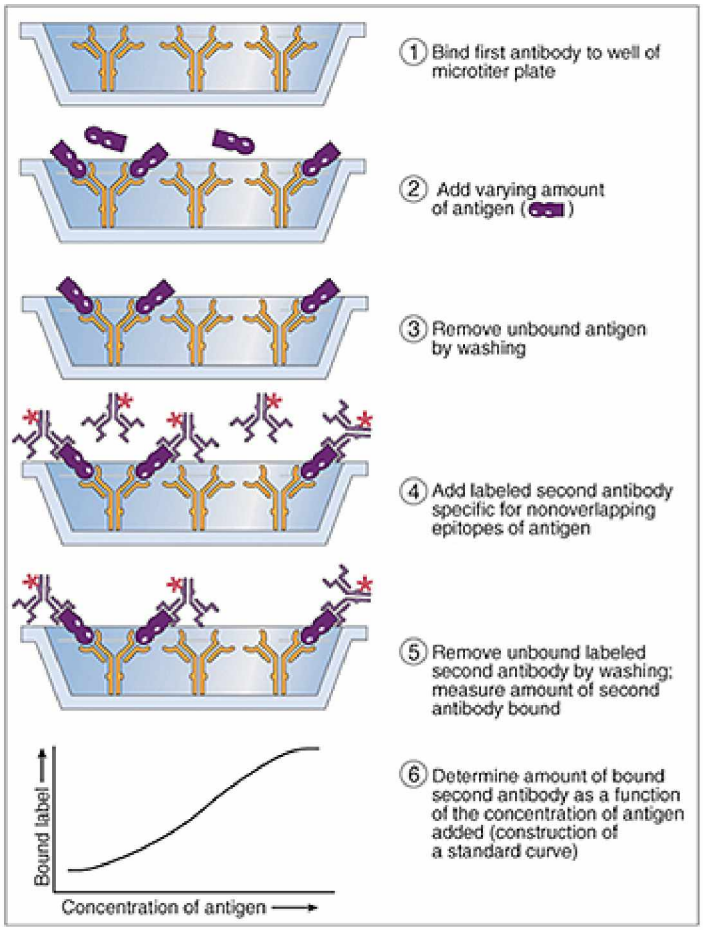


Figure 25. Sandwich enzyme-linked immunosorbent assay or radioimmunoassay. A fixed amount of one immobilized antibody is used to capture an antigen. The binding of a second, labelled antibody that recognizes a no overlapping determinant on the antigen will increase as the concentration of antigen increases and thus allow quantification of the antigen. Not copy-right issue.

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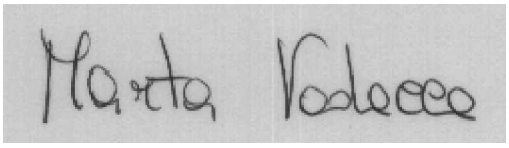
This limitation is frequently overcome by cytokine ELISPOT assay (Meierhoff G, 2002).

1.8.3 ELISPOT

1.8.3.1 ELISPOT methodology

It was demonstrated that single cell resolution cytokine enzyme-linked immunosorbent spot (ELISPOT) assays allow the direct detection of individual antigen specific T cells even if these occur at very low frequencies in the peripheral blood (Otto PA, 2004). The ELISPOT assay allows direct ex vivo measurement of antigen-reactive T cells, down to the 1:1,000,000 frequency range (Meierhoff G, 2002).

ELISPOT is principally a modification of ELISA with the important difference that upon their release by T cells, cytokines are captured directly on a surface in ELISPOT (Figure 26).



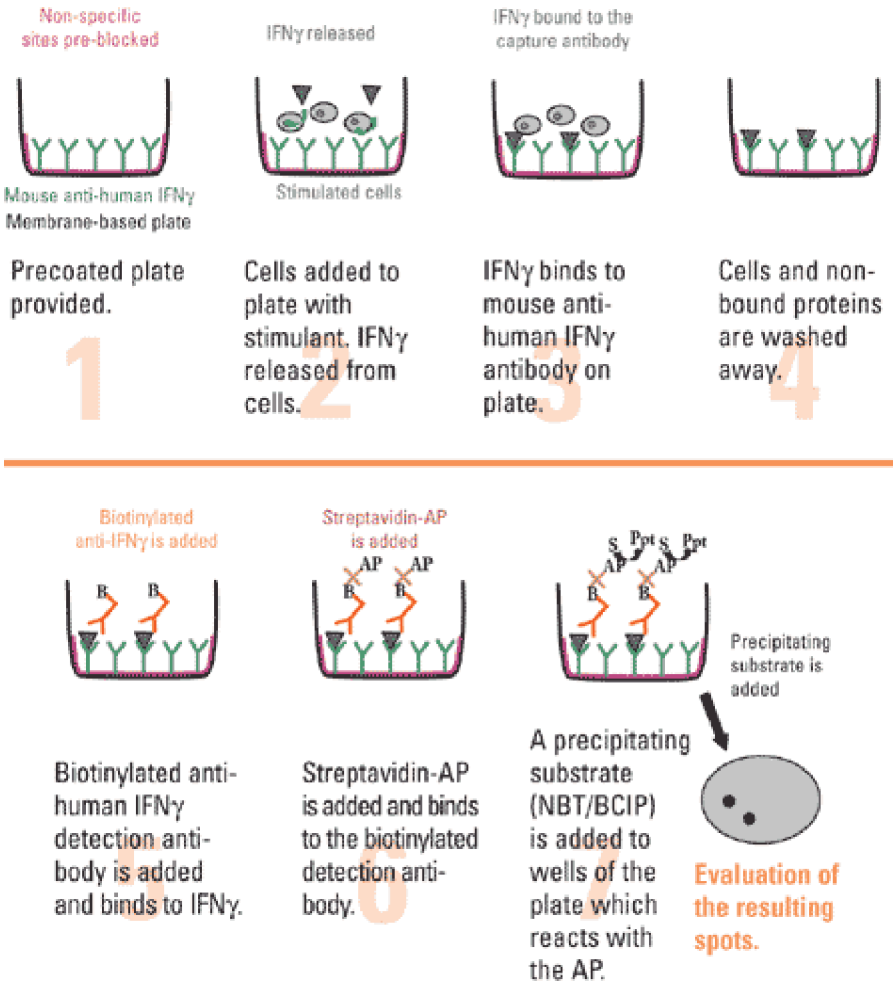
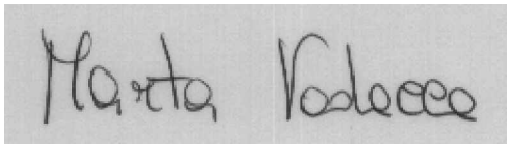


Figure 26.
How an ELISPOT works

Thereby, ELISPOT permits evaluation of antigen specific memory T cells at the single cell level with regard to the frequency (clonal size) and cytokine signature (Table 7). The cardinal ability of specific immunity being the capacity to clonally expand antigen specific cells, measuring clonal sizes is central for the understanding of T cell immunity in vivo. ELISPOT assay has been reported to be up to 400 times more sensitive than ELISA (Helms T, 2000).

Cytokine ELISPOT assay has become very attractive for clinical trials, because they assess not only the quality of the immune system but can also be used to estimate the

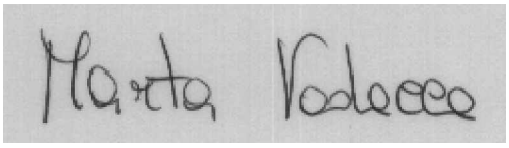


precursor frequency. Furthermore, distinctive protocols have been reported to work successfully with freeze-thawed human PBMC without loss of function.

Compared to LDA and proliferation assay, ELISPOT provides additional qualitative and quantitative information about antigen reactive precursor cells and their cytokine profile. ELISPOT assays also permit identifying the effector cells of antigen specific T cells and the relative contribution of each subset by relating the number of spots for one cytokine (e.g. INF-gamma) to another cytokine (e.g. IL-4).

1.8.3.2 ELISPOT assay in type 1 diabetes

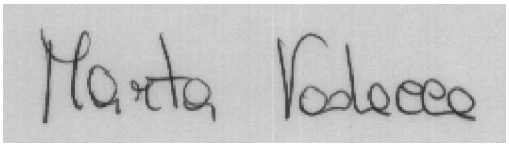
It was estimated that only 1 in 30,000 or less CD4+ T-cells in peripheral blood are GAD-65 reactive (Oling V, 2005; Peakman M, 2001). For these reasons, assays to study T cell responses must be highly sensitive. An assay of particular promise in this regard is the enzyme linked immunospot assay for the detection of cytokine secreting cells (CK ELISPOT). Several reports on the use of the CK ELISPOT assays to measure T cell response in T1D have been published (Kotani R, 2002; Karlsson Faresjo MGE, 2000; Alleva DG, 2001) and many more are sure to follow. In recent papers using an ELISPOT assay GAD-reactive Th1 cells in PBMC from T1D have been identified at a higher frequency than by other assays (Schloot NC, 2003; Meierhoff G, 2002). However, as has been the case for proliferation assay for the detection of autoreactive T cells, difficulties may arise when comparing results obtained in different studies, due to many factors including: assay format, nature of cell analyzed, choice of antigens, the method of plate reading and the analysis of the results. Recently, it has been reported a "wet" workshop for the comparisons of CK ELISPOT assays in T1D, with all participating using the blood from the same donors



and the same antigen preparations (Schloot NC, 2003). All assays were able to detect significant cytokine secretion in response to the polyclonal stimulators PHA and PMA/Ionomicyn. These stimuli are sufficiently powerful that in some cases accurate spot numbers where difficult to assign. In summary, this CK ELISPOT workshop demonstrated the detection of low-level autoreactive T cell responses. Differences between CK ELISPOT formats in terms of sensitivity will require further investigation and this will lead to the refinement of these assays as tools for understanding the complex mechanisms of beta cell destruction that lead to T1D (Schloot NC, 2003).

1.8.4 Flow cytometry analysis

Other approaches for cellular cytokine detection evolved from flow cytometry. Multiparametric flow cytometry enables simultaneous measurement of functional plus immuno-phenotypic parameters and therefore provides us with a very powerful tool (Table 7). The detection limit of flow cytometry to determine antigen-specific T cells typically ranges about 1:10,000. Multiparameter analysis of surface agents, such as cluster of differentiation markers, adds unique information not available by ELISPOT with regard to analysis of the producing cell type at single cell level. However, the sensitivity of standard methods for intracellular cytokine detection in antigen specific T cells is currently much lower compared to that for ELISPOT (Meierhoff G, 2002) (Figure 27).



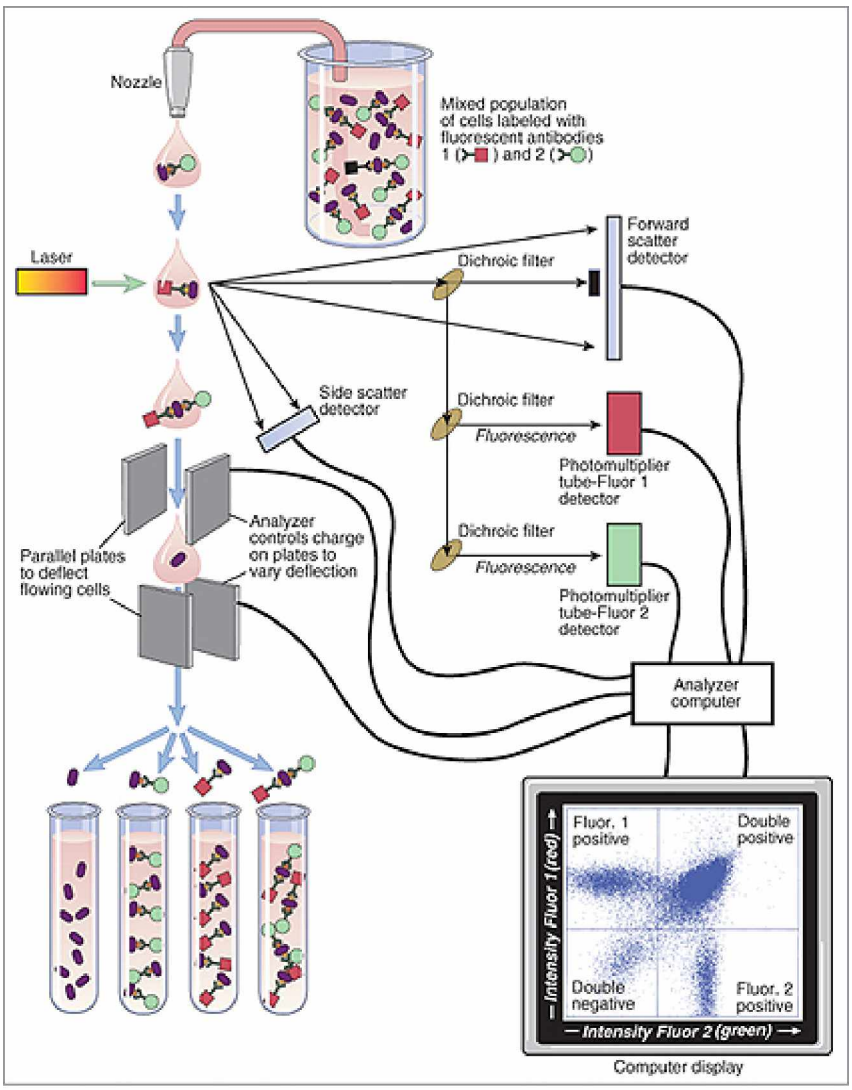
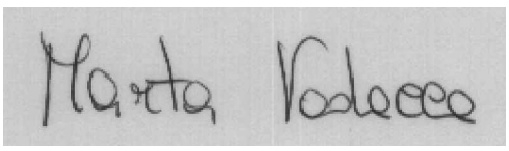


Figure 27.
Principle of flow cytometry and fluorescence-activated cell sorting. The incident laser beam is of a designated wavelength, and the light that emerges from the sample is analyzed for forward and side scatter as well as fluorescent light of two or more wavelengths that depend on the fluorochrome labels attached to the antibodies. The separation depicted here is based on two antigenic markers (two-colour sorting). Modern instruments can routinely analyze and separate cell populations on the basis of three or more different colored probes.

1.9 Therapeutic opportunities in type 1 diabetes

Immunotherapy of T1D in man and mouse models can occur at various levels including immunomanipulation, immunosuppression, using monoclonal antibodies, cytokines and various miscellaneous products including immunosuppressant,

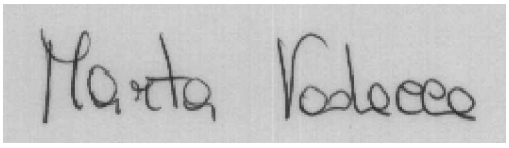


antioxidants, HSP60, insulin, dietary manipulation and various others (Pozzilli P, 2007).
Studies attempting to induce immuno tolerance, suppress the immune system and stimulate the immune system in human T1D have been carried out. Using a HSP immuno-modulatory peptide Diapep 277 in T1D, endogenous insulin secretion was preserved and a decreased need for exogenous insulin was observed (Schloot NC, 2007; Lazar L, 2007).

Basing on the ability of immunosuppressive agents to prevent diabetes in animal models i.e. cyclosporine A, various newer immunosuppressive agents including tacrolimus and sirolimus are currently undergoing clinical trials in humans (Parving HH, 1999; Aly T, 2005; Palmer JP, 2001). Anti CD3 antibodies also induced long-term remission of T1D in humans (Aly T, 2005; Pozzilli P, 2007).

1.9.1 Treatment of autoimmunity with recombinant invariant chain

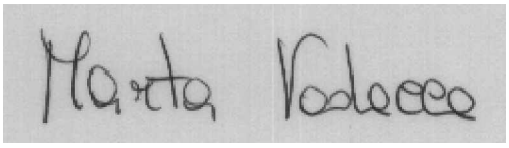
CD4 cells recognize peptides presented in association with MHC class II molecules on the surface of antigen presenting cells. Peptide loading of MHC class II molecules is regulated by the chaperone molecule invariant chain (Ii). Trimers of Ii form nanomeric complexes with newly synthesized MHC class II ab-heterodimers within the endoplasmic reticulum. In these complexes, the CLIP core region occupies the binding-grove of the MHC of class II. Genetically modified Ii proteins, in which the CLIP core region has been replaced by a T helper epitope, can be processed and presented in vitro to T cell clones by HLA-DR expressing cell lines. Genetically modified Ii proteins are superior to the peptide epitopes in triggering polyclonal, antigen-specific T cell populations in vitro and in vivo and most importantly, in modulating autoimmunity in vivo. It has been demonstrated that Ii proteins with the CLIP region replaced by antigenic epitopes represent an efficient way to activate and down-regulate specific T cells in vivo, induce antigen-specific tolerance and suppress autoimmunity. This approach may in addition be feasible for tolerance induction by



oral antigen application or DNA vaccination or to induce immunity against infectious agents or tumours (Bischof F, 2001).

1.9.2 Therapeutic opportunities with MHC class II-presented antigenic peptides

Two immunotherapy strategies focus on the control of antigen binding to the MHC class II antigenic peptide binding groove. The first focus on the identification and use of MHC class-II presented antigenic peptides to induce either an active response or antigenic specific tolerance. The second entails blocking the antigenic peptide binding groove to prevent or stop the binding of antigens that results in a harmful immune response. Numerous, clinically relevant, antigens have been identified. Some antigenic epitopes have been discovered after expressing the fragments of structural genes in APCs that is subsequently screened with a T cell line. Other antigenic epitopes have been identified by acid elution of immunopurified MHC of class II molecules followed by HPLC and tandem mass spectrometry. Yet other antigenic epitopes have been discovered after the synthesis and testing of libraries of overlapping 15 amino acids peptides of target antigenic proteins (Fleckenstein B, 1999). From these data, consensus motif for both MHC class I and II restricted epitopes have been proposed. While some peptides of potential clinical importance have been identified and specific immune responses have been observed in patients, good therapeutic efficacy has not been obtained. The main obstacles appear to be: 1. relatively modest stability of MHC class II antigenic peptides complexes does not allow an easy replacement of a designed antigenic peptide with bound of ambient peptides to form a new antigenic/MHC class II complex and 2. quick clearance of



peptide in vivo. To overcome these effects, antigenic peptides would have to be given at a saturating concentration, which is impracticable for clinical use. By enhancing binding of the antigenic epitopes in the peptide-binding groove of MHC class II molecules, Ii-key compounds, alone or in hybrids, opens the door to the discovery and clinical exploitation of many MHC class II presented epitopes, in a wide range of disease application (Xu M, 2001) (Figure 28).

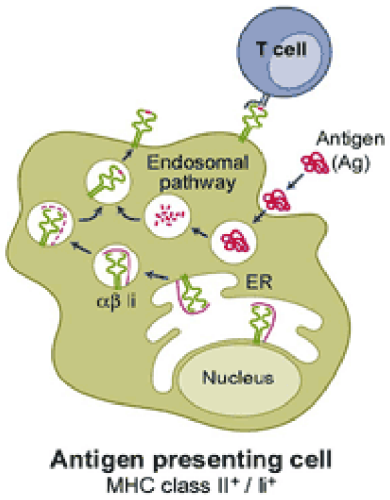
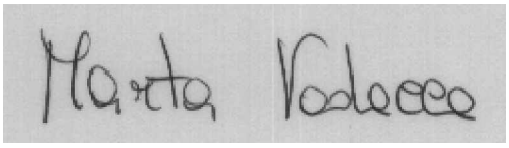


Figure 28.
MHC class II presented epitopes

1.9.3 Ii-Key/MHC class II epitope peptides as helper T cell vaccines

Ii-Key hybrids might accelerate the development of MHC class II-restricted antigenic peptide vaccines. Addition of Ii-Key spacer during the synthesis of peptide libraries, by combinatorial methods, will greatly increase the potency of the products. When the sensitivity of indicator assays are limiting, for various reasons, the greatly enhanced potency of the components in the peptide/peptidomimetic libraries will afford better pickups and/or SAR studies. This result is obtainable whether the readout is cellular proliferation or a biophysical measurement (Humphreys RE, 2000). One advantage of the Ii-Key hybrids method of immunodeviation is that the epitope itself is not altered. Synthesizing the Ii-Key-spacer motif into combinatorial libraries of putative MHC class II epitopes will enhance identification of epitopes in such libraries (Kallinteris NL, 2003).
The allosteric site on MHC class II molecules, at which Ii protein derived peptides and peptidomimetics act to alter the binding at the antigenic peptide binding site, is

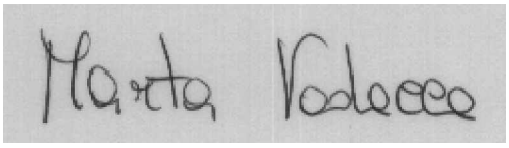


an important target for the design of drugs useful in a variety of indications. Compounds acting at this site can be used to induce the release of autoantigenic or allergic peptides, to be replaced either by ambient peptides or antigenic-site blockers. In addition, Ii-key drugs can promote the binding of vaccine peptides. Such Ii-key containing constructs can induce a T helper 1 response when administered in combination with inflammatory cytokines or adjuvants.
In the case of viral infections, the enhanced sensitivity afforded by Ii-Key hybrids in measuring T cell responses could be invaluable in early diagnosis of viral infections, such as the SARS agent or respiratory syncitial virus. The sensitivity of Ii-Key/HIV MHC class II epitope hybrids it ahs been demonstrated (Kallinteris NL, 2003). Such hybrids can also be used to vaccinate individuals, either as a stand-alone vaccine, or in preparation for a DNA vaccine, or before an attenuated virus vaccine (Table 8).

Allosteric ligand function	Therapeutic mechanism	Disease target
Ii-key as a spiller	Releasing autoantigenic peptides from MHC class II molecules	Autoimmune disease
Ii-key as a blocker-enhancer	Enhancing the binding of a blocking (no antigenic) peptide in the MHC class II antigenic peptide binding site	Autoimmune disease
Ii-key as a vaccine peptide enhancer	Enhancing the binding of an antigenic peptide in the MHC class II antigenic peptide binding site	Vaccine for cancer, infectious disease Tollerogen for allergy, autoimmune diseases, transplantation
Ii-key/blocker hybrid	Enhancing the binding of a blocking (nonatigenic)peptide in the MHC class II antigenic peptide binding site	Autoimmune disease
Ii-key/antigenic epitope hybrid	Enhancing the binding of an antigenic peptide in the MHC class II antigenic peptide binding site	Vaccine for cancer, infectious disease Tollerogen for allergy, Autoimmune disease, transplantation

Table 8. Proposed application for MHC class II allosteric site directed therapeutics.

Many data indicate that Ii-Key the technology is not only a novel method to significantly enhance the potency of peptide vaccines but also provides a novel tool for enhancing epitope-specific antibody production (Kallinteris NL, 2006).

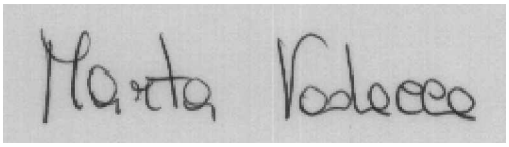


CHAPTER 2: INTRODUCTION TO THE STUDY

2.2 Background to the study

Elucidation of the specific epitopes recognised by T cells in T1D will be invaluable in developing diagnostic and therapeutic approaches to prevent the disease. At clinical onset of T1D approximately 80% of islet contains no insulin secreting cells and an infiltration of mononuclear cells is observed. These mononuclear cells include macrophages and T lymphocytes. Both CD4 and CD8 T cells are required for disease occurrence (Otto PA, 2005; Viglietta V, 2002). Genetic predisposition to type 1 diabetes is evident from population, family and twin studies as well as from identification of some key genetic regions associated with disease risk (Hawa MI, 2002). Environmental factors have been proposed as possible triggers of beta-cell autoimmunity in genetically predisposed individuals.

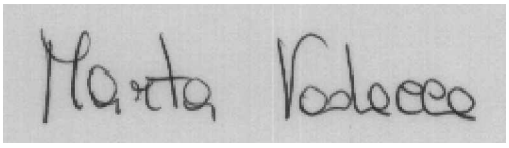
Of the familial risk for T1D, approximately 50% has been linked to the IDDM1 locus in the HLA class II region on chromosome 6p21 (Noble JA, 1996), whereas 10% has been linked to the IDD2M locus in the promoter region of the insulin gene on chromosome 11p 15.5 (Barrat BJ, 2004). In addition, several other loci have been suggested to contribute to the genetic susceptibility to T1D. The prevalence of the HLA-DR3-DQB1*0201/DR4-DQB1*0302 genotype reflects the age at onset of autoimmune diabetes, being more prevalent in childhood than adult-onset, thus T1D subjects older than 20 years at diagnosis are significantly less heterozygous for HLA -DR3-DQB1*0201/DR4-DQB1*0302 compared to childhood onset cases (Vandewalle CL, 1993).



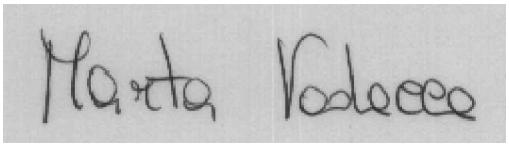
Before the clinical onset of T1D, immune changes can be detected in peripheral blood including antibodies to insulin (IAA), glutamic acid decarboxylase (GAD), and insulinoma-associated antigen (IA-2). This immune response involves both cellular and humoral changes that persist over a prolonged period of time up to diagnosis. However, the specific epitopes recognized by pathogenic T cells in this autoimmune disease remain poorly defined although numerous relevant antigenic peptides (epitopes) have been identified after the synthesis of overlapping amino acid peptides of target antigenic proteins (Schlosser M, 2005). IAA, GAD and IA-2 antibodies have become a useful marker for the progression of T1D and are the most informative biological markers for the prediction of the disease. Less is known on the specificity of cellular immune responsiveness and on T-cell profiling in T1D (Oling V, 2005).

2.2.1 T cell detection in type 1 diabetes

Because autoreactive T cells are assumed to be the key effector cells that destroy pancreatic β cells, the demonstration of autoantigen-reactive T cells could represent a good index of progressive beta-cell destruction (Kotani R, 2002). In the past years Nepom et al. have demonstrated that the use of soluble class II MHC tetramers can enable the identification of autoantigen specific T cells in the peripheral blood of T1D patients. The use of tetramer technique in the detection of autoreactive T cells is a powerful tool to gain understanding of mechanisms of the molecular basis of autoimmunity (Reijonen H, 2003). Tetramer MHC class II peptide complexes bind antigen specific CD4+ T-cells with a high degree of specificity. In a recent study it has been demonstrated that GAD-65 and proinsulin tetramer binding CD4+ T cells



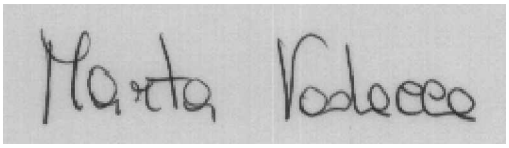
are detectable more often in peripheral blood of T1D patients and at-risk subjects than healthy individuals (Oling V, 2005). The measure of the clonal sizes of the islet reactive T cells provides direct information on the magnitude of the autoimmune response, and the cytokine signature of the autoreactive T cells provides information of the effector T cell class. In T1D it is widely assumed that the pathogenetic process is orchestrated by autoreactive T cells, however current studies in this respect are limited by the fact they make use of peripheral blood in which islet autoreactive T cells are likely to be rare and the epitopes remain poorly characterized. In T1D, exhaustion of the reactive T cell pool by the chronic stimulation with the endogenous antigen and the possible retention of the reactive T cells within the islets might contribute to the low frequency of autoreactive T cells in peripheral blood (Oling V, 2005). Several techniques have been used to identify autoreactive T cells in the peripheral blood of T1D. The difficulty to discriminate affected from control subjects has been reported in T cell workshops (Peakman M, 2001). It was estimated that only 1 in 30,000 or less $CD4^+$ T cells in peripheral blood are GAD-65 reactive (Novak EJ, 1999). For these reasons, assays to study T cell responses must be highly sensitive. Recently, it was demonstrated that single cell resolution cytokine enzyme - linked immunosorbent spot (ELISPOT) assays allow the direct detection of individual antigen specific T cells even if these occur at very low frequencies in the peripheral blood (Otto PA, 2004). The ELISPOT assay allows direct ex vivo measurement of antigen-reactive T cells, down to the 1:1,000,000 frequency range (Meierhoff G, 2002). In recent papers using an ELISPOT assay GAD reactive TH1 cells in PBMC from T1D have been identified at a higher frequency than by other assays (Meierhoff G, 2002; Schloot N, 2003).



2.2.2 Ii-key hybrids in infectious and cancer diseases

A novel technique to boost the potency of MHC class II-presented epitope peptides has become available in the form of Ii-key/MHC class II hybrid peptides (Xu M, 2001; Humphreys RE, 2000; Kallinteris NL, 2006). This is an entirely new strategy for augmenting the potency of MHC class-II restricted epitopes and results in a profound increase in both *in vitro and in vivo* T cell stimulation. It is also a powerful tool to efficiently expand $CD4^+$ T cells *ex vivo*. Clear data came from literature that Ii-Key hybrids are more potent and could be used for diagnostic purposes. In an initial preliminary study, HIV gag-hybrid peptides consistently elicited IFN-γ response. In breast cancer, the Ii-key modified HER-2/*neu* hybrid peptide greatly augmented proliferative responses in human PBMC and lymphocytes from metastatic lymph nodes (Gillohgly ME, 2005). Recent studies with DR4 transgenic mice demonstrated that Ii-key/ gp100 (46-58) MHC class II melanoma hybrid mediated a significantly greater magnitude of IFN-γ memory recall T cell responder cells comparable to those cells generated specific to the native peptide sequence (Kallinteris NL, 2005). Overall, Ii-key/MHC class II hybrid peptides induce strong Th1 responses to various MHC class II epitopes from HER-2/*neu*, NY-ESO and tyrosinase melanoma, and influenza H5 hemagglutinin proteins (unpublished observations).

Elucidation of the specific epitopes recognised by T cells in T1D will be invaluable in developing diagnostic and therapeutic approaches to prevent the disease.



2.3 Aim of this study

The overall aim of our proposal is to establish the efficacy of the Ii-key/MHC class II insulin/GAD epitope hybrids to detect anti-insulin and anti-GAD responses in subjects with T1D.

Specific aims of this study include:

a) to investigate the cellular immune response in T1D by characterising the cytokines secreted by peripheral blood mononuclear cells (PBMCs) in response to the exposure to Ii-key/MHC class II insulin/GAD epitope hybrids in an ELISPOT assay;

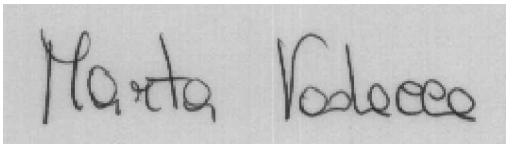
b) whether and how such immune response correlates with specific HLA types.

In order to determine whether the Ii-Key technology can enhance the presentation of specific epitopes in T1D, we have designed and synthesized a series of Ii-Key/proinsulin and GAD65 hybrids to test T1D subjects and healthy controls.

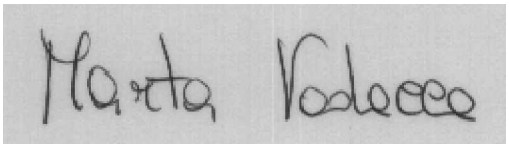
Data from this study will enable us to define the following parameters:

A) characterization of T cell immune response in subjects with T1D using the Ii-key/MHC class epitope hybrids technique. To validate the method, subjects with a positive response to one epitope hybrid will be tested again to confirm that a significant class II epitope for insulin and/or GAD has been identified.

B) identification of HLA types associated with a specific immune response in terms of epitope recognition and whether the response is T1D or HLA specific since we will study T1D and normal subjects matched for HLA class II.



To date, there are no studies using the recently introduced novel technique of Ii-key/MHC class II epitope hybrids in diabetes. Since extensive studies have confirmed the role of ELISPOT assay to detect early antigen-directed activation of lymphocyte subpopulations in T1D, we believe that the combined use of the key-hybrids technique and the ELISPOT assay might assure a more selective and sensitive cytokine detection in response to antigen stimulation.



CHAPTER 3: MATERIALS AND METHODS

3.3 Materials

3.3.1 List of Chemicals

Absolute alcohol 100 (Farcmec)

Acid acetic

AEC substrate reagent Set (Cat. No. 551951 BD)

Albumin standard

Biotinylated anti-human INF-γ (BD No. 51-1890KC)

Bovine serum albumin (BSA)

Bromophenol blue (Sigma)

Dextran (Sigma)

DMSO (Dimethyl sulfoxide)

EDTA (ethylene diaminetetra acetic acid)

Fetal bovine serum (FBS)

Ficoll Hypaque (ICN Biomedicals Inc., USA)

Glycerol (Sigma)

Hydrochloric acid (BD)

Ionomycin

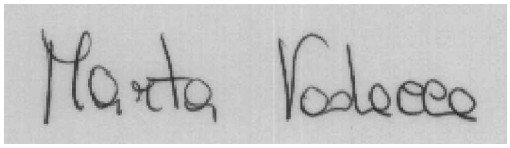
rhIL-12 (Sigma, Co. I2276)

IL-2 (BD Discovery Labwere Co. 354043)

Magnesium cloride (Sigma)

NaCl 0.1 M

PBS



Penicillin-Streptomycin-L-Glutamine (Gibco-BRL No. 10378-016)

Phytohemagglutinin (PHA)

Phorbol myristate acetate (PMA)

Purified anti-human INF-γ (NA/LE, sterile BD No. 51-2555KC)

RPMI 1640

Sodium chloride (Sigma)

Sodium phosphate buffer 0.01 M

Streptavidin-HRP (BD No. 51-9000209)

Tetramethyl benzidine (TMB)

Tween 20

3.3.2 List of Equipment

Autoclave (laboratory equipment)

Centrifuges

CO2 Chamber

Culture facilities (laboratory equipment)

Dark room (laboratory equipment)

Eli.Analyse 4.0 software (AEVS, Istituto Superiore di Sanità, Rome)

ELISA reader (laboratory equipment)

Flow-cytometer equipped with dual laser capability

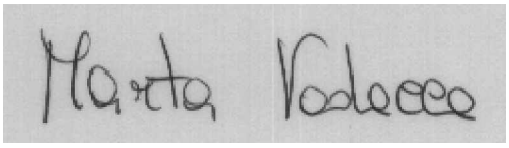
Hemocytometer (Neubauer)

Incubator

Inverted microscope

Irradiation facilities (University La Sapienza, Rome)

Liquid nitrogen cell storage



Microscope

Molecular and protein laboratories facility (laboratory equipment)

Networked computers

Personal desktop computer

Photo-documentation facility

Plate reader

Standup refrigerator/freezer (laboratory equipment)

Sterile hoods (laboratory equipment)

Tissue culture incubators

Ultracentrifuges (laboratory equipment)

Vortex mixer

-20°C freezers (laboratory equipment)

-80°C freezers (laboratory equipment)

3.3.3 List of Consumables

Alkaline Phosphatase Conjugate Substrate

ELISPOT plates (BD, Component No. 51-2447KC)

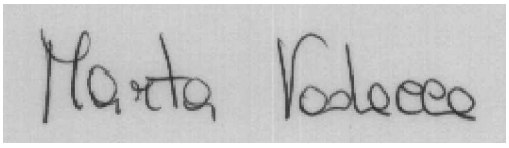
Liquid nitrogen cell storage

Microtitre plates (96 wells)

QIAmp DNA Blood Kit (QIAGEN Genomics Inc., Bothell, WA)

Tissue culture dish

3.1.4 Buffers, Media and Solutions



Blocking solution (RPMI 1640, 10% FBS, 1% Penicillin-Streptomycin-L-Glutamine)

Diluition buffer: 1 x PBS containing 10% FBS

Phosphate buffered saline (0.01 M sodium phosphate buffer, pH 7.2, 0.1 M NaCl)

Substrate solution: BD AEC substrate reagent set

Substrate solution can also be prepared as follows:

a. Prepare AEC (3-amino-9-ethyl-carbazole; Sigma A-5754) stock solution: 100 mg AEC in 10 ml DMF (N,N-Dimethylformamide; Sigma D-4551).

b. Prepare 0.1 M Acetate Solution: add 148 ml of 0.2 M acetic acid to 352 ml of 0.2 M sodium acetate. Adjust volume to 1L with water; adjust pH 5.0.

c. For final substrate solution add 333.3 µl of AEC stock solution to 10 ml 0.1 M acetate solution. Filter through 0.45 µm filter. Add 5 µl of H_20_2 (30%) and use immediately.

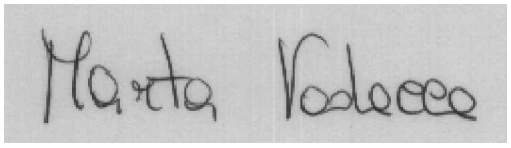
Wash buffer I: PBS containing 0.05% Tween-20

Wash buffer II: PBS

3.4 Methods

3.4.1 Autoantibodies

GAD-65, IAA and I-A2 autoantibody will be evaluated by University La Sapienza, Rome, using the radioimmunoprecipitation assays, as described elsewhere (Bingley PJ, 2003; Medici F, 1999). This laboratory, in collaboration with the Department of Diabetes and Metabolism of St. Bartholomew's Hospital in London, has participated in the ISD Proficiency Workshop; their assay has a specificity of 98% and a



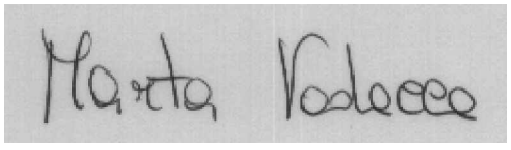
sensitivity of 80% for GAD-65, and a specificity of 100% and a sensitivity of 80% for IA-2.

3.4.2 **HLA genotyping**

For HLA typing genomic DNA will be extracted from peripheral EDTA -blood lymphocytes using the QIAmp DNA Blood Kit (QIAGEN Genomics Inc., Bothell, WA). Samples will be analyzed using the Applied Biosystem Snapshot and/or Taqman technologies, two of the cheapest and fastest methods of genotyping available today. The HLA-DRB1 polymorphism has been typed using a SSO Reverse Line Blot method established, and kindly provided by H.A. Erlich and T. Bugawan, already employed successfully in the laboratory in Rome (Galgani A, 2004). Briefly, a set of unlabelled oligonucleotide probes is immobilized onto backed nylon membrane sheets in a series of parallel strips. Cross-sections of the sheets can then be hybridised to PCR products labelled with biotin. A colorimetric detection system, which utilized the substrate tetramethyl benzidine (TMB) is used to detect hybridization. The hybridization and colour development has been performed either manually or automatically.

3.2.3 **Selection and identification of proinsulin and GAD peptides**

To select proinsulin and GAD epitopes we used using publishable information defining the epitopes (sequences and MHC class II restrictions) and testing the hypothesis that insertion of those epitopes into Ii-Key hybrids enhances potency of presentation. Predicted DRB1*0301 and DRB1*0401 epitopes have been identified by application of the Rammensee SYFPEITH program (http://syfpeithi.bmi-heidelberg.com/Scripts/ MHCServer.dll/EpPredict.htm) to the sequences of the



experimentally characterized peptides containing DR-presented sequences. Five proinsulin and five GAD peptides have been identified.

Further details are discussed in chapter 4.

3.2.3.1 Ii-key hybrids design

Ii-Key hybrid peptides are designed to contain: a) constant LRMK-*ava*- (where *ava* = amino-valeric acid; 5-aminopentanoic acid), and b) peptide sequences with a constant C-terminus, but varying at the N-terminus by one amino acid among members of the set, in a nested deletion pattern. N-termini are acetylated and C-termini are amidated to block catabolism by amino- and carboxy-peptidases, respectively. Within a homologous series, the longest and shortest hybrids are taken for initial synthesis plus the shortest peptide of 9 amino acids being considered to be a control. Further details are discussed in chapter 4.

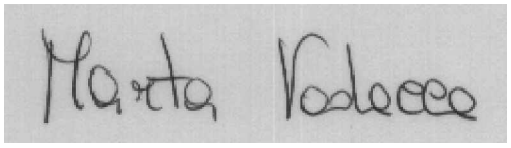
3.2.3.2 Ii-key hybrids dilution and long term storage

All peptides were dissolved in sterile distilled water (5 mg/ml) and stored at –80°C until used. Further details are discussed in Chapter 4 and 5.

3.2.4 Cell culture

3.2.4.1 Isolation of PBMC

Heparinized, freshly drawn blood samples from each subject were assayed within 6 hours. PBMC were isolated by Ficoll Hypaque density gradient centrifugation (ICN Biomedicals Inc., USA).



3.2.5 ELISPOT assay

ELISPOT assay were performed with BD Pharmingen set for IFN-γ (BD™ ELISPOT Human INF-γ ELISPOT Set, BD Bioscences, San Diego, CA, USA) (Kallinteris NL, 2003; Kallinteris NL, 2006). ELISPOT assays were performed in triplicate.

3.2.5.1 Coating antibody

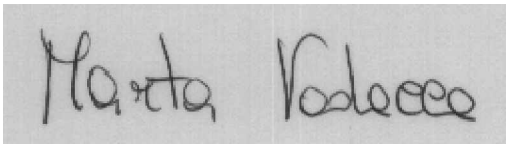
100 µl of purified anti-human INF-γ, diluted in sterile coating buffer (PBS, pH 7.2), were added to each well of the ELISPOT plate. Plate was coated overnight at 4 °C.

3.2.5.2 Blocking

On day +1, coating antibody was discarded. Wells were washed with 200 µl/well blocking solution [Cell culture medium (RPMI 1640) containing 10% human AB serum and 1% Penicillin-Streptomycin-Glutamine (Gibco-BRL)] and the plate was incubated for 2 hours at room temperature (RT) with blocking solution.

3.2.5.3 Cell activation

Irradiated autologous antigen presenting cells (APCs) pulsed (10^6 APCs/ml) were mixed with Ii-key hybrids (50 µg/ml), or epitope-controls (50 µg/ml), in complete medium and were dispensed in triplicate to the 96-well ELISA plate. 100 µl/well of PBMC suspensions (10^6 PBMC/ml) were added in each wells. IL-12 (25 pg/ml final concentration) was added to wells with medium alone and with hybrids. Positive control wells contained cell culture medium with PMA/Ionomycin (50 ng/ml and 1 µg/ml final concentration, respectively). ELISPOT plate was incubated at 37 ºC, in a 5% CO^2 and humidified incubator for 96 hours.



3.2.5.4 Detection antibody

On day +5, cell suspension was aspired and wells were washed twice with deionized (DI) water first and with 200 µl/well PBS containing 0.05% Tween-20 then. 100 µl per well of biotinylated anti-human INF-γ antibody were added. Plate was incubated for 2 hours at RT.

3.2.5.5 Enzyme conjugate

Wells were washed twice with 200 µl/well PBS. Streptavidin-HRP (100 µl/well) were dispensed into wells.

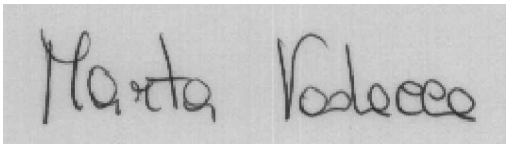
3.2.5.6 Substrate

After 1 hour at RT, 100 µl of final substrate solution (3-Amino-9-ethylcarbazole) were added to each well and incubated until the appearance of red spots in the wells (20-30 min). Plate was dried and stored in the dark, until it was analyzed.

Further details are discussed in chapter 6.

3.2.5.7 ELISPOT reader

Digitalized images of spots were analyzed with an *Eli.Analyse 4.0 software*. Criteria for spot size, circularity and color density were determined by comparing control and experimental wells. Partially overlapping spots were separated and noise signals caused by substrate precipitation and non-specific antibody binding eliminated. Relative IFN- γ production was estimated by total spot areas. Total spot area (mm^2) equals the product of mean spot size x the number of spots. Counting results have



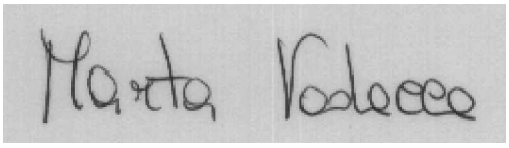
been validated by the human eye to judge whether all spots have been counted accurately and artifacts have been excluded. The number of cytokine-producing cells in antigen stimulated wells is measured in relation to the negative control wells (medium alone or epitope-controls). The difference between spot counts in the antigen-stimulated culture versus the control revealed how many antigen-specific T cells were present in the experimental wells. All data are expressed as means of triplicate determinations for each antigen.

Further details are discussed in chapter 6.

3.3 Statistical analysis

ELISPOT assays for IFN-γ production were performed in triplicate.

Statistical analysis will be performed using GraphPad statistical software, version 3.0. Results will be expressed as mean (SD) or median (range) where appropriate. Changes in the results will be considered significant at $p<0.05$. The number of cytokine producing cells in antigen stimulated wells and in negative control wells (irrelevant peptide) will be compared using the one-way ANOVA test. Kruskall-Wallis test will be performed if data is not Gaussian distributed. Bonferroni or Dunns post tests, in Gaussian or non Gaussian distribution, respectively, will be used where appropriate. Further details are discussed in chapter 6.



CHAPTER 4: SELECTION OF MHC CLASS II PRESENTED EPITOPES IN PROINSULIN AND GLUTAMIC ACID DECARBOXYLASE (GAD) IN TYPE 1 DIABETIC PATIENTS

4.1 Introduction

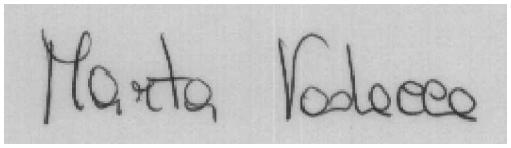
We focused on MHC Class II-presented epitopes in proinsulin and glutamic acid decarboxylase (GAD) because computer based analysis revealed a plethora of theoretical epitopes in the sequences of insulin or GAD peptides, which have been reported to contain active epitopes which simulate responses by $CD4^+$ T cells of type I diabetics. It was important to identify exactly which of the theoretical epitopes were being presented by various DR alleles and suballeles.

4.2 Materials and methods

To select proinsulin and GAD epitopes we used using publishable information defining the epitopes (sequences and MHC class II restrictions) and testing the hypothesis that insertion of those epitopes into Ii-Key hybrids enhances potency of presentation.

4.2.1 Selection of proinsulin epitopes for type 1 diabetic patients

Which of the theoretically predicted epitopes are actually recognized by a patient's immune response can be determined through experiments with panels of Ii-Key hybrids with systematic variations in N-terminal lengths of the epitope-containing segment. In some instances, up to 6 or 8 potential DR3- or DR4-presented, 9-amino-acids-long, algorithm-predicted epitopes are strung out within experimentally active



peptides of 9-24 amino acids in length. The specific 9 amino acid epitopes in such longer peptides, presented by any given DR allele, or suballele, have been reported.

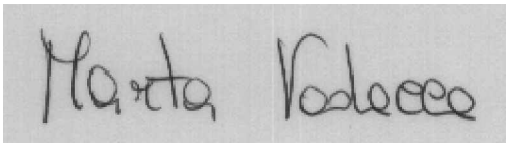
The steps taken to design Ii-Key peptides for this study are the following.

4.2.1.1 Selection of proinsulin epitopes from the literature

Peptides of proinsulin shown by others to be recognized by $CD4^+$ T cells of TID patients have been selected from the literature (Otto PA, 2004; Durinovic-Bello I, 2002; Mannering SI, 2004; Achenbach P, 2004; Padoa CJ, 2005; Alleva DG, 2001).

4.2.1.2 Sequence of human proinsulin

Sequences of the human proinsulin have been obtained from Genbank (Figure 29).



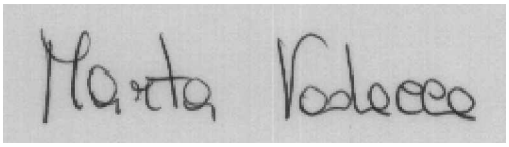


Figure 29. Sequences of proinsulin obtained from Genbank

1 malwmrllpl 1llallalwgpd 21paa afvnqhl 31cgshlvealy 41lvcgergffy 51tpktrreaed 61lqvgqvelgg 71gpgagslqpl 81alegslqkrg 91iveqcctsic 101slyqlenycn

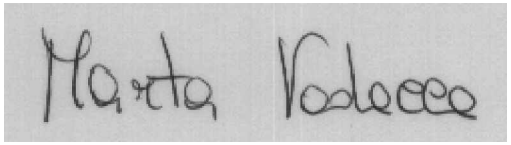
1-24 Signal peptid

25-54 Peptide B

55-89 Peptide C

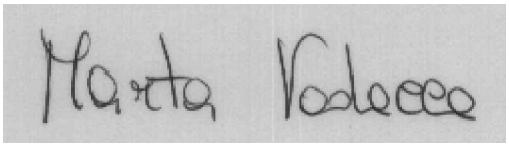
90-110 Peptide A

25-110 Proinsulin



4.2.1.3 Selection of peptides of proinsulin shown to be recognized by $CD4^+$ T cells of type 1 diabetic patients

- Predicted DRB1*0301 and DRB1*0401 epitopes have been identified by application of the Rammensee SYFPEITH program (http://syfpeithi.bmi-heidelberg.com/Scripts/MHCServer.dll/EpPredict.htm) to the sequences of the experimentally characterized peptides containing DR-presented sequences. In the case of relatively short peptides, the sequence for analysis by the program was extended by 5 amino acids at both the N- and C-termini.
- The sequence of the top-scoring, epitopes predicted for DRB1*0301 and DRB1*0401 have been plotted against the primary amino acid sequence of the experimentally studied peptides.
- Ii-Key hybrid peptides are designed to contain: a) constant LRMK-*ava*- (where *ava* = amino-valeric acid; 5-aminopentanoic acid), and b) peptide sequences with a constant C-terminus, but varying at the N-terminus by one amino acid among members of the set, in a nested deletion pattern. N-termini are acetylated and C-termini are amidated to block catabolism by amino- and carboxy-peptidases, respectively.
- Within a homologous series, the longest and shortest hybrids are taken for initial synthesis plus the shortest peptide of 9 amino acids being considered to be a control. Formally, since that peptide is the most C-terminal sequence, it might not comprise a biologically active epitope. That is, while it is a control, it might not be considered to be the appropriate epitope-only control for epitopes more N-terminal to it in a sequence. In addition, every other member

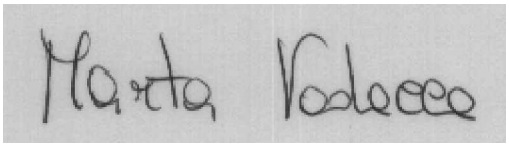


of the series has been synthesized. This limitation on the initially studied peptides within a series is made for considerations of cost and complexity of the assays, favouring instead the testing of additional possible epitopes.

- When members of a series of peptides show biological activity, the remaining unsynthesized members of a series are then synthesized, i.e., the every other hybrids which were bypassed in the first round of syntheses.

- Full runs of homologous peptides are tested within series for which at least some show some degree of activity, probably at 1:4 serial dilutions thorough a putative endpoint, if enough cells are available. Comparisons of percentage activity at one peptide concentration are potentially misleading, in contrast to the study of serial dilutions of the hybrids, which better portray potency differences among peptides within a series.

- MHC class II genotypes of the patients responding to some peptides are determined. Correlations of potency of presentation of each peptide in a homologous series with genotype (in particular DR genotype) are made. In initial studies with few patients, no statistically significant correlations are expected. As the study expands, significant epitope to genotype correlations might become apparent. The goal here is to test the hypothesis that some epitopes within hybrids become presented by certain DR suballeles which are low responders to the epitope-only peptide. The term suballele is applied here, for example to DRB1*0301, 0302, 0303 etc., within the DRB1*03 allele.



4.2.1.4 Application of the Rammensee SYFPEITH program (http://syfpeithi.bmi-eidelberg.com/Scripts/HCServer.dll/EpPredict.htm) to the sequences

NP_000198. proinsulin precur...[gi:4557671]
REVIEWED REFSEQ: This record has been curated by NCBI staff. The reference sequence was derived from X70508.1.

Summary: After removal of the precursor signal peptide, proinsulin is post-translationally cleaved into two chains (peptide A and peptide B) that are covalently linked via two disulfide bonds. Binding of this mature form of insulin to the insulin receptor (INSR) stimulates glucose uptake. A variety of mutant alleles with changes in the coding region have been identified.

FEATURES Location/Qualifiers
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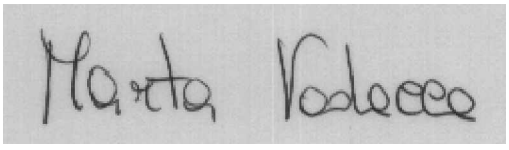
/organism="Homo sapiens"
/db_xref="taxon:9606"
/chromosome="11"
/map="11p15.5"
Protein 1..110
/product="proinsulin precursor"
proprotein 1..110
sig_peptide 1..24
mat_peptide 25..110
/product="proinsulin"
mat_peptide 25..54
/product="proinsulin peptide B"
Region 28..109
/region_name="Insulin/IGF/Relaxin family. Superfamily includes insulins"
/note="Insulin"
/db_xref="CDD:pfam00049"
mat_peptide 90..110
/product="proinsulin peptide A"
CDS 1..110
/gene="INS"
/coded_by="NM_000207.1:45..377"
/note="go_component: extracellular [goid 0005576] [evidence IEA];
go_function: insulin receptor binding [goid 0005158] [evidence TAS] [pmid 381941];
go_function: hormone activity [goid 0005179] [evidence IEA];
go_process: glucose metabolism [goid 0006006] [evidence



TAS] [pmid 381941];
go_process: lipid metabolism [goid 0006629] [evidence NR];
go_process: cell surface receptor linked signal transduction [goid 0007166] [evidence TAS] [pmid 381941];
go_process: energy pathways [goid 0006091] [evidence TAS] [pmid 381941];
go_process: cell-cell signaling [goid 0007267] [evidence NR];
go_process: physiological processes [goid 0007582] [evidence IEA]"
/db_xref="GeneID:3630"
/db_xref="LocusID:3630"
/db_xref="MIM:176730"
ORIGIN
1 malwmrllpl lallalwgpd paaafvnqhl cgshlvealy lvcgergffy tpktrreaed
61 lqvgqvelgg gpgagslqpl alegslqkrg iveqcctsic slyqlenycn
//
Summary of above:

1-24	Signal peptide
1-110	preproinsulin
25-110	proinsulin
25-54	peptide B
90-110	peptide A

C peptide (by deduction; not in above record)

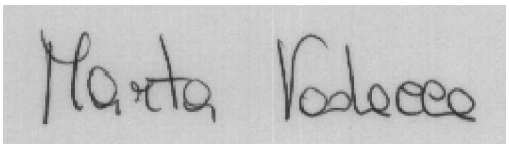
4.2.1.5 Extension of the sequence for analysis at both the N- and C-termini

DRB1*0301

14 _____
15 _____
13 _____
5 _____
3 _____
4 _____

DRB1*0401

3 _____
8 _____
9 _____
12 _____
14 _____
15 _____



AE#	EPI SEQ #	N-term	SEQ	C-term	1st aa	Syn
D1		Ac-LRMK-*ava*-	NQHLCGSHLVEALY	-NH_2	3	Y
D2		Ac-LRMK-*ava*-	QHLCGSHLVEALY	-NH_2	4	
D3		Ac-LRMK-*ava*-	HLCGSHLVEALY	-NH_2	5	Y
D4		Ac-LRMK-*ava*-	LCGSHLVEALY	-NH_2	6	
D5		Ac-	NQHLCGSHLVEALY	-NH_2	3	Y
D6		Ac-LRMK-*ava*-	VEALYLVCGERGFFYT	-NH_2	12	Y
D7		Ac-LRMK-*ava*-	EALYLVCGERGFFYT	-NH_2	13	
D8		Ac-LRMK-*ava*-	ALYLVCGERGFFYT	-NH_2	14	Y
D9		Ac-LRMK-*ava*-	LYLVCGERGFFYT	-NH_2	15	
D10		Ac-LRMK-*ava*-	YLVCGERGFFYT	-NH_2	16	Y
D11		Ac-	VEALYLVCGERGFFYT	-NH_2	12	Y

HLA-DRB1*0301 (DR17) 15 - mers

HLA-DRB1*0301 (DR17) 15 - mers

go to top

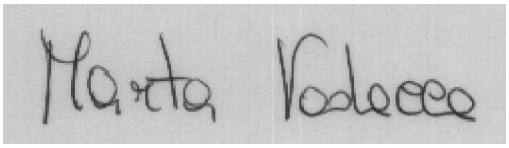
Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
14	**A** L Y **L** V **C** G E **R** G F F Y T P	26
15	**L** Y L **V** C **G** E R **G** F F Y T P K	18
13	**E** A L **Y** L **V** C G **E** R G F F Y T	17
5	**H** L C **G** S **H** L V **E** A L Y L V C	16
8	**G** S H **L** V **E** A L **Y** L V C G E R	14
3	**N** Q H **L** C **G** S H **L** V E A L Y L	11
4	**Q** H L **C** G **S** H L **V** E A L Y L V	11
6	**L** C G **S** H **L** V E **A** L Y L V C G	11
9	**S** H L **V** E **A** L Y **L** V C G E R G	11
12	**V** E A **L** Y **L** V C **G** E R G F F Y	11
2	**V** N Q **H** L **C** G S **H** L V E A L Y	4
1	**F** V N **Q** H **L** C G **S** H L V E A L	3
7	**C** G S **H** L **V** E A **L** Y L V C G E	3
10	**H** L V **E** A **L** Y L **V** C G E R G F	2
11	**L** V E **A** L **Y** L V **C** G E R G F F	2
16	**Y** L V **C** G **E** R G **F** F Y T P K T	2

HLA-DRB1*0401 (DR4Dw4) 15 - mers

HLA-DRB1*0401 (DR4Dw4) 15 - mers

go to top

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
3	**N** Q H **L** C **G** **S** H **L** V E A L Y L	14
8	**G** S H **L** V **E** **A** L **Y** L V C G E R	14
9	**S** H L **V** E **A** **L** Y **L** V C G E R G	14
12	**V** E A **L** Y **L** **V** C **G** E R G F F Y	14
14	**A** L Y **L** V **C** **G** E **R** G F F Y T P	14
15	**L** Y L **V** C **G** **E** R **G** F F Y T P K	14
1	**F** V N **Q** H **L** **C** G **S** H L V E A L	12
6	**L** C G **S** H **L** **V** E **A** L Y L V C G	12
7	**C** G S **H** L **V** **E** A **L** Y L V C G E	12
13	**E** A L **Y** L **V** **C** G **E** R G F F Y T	10
2	**V** N Q **H** L **C** **G** S **H** L V E A L Y	6
4	**Q** H L **C** G **S** **H** L **V** E A L Y L V	6
5	**H** L C **G** S **H** **L** V **E** A L Y L V C	6



11	**L** V E **A** L **Y** **L** V **C** G E R G F F	6
16	**Y** L V **C** G **E** **R** G **F** F Y T P K T	1

A g iveqcctsic slyqlenycn
Reported epitope in ____
g iveqcctsic slyqlenycn

DRB1*0301
7 _____
2 _____
3 _____
5 _____

DRB1*0401
7 _____
4 _____
1 _____

AE#	EPI SEQ #	N-term	SEQ	C-term	1st aa	Syn
D21		Ac-LRMK-*ava*-	GIVEQCCTSICSLYQ	-NH_2	1	Y
D22		Ac-LRMK-*ava*-	IVEQCCTSICSLYQ	-NH_2	2	
D23		Ac-LRMK-*ava*-	VEQCCTSICSLYQ	-NH_2	3	Y
D24		Ac-LRMK-*ava*-	EQCCTSICSLYQ	-NH_2	4	
D25		Ac-LRMK-*ava*-	QCCTSICSLYQ	-NH_2	5	Y
D26		Ac-LRMK-*ava*-	CCTSICSLYQ	-NH_2	6	
D27		Ac-LRMK-*ava*-	CTSICSLYQ	-NH_2	7	Y
D28		Ac-	GIVEQCCTSICSLYQ	-NH_2	1	Y

HLA-DRB1*0301 (DR17) 15 - mers

HLA-DRB1*0301 (DR17) 15 – mers

go to top

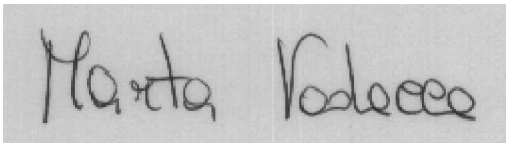
Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
7	**C** T S **I** C **S** L Y **Q** L E N Y C N	18
2	**I** V E **Q** C **C** T S **I** C S L Y Q L	9
3	**V** E Q **C** C **T** S I **C** S L Y Q L E	9
5	**Q** C C **T** S **I** C S **L** Y Q L E N Y	9
6	**C** C T **S** I **C** S L **Y** Q L E N Y C	4
4	**E** Q C **C** T **S** I C **S** L Y Q L E N	3

HLA-DRB1*0401 (DR4Dw4) 15 - mers

HLA-DRB1*0401 (DR4Dw4) 15 - mers

go to top

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
7	**C** T S **I** C **S** **L** Y **Q** L E N Y C N	26
4	**E** Q C **C** T **S** **I** C **S** L Y Q L E N	18



1	**G** I V **E** Q **C** **C** T **S** I C S L Y Q	12
2	**I** V E **Q** C **C** **T** S **I** C S L Y Q L	6
5	**Q** C C **T** S **I** C S **L** Y Q L E N Y	6

C rreaed lqvgqvelgg gpgagslqpl alegslqkr
Reported epitope in C8-24: lgg gpgagslqpl alegslqkr

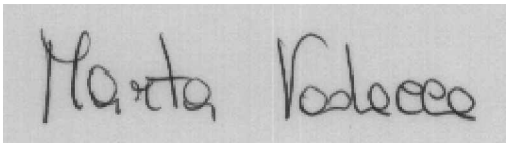
DRB1*0301
rreaed lqvgqvelgg gpgagslqpl alegslqkr

3 _____
9 _____
11 _____
4 _____
6 _____

DRB1*0401

3 _____
6 _____
9 _____
1 _____
11 _____
14 _____
16 _____
17 _____

AE#	EPI SEQ #	N-term	SEQ	C-term	1st aa	Syn
D31		Ac-LRMK-*ava*-	RREAEDLQVGGVEL	-NH_2	1	Y
D32		Ac-LRMK-*ava*-	REAEDLQVGGVEL	-NH_2	2	
D33		Ac-LRMK-*ava*-	EAEDLQVGGVEL	-NH_2	3	Y
D34		Ac-LRMK-*ava*-	AEDLQVGGVEL	-NH_2	4	
D35		Ac-LRMK-*ava*-	EDLQVGGVEL	-NH_2	5	Y
D36		Ac-	RREAEDLQVGGVEL	-NH_2	1	Y
D37		Ac-LRMK-*ava*-	LQVGGVELGGGPGA	-NH_2	7	Y
D38		Ac-LRMK-*ava*-	QVGGVELGGGPGA	-NH_2	8	
D39		Ac-LRMK-*ava*-	VGGVELGGGPGA	-NH_2	9	Y



D40		Ac-LRMK-*ava*-	GGVELGGGPGA	-NH_2	10	
D41		Ac-LRMK-*ava*-	GVELGGGPGA	-NH_2	11	Y
D42		Ac-	LQVGGVELGGGPGA	-NH_2	7	Y

HLA-DRB1*0301 (DR17) 15 - mers

HLA-DRB1*0301 (DR17) 15 - mers

go to top

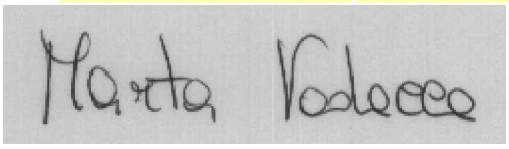
Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	Score
3	**E** A E **D** L **Q** V G **Q** V E L G G G	18
21	**G** S L **Q** P **L** A L **E** G S L Q K R	16
9	**V** G Q **V** E **L** G G **G** P G A G S L	12
11	**Q** V E **L** G **G** G P **G** A G S L Q P	12
4	**A** E D **L** Q **V** G Q **V** E L G G G P	11
6	**D** L Q **V** G **Q** V E **L** G G G P G A	11
17	**G** P G **A** G **S** L Q **P** L A L E G S	11
20	**A** G S **L** Q **P** L A **L** E G S L Q K	11
15	**G** G G **P** G **A** G S **L** Q P L A L E	10
12	**V** E L **G** G **G** P G **A** G S L Q P L	9
5	**E** D L **Q** V **G** Q V **E** L G G G P G	8
16	**G** G P **G** A **G** S L **Q** P L A L E G	8
10	**G** Q V **E** L **G** G G **P** G A G S L Q	4
18	**P** G A **G** S **L** Q P **L** A L E G S L	4
19	**G** A G **S** L **Q** P L **A** L E G S L Q	4
8	**Q** V G **Q** V **E** L G **G** G P G A G S	3
2	**R** E A **E** D **L** Q V **G** Q V E L G G	2
13	**E** L G **G** G **P** G A **G** S L Q P L A	2
14	**L** G G **G** P **G** A G **S** L Q P L A L	2
7	**L** Q V **G** Q **V** E L **G** G G P G A G	1

HLA-DRB1*0401 (DR4Dw4) 15 - mers

HLA-DRB1*0401 (DR4Dw4) 15 - mers

go to top

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	Score
20	**A** G S **L** Q **P** **L** A **L** E G S L Q K	20
3	**E** A E **D** L **Q** **V** G **Q** V E L G G G	18
6	**D** L Q **V** G **Q** **V** E **L** G G G P G A	14
9	**V** G Q **V** E **L** **G** G **G** P G A G S L	14
11	**Q** V E **L** G **G** **G** P **G** A G S L Q P	14
1	**R** R E **A** E **D** **L** Q **V** G Q V E L G	12
14	**L** G G **G** P **G** **A** G **S** L Q P L A L	12
16	**G** G P **G** A **G** **S** L **Q** P L A L E G	12
17	**G** P G **A** G **S** **L** Q **P** L A L E G S	12
21	**G** S L **Q** P **L** **A** L **E** G S L Q K R	12



4	**A** E D **L** Q **V** **G** Q **V** E L G G G P	8
7	**L** Q V **G** Q **V** **E** L **G** G G P G A G	6
8	**Q** V G **Q** V **E** **L** G **G** G P G A G S	6
12	**V** E L **G** G **G** **P** G **A** G S L Q P L	6
13	**E** L G **G** G **P** **G** A **G** S L Q P L A	6
15	**G** G G **P** G **A** **G** S **L** Q P L A L E	6

4.2.2 Selection of GAD epitopes for type 1 diabetic patients

In the last two years many GAD 65 peptides have been shown to be efficiently processed immunodominant epitopes in patients with type 1 diabetes.

4.2.2.1 Selection of GAD epitopes from the literature

Here it has been reported a panel of epitopes reported to be of relevance to islet autoimmunity (Banga JP, 2004; Gebe JA, 2004; Capitani G, 2005; Otto PA, 2005; Kanaani J, 2002; Nepom GT, 2001; Kotani R, 2002; Viglietta V, 2002; Jaume JC, 2002).

GAD 115-125

GAD 217-236

GAD 243-444

p247-279

p270-283

p274-286

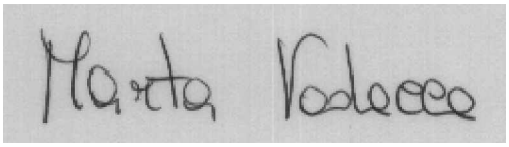
p286-300

GAD 479-498

GAD 500-585

p509-528

p515-524



p525-543

p554-575

p555-567

p556-575

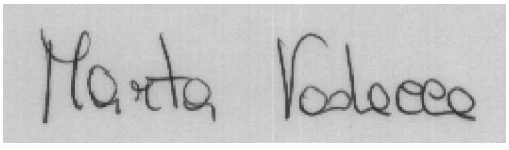
p570-585

Correspondence of peptide sequences in literature with peptide sequences in Genbank for GAD 65.

Seq no	Ref	Seq in references aa numbers	Seq in references Amino acids	aa numbers of that sequence In Genbank
270-283	1	270-283	LPRLIAFTSEHF	270-283
286-300	2	286-300	Not in this ref	
525-543	3	524-543	SRLSKVAPVIKARMMEYGTT	525-544
555-567	4	555-567	NFFRMVISNPAAT	555-567

MHC class II epitopes (9 amino acids from first position in SYFPETHI predictions) for the above segments of GAD 65.

Seq.	270-283		286-300		524-543		555-567	
DR-1	273	25	288	21	534	24	554	33
	270	22	289	19	527	23	556	18
			287	18	535	22		
					538	21		
					544	20		
					533	18		
DR-3	271	17			546	22	557	14
					529	19	556	13
					526	18		
					545	17		
					547	16		
					541	13		



DR-4	273	28	288	18	526	20	554	28
	270	26			534	20		
					535	20		
					537	16		
DR-7	271	22	288	22	534	24	554	22
	273	18			537	18		
	270	16			526	16		
					535	16		
					544	16		
DR-11	273	28			534	28	555	16
	270	28			535	24		
					547	24		
DR-15	273	16	291	20	541	30	553	25
					547	24	554	16
					529	16		

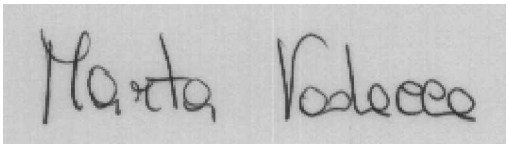
4.2.2.2 Sequence of human GAD

The following sequences are deduced from the deduced protein sequence in the Genbank entry appended below.

ORIGIN
1 maspgsgfws fgsedgsgds enpgtarawc qvaqkftggi gnklcallyg daekpaesgg
61 sqppraaark aacacdqkpc scskvdvnya flhatdllpa cdgerptlaf lqdvmnillq
121 yvvksfdrst kvidfhypne llqeynwela dqpqnleeil mhcqttlkya iktghpryfn
181 qlstgldmvg laadwltsta ntnmftyeia pvfvlleyvt lkkmreiigw pggsgdgifs
241 pggaisnmya mmiarfkmfp evkekgmaal prliaftseh shfslkkgaa algigtdsvi
301 likcdergkm ipsdlerril eakqkgfvpf lvsatagttv ygafdpllav adickkykiw
361 mhvdaawggg llmsrkhkwk lsgveransv twnphkmmgv plqcsallvr eeglmqncnq
421 mhasylfqqd khydlsydtg dkalqcgrhv dvfklwlmwr akgttgfeah vdkclelaey
481 lyniiknreg yemvfdgkpq htnvcfwyip pslrtledne ermsrlskva pvikarmmey
541 gttmvsyqpl gdkvnffrmv isnpaathqd idflieeier lgqdl
//

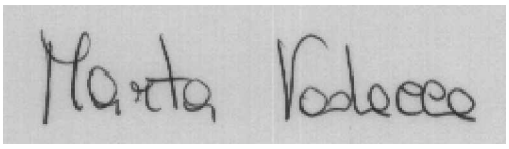
4.2.2.3 Selection of peptides of GAD shown to be recognized by $CD4^+$ T cells of type 1 diabetic patients

LOCUS Q05329 585 aa linear PRI 24-JAN-2006
DEFINITION Glutamate decarboxylase 2 (Glutamate decarboxylase, 65 kDa isoform)



(GAD-65) (65 kDa glutamic acid decarboxylase).
ACCESSION Q05329
VERSION Q05329 GI:1352216
DBSOURCE swissprot: locus DCE2_HUMAN, accession Q05329;
class: standard.
extra accessions:Q9UD87,created: Feb 1, 1996.
sequence updated: Feb 1, 1996.
annotation updated: Jan 24, 2006.
xrefs: M81882.1, AAA62367.1, M74826.1, AAA58491.1, AY340073.1, AAP88040.1, X69936.1, CAA49554.1, M70435.1, AAA52513.1, A41292, 1ES0B
xrefs (non-sequence databases): EnsemblENSG00000136750, HGNCHGNC:4093, MIM138275, GO0005515, GO0006540, GO0007268, InterProIPR002129, PANTHERPTHR11999, PfamPF00282, PROSITEPS00392
KEYWORDS 3D-structure; Decarboxylase; Lipoprotein; Lyase; Multigene family; Neurotransmitter biosynthesis; Palmitate; Phosphorylation; Polymorphism; Pyridoxal phosphate.
SOURCE Homo sapiens (human)
ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Catarrhini; Hominidae; Homo.
COMMENT On Mar 15, 2005 this sequence version replaced gi:106112.
[FUNCTION] Catalyzes the production of GABA.
[CATALYTIC ACTIVITY] L-glutamate = 4-aminobutanoate + CO(2).
[COFACTOR] Pyridoxal phosphate.
[BIOPHYSICOCHEMICAL PROPERTIES] Kinetic parameters: KM=2.15 mM for
glutamate.
[SUBUNIT] Homodimer (By similarity).
[SUBCELLULAR LOCATION] Cytosolic, and associated to cytoplasmic vesicles. In neurons, cytosolic leaflet of Golgi membranes and presynaptic clusters.
[PTM] Phosphorylated; which does not affect kinetic parameters or subcellular location.
[PTM] Palmitoylated; which is required for presynaptic clustering.
[SIMILARITY] Belongs to the group II decarboxylase family.

4.2.2.4 Application of the Rammensee SYFPEITH program (http://syfpeithi.bmideidelberg.com/Scripts/MHCServer.dll/EpPredict.htm) to the sequences



The deduced protein sequence for Genbank entry below was run throught SYFPETHI (http://www.syfpeithi.de/Scripts/MHCServer.dll/Info.htm) and the top MHC II epitopes for each scored DR allele are listed.

FEATURES Location/Qualifiers
source 1..585
/organism="Homo sapiens"
/db_xref="taxon:9606"
gene 1..585
/gene="GAD2"
/note="synonym: GAD65"
Protein 1..585
/gene="GAD2"
/product="Glutamate decarboxylase 2"
/EC_number="4.1.1.15"
Site 3
/gene="GAD2"
/site_type="modified"
/experiment="experimental evidence, no additional details recorded"
/note="Phosphoserine."
Site 6
/gene="GAD2"
/site_type="modified"
/experiment="experimental evidence, no additional details recorded"
/note="Phosphoserine."
Site 10
/gene="GAD2"
/site_type="modified"
/experiment="experimental evidence, no additional details recorded"
/note="Phosphoserine."
Region 12
/gene="GAD2"
/region_name="Variant"
/experiment="experimental evidence, no additional details recorded"
/note="G -> R (in dbSNP:8190591). /FTId=VAR_018821."
Site 13
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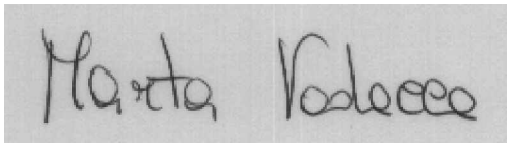
/site_type="modified"
/experiment="experimental evidence, no additional details recorded"
/note="Phosphoserine."
Site 30



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                /gene="GAD2"
                /site_type="lipid-binding"
                /experiment="experimental evidence, no additional details
                recorded"
                /note="S-palmitoyl cysteine."
     Site       45
                /gene="GAD2"
                /site_type="lipid-binding"
                /experiment="experimental evidence, no additional details
                recorded"
                /note="S-palmitoyl cysteine."
     Region     124
                /gene="GAD2"
                /region_name="Variant"
                /experiment="experimental evidence, no additional details
                recorded"
                /note="K -> N (in dbSNP:8190600). /FTId=VAR_018822."
     Region     286
                /gene="GAD2"
                /region_name="Variant"
                /experiment="experimental evidence, no additional details
                recorded"
                /note="K -> R (in dbSNP:8190671). /FTId=VAR_018823."
     Region     375
                /gene="GAD2"
                /region_name="Variant"
                /experiment="experimental evidence, no additional details
                recorded"
                /note="R -> Q (in dbSNP:8190730). /FTId=VAR_018824."
     Site       396
                /gene="GAD2"
                /site_type="binding"
                /inference="non-experimental evidence, no additional
                details recorded"
                /note="Pyridoxal phosphate (covalent) (By similarity)."
ORIGIN
        1 maspgsgfws fgsedgsgds enpgtarawc qvaqkftggi gnklcallyg daekpaesgg
       61 sqppraaark aacacdqkpc scskvdvnya flhatdllpa cdgerptlaf lqdvmnillq
      121 yvvksfdrst kvidfhypne llqeynwela dqpqnleeil mhcqttlkya iktghpryfn
      181 qlstgldmvg laadwltsta ntnmftyeia pvfvlleyvt lkkmreiigw pggsgdgifs
      241 pggaisnmya mmiarfkmfp evkekgmaal prliaftseh shfslkkgaa algigtdsvi
      301 likcdergkm ipsdlerril eakqkgfvpf lvsatagttv ygafdpllav adickkykiw
      361 mhvdaawggg llmsrkhkwk lsgveransv twnphkmmgv plqcsallvr eeglmqncnq
      421 mhasylfqqd khydlsydtg dkalqcgrhv dvfklwlmwr akgttgfeah vdkclelaey
      481 lyniiknreg yemvfdgkpq htnvcfwyip pslrtledne ermsrlskva pvikarmmey
      541 gttmvsyqpl gdkvnffrmv isnpaathqd idflieeier lgqdl
//
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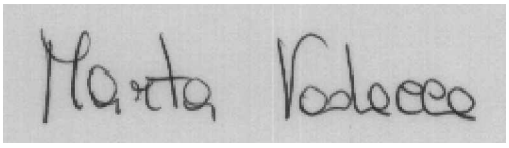


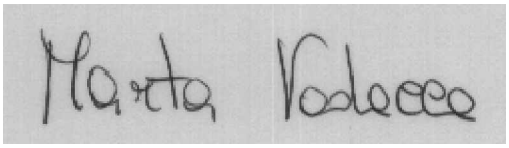
4.2.2.5 Extension of the sequence for analysis at both the N- and C-termini.

MHC Class II epitopes predicted for selected common HLA-DR alleles are reported below.

HLA-DRB1*0101 15 - mers

HLA-DRB1*0101 15 - mers go to top

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
86	**D** V N **Y** A **F** L H **A** T D L L P A	36
175	**H** P R **Y** F **N** Q L **S** T G L D M V	33
185	**G** L D **M** V **G** L A **A** D W L T S T	33
554	**V** N F **F** R **M** V I **S** N P A A T H	33
267	**M** A A **L** P **R** L I **A** F T S E H S	32
325	**K** G F **V** P **F** L V **S** A T A G T T	31
242	**G** G A **I** S **N** M Y **A** M M I A R F	30
456	**W** L M **W** R **A** K G **T** T G F E A H	30
210	**A** P V **F** V **L** L E **Y** V T L K K M	29
397	**M** M G **V** P **L** Q C **S** A L L V R E	29
33	**A** Q K **F** T **G** G I **G** N K L C A L	28
112	**Q** D V **M** N **I** L L **Q** Y V V K S F	28
227	**I** I G **W** P **G** G S **G** D G I F S P	28
142	**L** Q E **Y** N **W** E L **A** D Q P Q N L	27
202	**T** N M **F** T **Y** E I **A** P V F V L L	27
256	**F** K M **F** P **E** V K **E** K G M A A L	27
280	**H** S H **F** S **L** K K **G** A A A L G I	27
246	**S** N M **Y** A **M** M I **A** R F K M F P	26
341	**Y** G A **F** D **P** L L **A** V A D I C K	26
360	**W** M H **V** D **A** A W **G** G G L L M S	26
482	**Y** N I **I** K **N** R E **G** Y E M V F D	26
488	**R** E G **Y** E **M** V F **D** G K P Q H T	26
44	**L** C A **L** L **Y** G D **A** E K P A E S	25
176	**P** R Y **F** N **Q** L S **T** G L D M V G	25
235	**G** D G **I** F **S** P G **G** A I S N M Y	25
273	**L** I A **F** T **S** E H **S** H F S L K K	25
329	**P** F L **V** S **A** T A **G** T T V Y G A	25
432	**H** Y D **L** S **Y** D T **G** D K A L Q C	25
29	**W** C Q **V** A **Q** K F **T** G G I G N K	24
41	**G** N K **L** C **A** L L **Y** G D A E K P	24
95	**T** D L **L** P **A** C D **G** E R P T L A	24
114	**V** M N **I** L **L** Q Y **V** V K S F D R	24
168	**K** Y A **I** K **T** G H **P** R Y F N Q L	24
213	**F** V L **L** E **Y** V T **L** K K M R E I	24
221	**L** K K **M** R **E** I I **G** W P G G S G	24
264	**E** K G **M** A **A** L P **R** L I A F T S	24
318	**R** I L **E** A **K** Q K **G** F V P F L V	24
338	**T** T V **Y** G **A** F D **P** L L A V A D	24
357	**Y** K I **W** M **H** V D **A** A W G G G L	24



391	**T** W N **P** H **K** M M **G** V P L Q C S	24
439	**T** G D **K** A **L** Q C **G** R H V D V F	24
520	**E** E R **M** S **R** L S **K** V A P V I K	24
534	**K** A R **M** M **E** Y G **T** T M V S Y Q	24
553	**K** V N **F** F **R** M V **I** S N P A A T	24
25	**T** A R **A** W **C** Q V **A** Q K F T G G	23
130	**T** K V **I** D **F** H Y **P** N E L L Q E	23
314	**D** L E **R** R **I** L E **A** K Q K G F V	23
378	**K** W K **L** S **G** V E **R** A N S V T W	23
415	**M** Q N **C** N **Q** M H **A** S Y L F Q Q	23
527	**S** K V **A** P **V** I K **A** R M M E Y G	23
108	**L** A F **L** Q **D** V M **N** I L L Q Y V	22
111	**L** Q D **V** M **N** I L **L** Q Y V V K S	22
182	**L** S T **G** L **D** M V **G** L A A D W L	22
245	**I** S N **M** Y **A** M M **I** A R F K M F	22
270	**L** P R **L** I **A** F T **S** E H S H F S	22
304	**C** D E **R** G **K** M I **P** S D L E R R	22
354	**C** K K **Y** K **I** W M **H** V D A A W G	22
394	**P** H K **M** M **G** V P **L** Q C S A L L	22
453	**F** K L **W** L **M** W R **A** K G T T G F	22
503	**N** V C **F** W **Y** I P **P** S L R T L E	22
523	**M** S R **L** S **K** V A **P** V I K A R M	22
526	**L** S K **V** A **P** V I **K** A R M M E Y	22
535	**A** R M **M** E **Y** G T **T** M V S Y Q P	22
570	**D** I D **F** L **I** E E **I** E R L G Q D	22
6	**S** G F **W** S **F** G S **E** D G S G D S	21
56	**A** E S **G** G **S** Q P **P** R A A A R K	21
158	**E** I L **M** H **C** Q T **T** L K Y A I K	21
166	**T** L K **Y** A **I** K T **G** H P R Y F N	21
198	**S** T A **N** T **N** M F **T** Y E I A P V	21
204	**M** F T **Y** E **I** A P **V** F V L L E Y	21
288	**G** A A **A** L **G** I G **T** D S V I L I	21
376	**K** H K **W** K **L** S G **V** E R A N S V	21
389	**S** V T **W** N **P** H K **M** M G V P L Q	21
479	**E** Y L **Y** N **I** I K **N** R E G Y E M	21
538	**M** E Y **G** T **T** M V **S** Y Q P L G D	21
8	**F** W S **F** G **S** E D **G** S G D S E N	20
165	**T** T L **K** Y **A** I K **T** G H P R Y F	20
203	**N** M F **T** Y **E** I A **P** V F V L L E	20
248	**M** Y A **M** M **I** A R **F** K M F P E V	20
450	**V** D V **F** K **L** W L **M** W R A K G T	20
544	**M** V S **Y** Q **P** L G **D** K V N F F R	20
38	**G** G I **G** N **K** L C **A** L L Y G D A	19
118	**L** L Q **Y** V **V** K S **F** D R S T K V	19
180	**N** Q L **S** T **G** L D **M** V G L A A D	19
215	**L** L E **Y** V **T** L K **K** M R E I I G	19
216	**L** E Y **V** T **L** K K **M** R E I I G W	19
223	**K** M R **E** I **I** G W **P** G G S G D G	19
225	**R** E I **I** G **W** P G **G** S G D G I F	19
233	**G** S G **D** G **I** F S **P** G G A I S N	19
236	**D** G I **F** S **P** G G **A** I S N M Y A	19
258	**M** F P **E** V **K** E K **G** M A A L P R	19
281	**S** H F **S** L **K** K G **A** A A L G I G	19
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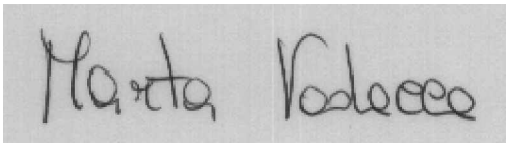

283	**F** S L **K** K **G** A A **A** L G I G T D	19
289	**A** A A **L** G **I** G T **D** S V I L I K	19
299	**V** I L **I** K **C** D E **R** G K M I P S	19
316	**E** R R **I** L **E** A K **Q** K G F V P F	19
464	**T** T G **F** E **A** H V **D** K C L E L A	19
504	**V** C F **W** Y **I** P P **S** L R T L E D	19
505	**C** F W **Y** I **P** P S **L** R T L E D N	19
510	**P** P S **L** R **T** L E **D** N E E R M S	19
50	**G** D A **E** K **P** A E **S** G G S Q P P	18
60	**G** S Q **P** P **R** A A **A** R K A A C A	18
88	**N** Y A **F** L **H** A T **D** L L P A C D	18
107	**T** L A **F** L **Q** D V **M** N I L L Q Y	18
156	**L** E E **I** L **M** H C **Q** T T L K Y A	18
192	**A** A D **W** L **T** S T **A** N T N M F T	18
193	**A** D W **L** T **S** T A **N** T N M F T Y	18
259	**F** P E **V** K **E** K G **M** A A L P R L	18
260	**P** E V **K** E **K** G M **A** A L P R L I	18
287	**K** G A **A** A **L** G I **G** T D S V I L	18
309	**K** M I **P** S **D** L E **R** R I L E A K	18
327	**F** V P **F** L **V** S A **T** A G T T V Y	18
335	**T** A G **T** T **V** Y G **A** F D P L L A	18
350	**V** A D **I** C **K** K Y **K** I W M H V D	18
358	**K** I W **M** H **V** D A **A** W G G G L L	18
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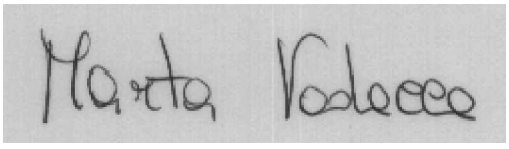
387	**A** N S **V** T **W** N P **H** K M M G V P	18
405	**S** A L **L** V **R** E E **G** L M Q N C N	18
422	**H** A S **Y** L **F** Q Q **D** K H Y D L S	18
424	**S** Y L **F** Q **Q** D K **H** Y D L S Y D	18
449	**H** V D **V** F **K** L W **L** M W R A K G	18
513	**L** R T **L** E **D** N E **E** R M S R L S	18
533	**I** K A **R** M **M** E Y **G** T T M V S Y	18
551	**G** D K **V** N **F** F R **M** V I S N P A	18
556	**F** F R **M** V **I** S N **P** A A T H Q D	18

HLA-DRB1*0301 (DR17) 15 - mers

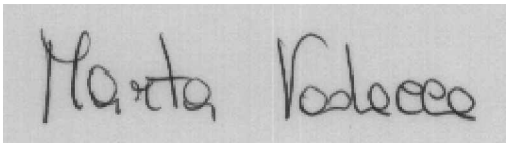
HLA-DRB1*0301 (DR17) 15 - mers

go to top

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
107	**T** L A **F** L **Q** D V **M** N I L L Q Y	29
299	**V** I L **I** K **C** D E **R** G K M I P S	29
308	**G** K M **I** P **S** D L **E** R R I L E A	28
316	**E** R R **I** L **E** A K **Q** K G F V P F	28
490	**G** Y E **M** V **F** D G **K** P Q H T N V	28
156	**L** E E **I** L **M** H C **Q** T T L K Y A	27
424	**S** Y L **F** Q **Q** D K **H** Y D L S Y D	27
45	**C** A L **L** Y **G** D A **E** K P A E S G	26
80	**C** S C **S** K **V** D V **N** Y A F L H A	26
138	**P** N E **L** L **Q** E Y **N** W E L A D Q	26
108	**L** A F **L** Q **D** V M **N** I L L Q Y V	25
370	**G** L L **M** S **R** K H **K** W K L S G V	25
259	**F** P E **V** K **E** K G **M** A A L P R L	23
145	**Y** N W **E** L **A** D Q **P** Q N L E E I	22
291	**A** L G **I** G **T** D S **V** I L I K C D	22
546	**S** Y Q **P** L **G** D K **V** N F F R M V	22



37	**T** G G **I** G **N** K L **C** A L L Y G D	21
397	**M** M G **V** P **L** Q C **S** A L L V R E	21
432	**H** Y D **L** S **Y** D T **G** D K A L Q C	21
115	**M** N I **L** L **Q** Y V **V** K S F D R S	20
158	**E** I L **M** H **C** Q T **T** L K Y A I K	20
188	**M** V G **L** A **A** D W **L** T S T A N T	20
210	**A** P V **F** V **L** L E **Y** V T L K K M	20
346	**P** L L **A** V **A** D I **C** K K Y K I W	20
358	**K** I W **M** H **V** D A **A** W G G G L L	20
368	**G** G G **L** L **M** S R **K** H K W K L S	20
404	**C** S A **L** L **V** R E **E** G L M Q N C	20
466	**G** F E **A** H **V** D K **C** L E L A E Y	20
33	**A** Q K **F** T **G** G I **G** N K L C A L	19
130	**T** K V **I** D **F** H Y **P** N E L L Q E	19
146	**N** W E **L** A **D** Q P **Q** N L E E I L	19
168	**K** Y A **I** K **T** G H **P** R Y F N Q L	19
185	**G** L D **M** V **G** L A **A** D W L T S T	19
193	**A** D W **L** T **S** T A **N** T N M F T Y	19
245	**I** S N **M** Y **A** M M **I** A R F K M F	19
253	**I** A R **F** K **M** F P **E** V K E K G M	19
337	**G** T T **V** Y **G** A F **D** P L L A V A	19
378	**K** W K **L** S **G** V E **R** A N S V T W	19
452	**V** F K **L** W **L** M W **R** A K G T T G	19
468	**E** A H **V** D **K** C L **E** L A E Y L Y	19
506	**F** W Y **I** P **P** S L **R** T L E D N E	19
512	**S** L R **T** L **E** D N **E** E R M S R L	19
520	**E** E R **M** S **R** L S **K** V A P V I K	19
529	**V** A P **V** I **K** A R **M** M E Y G T T	19
570	**D** I D **F** L **I** E E **I** E R L G Q D	19
571	**I** D F **L** I **E** E I **E** R L G Q D L	19
13	**S** E D **G** S **G** D S **E** N P G T A R	18
120	**Q** Y V **V** K **S** F D **R** S T K V I D	18
248	**M** Y A **M** M **I** A R **F** K M F P E V	18
255	**R** F K **M** F **P** E V **K** E K G M A A	18
264	**E** K G **M** A **A** L P **R** L I A F T S	18
360	**W** M H **V** D **A** A W **G** G G L L M S	18
471	**V** D K **C** L **E** L A **E** Y L Y N I I	18
526	**L** S K **V** A **P** V I **K** A R M M E Y	18
96	**D** L L **P** A **C** D G **E** R P T L A F	17
112	**Q** D V **M** N **I** L L **Q** Y V V K S F	17
164	**Q** T T **L** K **Y** A I **K** T G H P R Y	17
202	**T** N M **F** T **Y** E I **A** P V F V L L	17
209	**I** A P **V** F **V** L L **E** Y V T L K K	17
218	**Y** V T **L** K **K** M R **E** I I G W P G	17
249	**Y** A M **M** I **A** R F **K** M F P E V K	17
271	**P** R L **I** A **F** T S **E** H S H F S L	17
298	**S** V I **L** I **K** C D **E** R G K M I P	17
395	**H** K M **M** G **V** P L **Q** C S A L L V	17
403	**Q** C S **A** L **L** V R **E** E G L M Q N	17
411	**E** E G **L** M **Q** N C **N** Q M H A S Y	17
478	**A** E Y **L** Y **N** I I **K** N R E G Y E	17
481	**L** Y N **I** I **K** N R **E** G Y E M V F	17
513	**L** R T **L** E **D** N E **E** R M S R L S	17
545	**V** S Y **Q** P **L** G D **K** V N F F R M	17



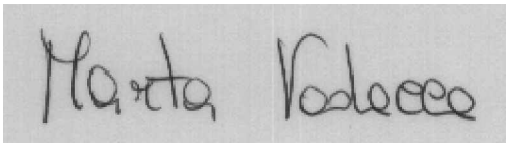
70	**K** A A **C** A **C** D Q **K** P C S C S K	16
116	**N** I L **L** Q **Y** V V **K** S F D R S T	16
131	**K** V I **D** F **H** Y P **N** E L L Q E Y	16
132	**V** I D **F** H **Y** P N **E** L L Q E Y N	16
256	**F** K M **F** P **E** V K **E** K G M A A L	16
317	**R** R I **L** E **A** K Q **K** G F V P F L	16
347	**L** L A **V** A **D** I C **K** K Y K I W M	16
350	**V** A D **I** C **K** K Y **K** I W M H V D	16
412	**E** G L **M** Q **N** C N **Q** M H A S Y L	16
423	**A** S Y **L** F **Q** Q D **K** H Y D L S Y	16
446	**C** G R **H** V **D** V F **K** L W L M W R	16
454	**K** L W **L** M **W** R A **K** G T T G F E	16
464	**T** T G **F** E **A** H V **D** K C L E L A	16
484	**I** I K **N** R **E** G Y **E** M V F D G K	16
547	**Y** Q P **L** G **D** K V **N** F F R M V I	16
568	**H** Q D **I** D **F** L I **E** E I E R L G	16
97	**L** L P **A** C **D** G E **R** P T L A F L	15
114	**V** M N **I** L **L** Q Y **V** V K S F D R	15
123	**V** K S **F** D **R** S T **K** V I D F H Y	15
149	**L** A D **Q** P **Q** N L **E** E I L M H C	15
212	**V** F V **L** L **E** Y V **T** L K K M R E	15
309	**K** M I **P** S **D** L E **R** R I L E A K	15
480	**Y** L Y **N** I **I** K N **R** E G Y E M V	15
492	**E** M V **F** D **G** K P **Q** H T N V C F	15
495	**F** D G **K** P **Q** H T **N** V C F W Y I	15
183	**S** T G **L** D **M** V G **L** A A D W L T	14
211	**P** V F **V** L **L** E Y **V** T L K K M R	14
557	**F** R M **V** I **S** N P **A** A T H Q D I	14
44	**L** C A **L** L **Y** G D **A** E K P A E S	13
94	**A** T D **L** L **P** A C **D** G E R P T L	13
206	**T** Y E **I** A **P** V F **V** L L E Y V T	13
216	**L** E Y **V** T **L** K K **M** R E I I G W	13
235	**G** D G **I** F **S** P G **G** A I S N M Y	13
281	**S** H F **S** L **K** K G **A** A A L G I G	13
282	**H** F S **L** K **K** G A **A** A L G I G T	13
296	**T** D S **V** I **L** I K **C** D E R G K M	13
339	**T** V Y **G** A **F** D P **L** L A V A D I	13
356	**K** Y K **I** W **M** H V **D** A A W G G G	13
405	**S** A L **L** V **R** E E **G** L M Q N C N	13
406	**A** L L **V** R **E** E G **L** M Q N C N Q	13
435	**L** S Y **D** T **G** D K **A** L Q C G R H	13
472	**D** K C **L** E **L** A E **Y** L Y N I I K	13
474	**C** L E **L** A **E** Y L **Y** N I I K N R	13
510	**P** P S **L** R **T** L E **D** N E E R M S	13
541	**G** T T **M** V **S** Y Q **P** L G D K V N	13
556	**F** F R **M** V **I** S N **P** A A T H Q D	13

HLA-DRB1*0401 (DR4Dw4) 15 - mers

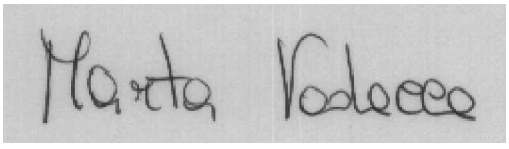
HLA-DRB1*0401 (DR4Dw4) 15 - mers

go to top

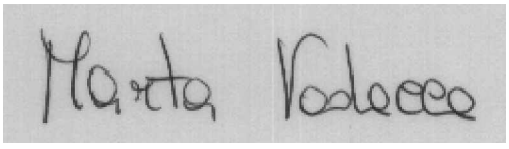
Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
26	**A** R A **W** C **Q** **V** A **Q** K F T G G I	28
144	**E** Y N **W** E **L** **A** D **Q** P Q N L E E	28
273	**L** I A **F** T **S** **E** H **S** H F S L K K	28



424	**S** Y L **F** Q **Q** **D** K **H** Y D L S Y D	28
554	**V** N F **F** R **M** **V** I **S** N P A A T H	28
108	**L** A F **L** Q **D** **V** M **N** I L L Q Y V	26
112	**Q** D V **M** N **I** **L** L **Q** Y V V K S F	26
120	**Q** Y V **V** K **S** **F** D **R** S T K V I D	26
138	**P** N E **L** L **Q** **E** Y **N** W E L A D Q	26
264	**E** K G **M** A **A** **L** P **R** L I A F T S	26
270	**L** P R **L** I **A** **F** T **S** E H S H F S	26
299	**V** I L **I** K **C** **D** E **R** G K M I P S	26
325	**K** G F **V** P **F** **L** V **S** A T A G T T	26
378	**K** W K **L** S **G** **V** E **R** A N S V T W	26
5	**G** S G **F** W **S** **F** G **S** E D G S G D	22
86	**D** V N **Y** A **F** **L** H **A** T D L L P A	22
107	**T** L A **F** L **Q** **D** V **M** N I L L Q Y	22
175	**H** P R **Y** F **N** **Q** L **S** T G L D M V	22
176	**P** R Y **F** N **Q** **L** S **T** G L D M V G	22
204	**M** F T **Y** E **I** **A** P **V** F V L L E Y	22
210	**A** P V **F** V **L** **L** E **Y** V T L K K M	22
256	**F** K M **F** P **E** **V** K **E** K G M A A L	22
324	**Q** K G **F** V **P** **F** L **V** S A T A G T	22
327	**F** V P **F** L **V** **S** A **T** A G T T V Y	22
338	**T** T V **Y** G **A** **F** D **P** L L A V A D	22
354	**C** K K **Y** K **I** **W** M **H** V D A A W G	22
479	**E** Y L **Y** N **I** **I** K **N** R E G Y E M	22
504	**V** C F **W** Y **I** **P** P **S** L R T L E D	22
544	**M** V S **Y** Q **P** **L** G **D** K V N F F R	22
570	**D** I D **F** L **I** **E** E **I** E R L G Q D	22
41	**G** N K **L** C **A** **L** L **Y** G D A E K P	20
45	**C** A L **L** Y **G** **D** A **E** K P A E S G	20
84	**K** V D **V** N **Y** **A** F **L** H A T D L L	20
105	**R** P T **L** A **F** **L** Q **D** V M N I L L	20
111	**L** Q D **V** M **N** **I** L **L** Q Y V V K S	20
153	**P** Q N **L** E **E** **I** L **M** H C Q T T L	20
156	**L** E E **I** L **M** **H** C **Q** T T L K Y A	20
157	**E** E I **L** M **H** **C** Q **T** T L K Y A I	20
185	**G** L D **M** V **G** **L** A **A** D W L T S T	20
186	**L** D M **V** G **L** **A** A **D** W L T S T A	20
188	**M** V G **L** A **A** **D** W **L** T S T A N T	20
193	**A** D W **L** T **S** **T** A **N** T N M F T Y	20
209	**I** A P **V** F **V** **L** L **E** Y V T L K K	20
211	**P** V F **V** L **L** **E** Y **V** T L K K M R	20
212	**V** F V **L** L **E** **Y** V **T** L K K M R E	20
213	**F** V L **L** E **Y** **V** T **L** K K M R E I	20
267	**M** A A **L** P **R** **L** I **A** F T S E H S	20
291	**A** L G **I** G **T** **D** S **V** I L I K C D	20
308	**G** K M **I** P **S** **D** L **E** R R I L E A	20
316	**E** R R **I** L **E** **A** K **Q** K G F V P F	20
328	**V** P F **L** V **S** **A** T **A** G T T V Y G	20
337	**G** T T **V** Y **G** **A** F **D** P L L A V A	20
344	**F** D P **L** L **A** **V** A **D** I C K K Y K	20
345	**D** P L **L** A **V** **A** D **I** C K K Y K I	20
347	**L** L A **V** A **D** **I** C **K** K Y K I W M	20
360	**W** M H **V** D **A** **A** W **G** G G L L M S	20
381	**L** S G **V** E **R** **A** N **S** V T W N P H	20



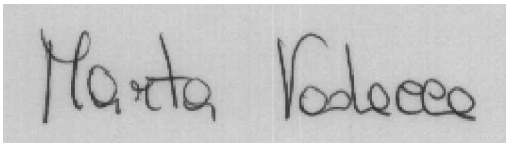
387	**A** N S **V** T **W** **N** P **H** K M M G V P	20
394	**P** H K **M** M **G** **V** P **L** Q C S A L L	20
395	**H** K M **M** G **V** **P** L **Q** C S A L L V	20
397	**M** M G **V** P **L** **Q** C **S** A L L V R E	20
405	**S** A L **L** V **R** **E** E **G** L M Q N C N	20
406	**A** L L **V** R **E** **E** G **L** M Q N C N Q	20
412	**E** G L **M** Q **N** **C** N **Q** M H A S Y L	20
432	**H** Y D **L** S **Y** **D** T **G** D K A L Q C	20
441	**D** K A **L** Q **C** **G** R **H** V D V F K L	20
447	**G** R H **V** D **V** **F** K **L** W L M W R A	20
472	**D** K C **L** E **L** **A** E **Y** L Y N I I K	20
523	**M** S R **L** S **K** **V** A **P** V I K A R M	20
526	**L** S K **V** A **P** **V** I **K** A R M M E Y	20
534	**K** A R **M** M **E** **Y** G **T** T M V S Y Q	20
535	**A** R M **M** E **Y** **G** T **T** M V S Y Q P	20
551	**G** D K **V** N **F** **F** R **M** V I S N P A	20
2	**A** S P **G** S **G** **F** W **S** F G S E D G	18
9	**W** S F **G** S **E** **D** G **S** G D S E N P	18
50	**G** D A **E** K **P** **A** E **S** G G S Q P P	18
61	**S** Q P **P** R **A** **A** A **R** K A A C A C	18
104	**E** R P **T** L **A** **F** L **Q** D V M N I L	18
121	**Y** V V **K** S **F** **D** R **S** T K V I D F	18
128	**R** S T **K** V **I** **D** F **H** Y P N E L L	18
135	**F** H Y **P** N **E** **L** L **Q** E Y N W E L	18
154	**Q** N L **E** E **I** **L** M **H** C Q T T L K	18
173	**T** G H **P** R **Y** **F** N **Q** L S T G L D	18
190	**G** L A **A** D **W** **L** T **S** T A N T N M	18
239	**F** S P **G** G **A** **I** S **N** M Y A M M I	18
247	**N** M Y **A** M **M** **I** A **R** F K M F P E	18
288	**G** A A **A** L **G** **I** G **T** D S V I L I	18
309	**K** M I **P** S **D** **L** E **R** R I L E A K	18
408	**L** V R **E** E **G** **L** M **Q** N C N Q M H	18
420	**Q** M H **A** S **Y** **L** F **Q** Q D K H Y D	18
428	**Q** Q D **K** H **Y** **D** L **S** Y D T G D K	18
462	**K** G T **T** G **F** **E** A **H** V D K C L E	18
475	**L** E L **A** E **Y** **L** Y **N** I I K N R E	18
550	**L** G D **K** V **N** **F** F **R** M V I S N P	18
555	**N** F F **R** M **V** **I** S **N** P A A T H Q	18
560	**V** I S **N** P **A** **A** T **H** Q D I D F L	18
492	**E** M V **F** D **G** **K** P **Q** H T N V C F	17
6	**S** G F **W** S **F** **G** S **E** D G S G D S	16
8	**F** W S **F** G **S** **E** D **G** S G D S E N	16
33	**A** Q K **F** T **G** **G** I **G** N K L C A L	16
46	**A** L L **Y** G **D** **A** E **K** P A E S G G	16
88	**N** Y A **F** L **H** **A** T **D** L L P A C D	16
132	**V** I D **F** H **Y** **P** N **E** L L Q E Y N	16
134	**D** F H **Y** P **N** **E** L **L** Q E Y N W E	16
142	**L** Q E **Y** N **W** **E** L **A** D Q P Q N L	16
192	**A** A D **W** L **T** **S** T **A** N T N M F T	16
202	**T** N M **F** T **Y** **E** I **A** P V F V L L	16
215	**L** L E **Y** V **T** **L** K **K** M R E I I G	16
227	**I** I G **W** P **G** **G** S **G** D G I F S P	16
236	**D** G I **F** S **P** **G** G **A** I S N M Y A	16
246	**S** N M **Y** A **M** **M** I **A** R F K M F P	16



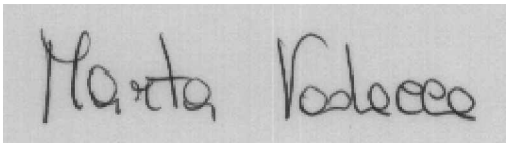
253	**I** A R **F** K **M** **F** P **E** V K E K G M	16
341	**Y** G A **F** D **P** **L** L **A** V A D I C K	16
357	**Y** K I **W** M **H** **V** D **A** A W G G G L	16
376	**K** H K **W** K **L** **S** G **V** E R A N S V	16
389	**S** V T **W** N **P** **H** K **M** M G V P L Q	16
434	**D** L S **Y** D **T** **G** D **K** A L Q C G R	16
450	**V** D V **F** K **L** **W** L **M** W R A K G T	16
453	**F** K L **W** L **M** **W** R **A** K G T T G F	16
464	**T** T G **F** E **A** **H** V **D** K C L E L A	16
488	**R** E G **Y** E **M** **V** F **D** G K P Q H T	16
503	**N** V C **F** W **Y** **I** P **P** S L R T L E	16
505	**C** F W **Y** I **P** **P** S **L** R T L E D N	16
537	**M** M E **Y** G **T** **T** M **V** S Y Q P L G	16

HLA-DRB1*0701 15 - mers go to top

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
193	**A** D W **L** T **S** T A **N** T N M F T Y	30
337	**G** T T **V** Y **G** A F **D** P L L A V A	28
381	**L** S G **V** E **R** A N **S** V T W N P H	28
175	**H** P R **Y** F **N** Q L **S** T G L D M V	26
29	**W** C Q **V** A **Q** K F **T** G G I G N K	24
204	**M** F T **Y** E **I** A P **V** F V L L E Y	24
256	**F** K M **F** P **E** V K **E** K G M A A L	24
290	**A** A L **G** I **G** T D **S** V I L I K C	24
327	**F** V P **F** L **V** S A **T** A G T T V Y	24
329	**P** F L **V** S **A** T A **G** T T V Y G A	24
397	**M** M G **V** P **L** Q C **S** A L L V R E	24
456	**W** L M **W** R **A** K G **T** T G F E A H	24
464	**T** T G **F** E **A** H V **D** K C L E L A	24
534	**K** A R **M** M **E** Y G **T** T M V S Y Q	24
88	**N** Y A **F** L **H** A T **D** L L P A C D	22
108	**L** A F **L** Q **D** V M **N** I L L Q Y V	22
111	**L** Q D **V** M **N** I L **L** Q Y V V K S	22
138	**P** N E **L** L **Q** E Y **N** W E L A D Q	22
158	**E** I L **M** H **C** Q T **T** L K Y A I K	22
271	**P** R L **I** A **F** T S **E** H S H F S L	22
288	**G** A A **A** L **G** I G **T** D S V I L I	22
289	**A** A A **L** G **I** G T **D** S V I L I K	22
308	**G** K M **I** P **S** D L **E** R R I L E A	22
360	**W** M H **V** D **A** A W **G** G G L L M S	22
404	**C** S A **L** L **V** R E **E** G L M Q N C	22
422	**H** A S **Y** L **F** Q Q **D** K H Y D L S	22
430	**D** K H **Y** D **L** S Y **D** T G D K A L	22
433	**Y** D L **S** Y **D** T G **D** K A L Q C G	22
479	**E** Y L **Y** N **I** I K **N** R E G Y E M	22
554	**V** N F **F** R **M** V I **S** N P A A T H	22
89	**Y** A F **L** H **A** T D **L** L P A C D G	20
94	**A** T D **L** L **P** A C **D** G E R P T L	20
119	**L** Q Y **V** V **K** S F **D** R S T K V I	20
186	**L** D M **V** G **L** A A **D** W L T S T A	20
194	**D** W L **T** S **T** A N **T** N M F T Y E	20
238	**I** F S **P** G **G** A I **S** N M Y A M M	20
248	**M** Y A **M** M **I** A R **F** K M F P E V	20



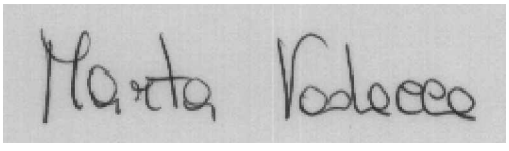
316	**E** R R **I** L **E** A K **Q** K G F V P F	20
325	**K** G F **V** P **F** L V **S** A T A G T T	20
330	**F** L V **S** A **T** A G **T** T V Y G A F	20
356	**K** Y K **I** W **M** H V **D** A A W G G G	20
368	**G** G G **L** L **M** S R **K** H K W K L S	20
376	**K** H K **W** K **L** S G **V** E R A N S V	20
529	**V** A P **V** I **K** A R **M** M E Y G T T	20
5	**G** S G **F** W **S** F G **S** E D G S G D	18
33	**A** Q K **F** T **G** G I **G** N K L C A L	18
45	**C** A L **L** Y **G** D A **E** K P A E S G	18
86	**D** V N **Y** A **F** L H **A** T D L L P A	18
107	**T** L A **F** L **Q** D V **M** N I L L Q Y	18
176	**P** R Y **F** N **Q** L S **T** G L D M V G	18
210	**A** P V **F** V **L** L E **Y** V T L K K M	18
227	**I** I G **W** P **G** G S **G** D G I F S P	18
228	**I** G W **P** G **G** S G **D** G I F S P G	18
273	**L** I A **F** T **S** E H **S** H F S L K K	18
389	**S** V T **W** N **P** H K **M** M G V P L Q	18
424	**S** Y L **F** Q **Q** D K **H** Y D L S Y D	18
504	**V** C F **W** Y **I** P P **S** L R T L E D	18
505	**C** F W **Y** I **P** P S **L** R T L E D N	18
537	**M** M E **Y** G **T** T M **V** S Y Q P L G	18
570	**D** I D **F** L **I** E E **I** E R L G Q D	18
6	**S** G F **W** S **F** G S **E** D G S G D S	16
37	**T** G G **I** G **N** K L **C** A L L Y G D	16
46	**A** L L **Y** G **D** A E **K** P A E S G G	16
87	**V** N Y **A** F **L** H A **T** D L L P A C	16
112	**Q** D V **M** N **I** L L **Q** Y V V K S F	16
121	**Y** V V **K** S **F** D R **S** T K V I D F	16
122	**V** V K **S** F **D** R S **T** K V I D F H	16
123	**V** K S **F** D **R** S T **K** V I D F H Y	16
130	**T** K V **I** D **F** H Y **P** N E L L Q E	16
132	**V** I D **F** H **Y** P N **E** L L Q E Y N	16
134	**D** F H **Y** P **N** E L **L** Q E Y N W E	16
144	**E** Y N **W** E **L** A D **Q** P Q N L E E	16
156	**L** E E **I** L **M** H C **Q** T T L K Y A	16
157	**E** E I **L** M **H** C Q **T** T L K Y A I	16
160	**L** M H **C** Q **T** T L **K** Y A I K T G	16
178	**Y** F N **Q** L **S** T G **L** D M V G L A	16
185	**G** L D **M** V **G** L A **A** D W L T S T	16
191	**L** A A **D** W **L** T S **T** A N T N M F	16
192	**A** A D **W** L **T** S T **A** N T N M F T	16
198	**S** T A **N** T **N** M F **T** Y E I A P V	16
201	**N** T N **M** F **T** Y E **I** A P V F V L	16
202	**T** N M **F** T **Y** E I **A** P V F V L L	16
212	**V** F V **L** L **E** Y V **T** L K K M R E	16
213	**F** V L **L** E **Y** V T **L** K K M R E I	16
216	**L** E Y **V** T **L** K K **M** R E I I G W	16
239	**F** S P **G** G **A** I S **N** M Y A M M I	16
242	**G** G A **I** S **N** M Y **A** M M I A R F	16
253	**I** A R **F** K **M** F P **E** V K E K G M	16
259	**F** P E **V** K **E** K G **M** A A L P R L	16
270	**L** P R **L** I **A** F T **S** E H S H F S	16
291	**A** L G **I** G **T** D S **V** I L I K C D	16



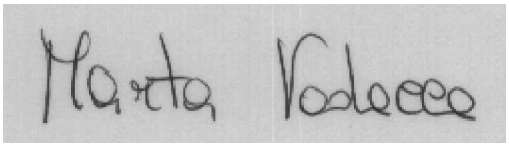
299	**V** I L **I** K **C** D E **R** G K M I P S	16
317	**R** R I **L** E **A** K Q **K** G F V P F L	16
350	**V** A D **I** C **K** K Y **K** I W M H V D	16
370	**G** L L **M** S **R** K H **K** W K L S G V	16
387	**A** N S **V** T **W** N P **H** K M M G V P	16
441	**D** K A **L** Q **C** G R **H** V D V F K L	16
447	**G** R H **V** D **V** F K **L** W L M W R A	16
474	**C** L E **L** A **E** Y L **Y** N I I K N R	16
482	**Y** N I **I** K **N** R E **G** Y E M V F D	16
488	**R** E G **Y** E **M** V F **D** G K P Q H T	16
503	**N** V C **F** W **Y** I P **P** S L R T L E	16
526	**L** S K **V** A **P** V I **K** A R M M E Y	16
535	**A** R M **M** E **Y** G T **T** M V S Y Q P	16
544	**M** V S **Y** Q **P** L G **D** K V N F F R	16
555	**N** F F **R** M **V** I S **N** P A A T H Q	16

HLA-DRB1*1101 15 - mers go to top

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
264	**E** K G **M** A **A** L P **R** L I A F T S	27
452	**V** F K **L** W **L** M W **R** A K G T T G	27
215	**L** L E **Y** V **T** L K **K** M R E I I G	26
378	**K** W K **L** S **G** V E **R** A N S V T W	26
520	**E** E R **M** S **R** L S **K** V A P V I K	26
553	**K** V N **F** F **R** M V **I** S N P A A T	25
255	**R** F K **M** F **P** E V **K** E K G M A A	24
354	**C** K K **Y** K **I** W M **H** V D A A W G	24
479	**E** Y L **Y** N **I** I K **N** R E G Y E M	23
488	**R** E G **Y** E **M** V F **D** G K P Q H T	23
116	**N** I L **L** Q **Y** V V **K** S F D R S T	22
357	**Y** K I **W** M **H** V D **A** A W G G G L	22
402	**L** Q C **S** A **L** L V **R** E E G L M Q	22
478	**A** E Y **L** Y **N** I I **K** N R E G Y E	22
526	**L** S K **V** A **P** V I **K** A R M M E Y	22
554	**V** N F **F** R **M** V I **S** N P A A T H	22
279	**E** H S **H** F **S** L K **K** G A A A L G	21
249	**Y** A M **M** I **A** R F **K** M F P E V K	20
325	**K** G F **V** P **F** L V **S** A T A G T T	20
347	**L** L A **V** A **D** I C **K** K Y K I W M	20
118	**L** L Q **Y** V **V** K S **F** D R S T K V	19
123	**V** K S **F** D **R** S T **K** V I D F H Y	19
218	**Y** V T **L** K **K** M R **E** I I G W P G	19
434	**D** L S **Y** D **T** G D **K** A L Q C G R	19
41	**G** N K **L** C **A** L L **Y** G D A E K P	18
46	**A** L L **Y** G **D** A E **K** P A E S G G	18
221	**L** K K **M** R **E** I I **G** W P G G S G	18
267	**M** A A **L** P **R** L I **A** F T S E H S	18
273	**L** I A **F** T **S** E H **S** H F S L K K	18
376	**K** H K **W** K **L** S G **V** E R A N S V	18
394	**P** H K **M** M **G** V P **L** Q C S A L L	18
424	**S** Y L **F** Q **Q** D K **H** Y D L S Y D	18
450	**V** D V **F** K **L** W L **M** W R A K G T	18
88	**N** Y A **F** L **H** A T **D** L L P A C D	17
176	**P** R Y **F** N **Q** L S **T** G L D M V G	17
246	**S** N M **Y** A **M** M I **A** R F K M F P	17



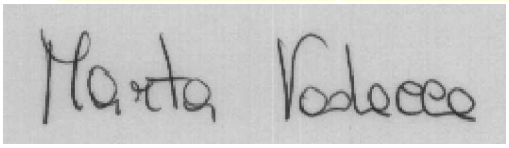
338	**T** T V **Y** G **A** F D **P** L L A V A D	17
503	**N** V C **F** W **Y** I P **P** S L R T L E	17
544	**M** V S **Y** Q **P** L G **D** K V N F F R	17
5	**G** S G **F** W **S** F G **S** E D G S G D	16
6	**S** G F **W** S **F** G S **E** D G S G D S	16
26	**A** R A **W** C **Q** V A **Q** K F T G G I	16
86	**D** V N **Y** A **F** L H **A** T D L L P A	16
142	**L** Q E **Y** N **W** E L **A** D Q P Q N L	16
210	**A** P V **F** V **L** L E **Y** V T L K K M	16
256	**F** K M **F** P **E** V K **E** K G M A A L	16
280	**H** S H **F** S **L** K K **G** A A A L G I	16
309	**K** M I **P** S **D** L E **R** R I L E A K	16
341	**Y** G A **F** D **P** L L **A** V A D I C K	16
387	**A** N S **V** T **W** N P **H** K M M G V P	16
538	**M** E Y **G** T **T** M V **S** Y Q P L G D	16
27	**R** A W **C** Q **V** A Q **K** F T G G I G	15
209	**I** A P **V** F **V** L L **E** Y V T L K K	15
247	**N** M Y **A** M **M** I A **R** F K M F P E	15
257	**K** M F **P** E **V** K E **K** G M A A L P	15
299	**V** I L **I** K **C** D E **R** G K M I P S	15
301	**L** I K **C** D **E** R G **K** M I P S D L	15
315	**L** E R **R** I **L** E A **K** Q K G F V P	15
368	**G** G G **L** L **M** S R **K** H K W K L S	15
370	**G** L L **M** S **R** K H **K** W K L S G V	15
423	**A** S Y **L** F **Q** Q D **K** H Y D L S Y	15
465	**T** G F **E** A **H** V D **K** C L E L A E	15
506	**F** W Y **I** P **P** S L **R** T L E D N E	15
35	**K** F T **G** G **I** G N **K** L C A L L Y	14
57	**E** S G **G** S **Q** P P **R** A A A R K A	14
61	**S** Q P **P** R **A** A A **R** K A A C A C	14
70	**K** A A **C** A **C** D Q **K** P C S C S K	14
95	**T** D L **L** P **A** C D **G** E R P T L A	14
120	**Q** Y V **V** K **S** F D **R** S T K V I D	14
154	**Q** N L **E** E **I** L M **H** C Q T T L K	14
164	**Q** T T **L** K **Y** A I **K** T G H P R Y	14
182	**L** S T **G** L **D** M V **G** L A A D W L	14
235	**G** D G **I** F **S** P G **G** A I S N M Y	14
245	**I** S N **M** Y **A** M M **I** A R F K M F	14
278	**S** E H **S** H **F** S L **K** K G A A A L	14
295	**G** T D **S** V **I** L I **K** C D E R G K	14
297	**D** S V **I** L **I** K C **D** E R G K M I	14
317	**R** R I **L** E **A** K Q **K** G F V P F L	14
350	**V** A D **I** C **K** K Y **K** I W M H V D	14
367	**W** G G **G** L **L** M S **R** K H K W K L	14
369	**G** G L **L** M **S** R K **H** K W K L S G	14
372	**L** M S **R** K **H** K W **K** L S G V E R	14
388	**N** S V **T** W **N** P H **K** M M G V P L	14
441	**D** K A **L** Q **C** G R **H** V D V F K L	14
446	**C** G R **H** V **D** V F **K** L W L M W R	14
454	**K** L W **L** M **W** R A **K** G T T G F E	14
490	**G** Y E **M** V **F** D G **K** P Q H T N V	14
517	**E** D N **E** E **R** M S **R** L S K V A P	14
571	**I** D F **L** I **E** E I **E** R L G Q D L	14



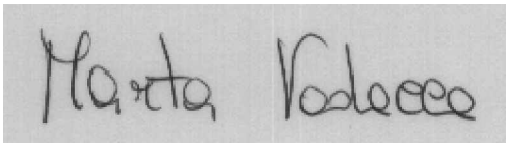
HLA-DRB1*1501 (DR2b) 15 - mers

go to top

Pos	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5	score
212	**V** F V **L** L E **Y** V T L K K M R E	34
541	**G** T T **M** V S **Y** Q P L G D K V N	30
551	**G** D K **V** N F **F** R M V I S N P A	30
224	**M** R E **I** I G **W** P G G S G D G I	28
270	**L** P R **L** I A **F** T S E H S H F S	28
534	**K** A R **M** M E **Y** G T T M V S Y Q	28
5	**G** S G **F** W S **F** G S E D G S G D	26
250	**A** M M **I** A R **F** K M F P E V K E	26
2	**A** S P **G** S G **F** W S F G S E D G	24
82	**C** S K **V** D V **N** Y A F L H A T D	24
108	**L** A F **L** Q D **V** M N I L L Q Y V	24
120	**Q** Y V **V** K S **F** D R S T K V I D	24
139	**N** E L **L** Q E **Y** N W E L A D Q P	24
201	**N** T N **M** F T **Y** E I A P V F V L	24
242	**G** G A **I** S N **M** Y A M M I A R F	24
253	**I** A R **F** K M **F** P E V K E K G M	24
267	**M** A A **L** P R **L** I A F T S E H S	24
321	**E** A K **Q** K G **F** V P F L V S A T	24
335	**T** A G **T** T V **Y** G A F D P L L A	24
338	**T** T V **Y** G A **F** D P L L A V A D	24
405	**S** A L **L** V R **E** E G L M Q N C N	24
447	**G** R H **V** D V **F** K L W L M W R A	24
474	**C** L E **L** A E **Y** L Y N I I K N R	24
501	**H** T N **V** C F **W** Y I P P S L R T	24
523	**M** S R **L** S K **V** A P V I K A R M	24
535	**A** R M **M** E Y **G** T T M V S Y Q P	24
547	**Y** Q P **L** G D **K** V N F F R M V I	24
236	**D** G I **F** S P **G** G A I S N M Y A	22
324	**Q** K G **F** V P **F** L V S A T A G T	22
450	**V** D V **F** K L **W** L M W R A K G T	22
23	**P** G T **A** R A **W** C Q V A Q K F T	20
83	**S** K V **D** V N **Y** A F L H A T D L	20
89	**Y** A F **L** H A **T** D L L P A C D G	20
105	**R** P T **L** A F **L** Q D V M N I L L	20
114	**V** M N **I** L L **Q** Y V V K S F D R	20
115	**M** N I **L** L Q **Y** V V K S F D R S	20
129	**S** T K **V** I D **F** H Y P N E L L Q	20
158	**E** I L **M** H C **Q** T T L K Y A I K	20
173	**T** G H **P** R Y **F** N Q L S T G L D	20
179	**F** N Q **L** S T **G** L D M V G L A A	20
218	**Y** V T **L** K K **M** R E I I G W P G	20
243	**G** A I **S** N M **Y** A M M I A R F K	20
249	**Y** A M **M** I A **R** F K M F P E V K	20
264	**E** K G **M** A A **L** P R L I A F T S	20
291	**A** L G **I** G T **D** S V I L I K C D	20
344	**F** D P **L** L A **V** A D I C K K Y K	20
350	**V** A D **I** C K **K** Y K I W M H V D	20
354	**C** K K **Y** K I **W** M H V D A A W G	20
381	**L** S G **V** E R **A** N S V T W N P H	20
399	**G** V P **L** Q C **S** A L L V R E E G	20
406	**A** L L **V** R E **E** G L M Q N C N Q	20
412	**E** G L **M** Q N **C** N Q M H A S Y L	20



418	**C** N Q **M** H A **S** Y L F Q Q D K H	20
441	**D** K A **L** Q C **G** R H V D V F K L	20
449	**H** V D **V** F K **L** W L M W R A K G	20
472	**D** K C **L** E L **A** E Y L Y N I I K	20
476	**E** L A **E** Y L **Y** N I I K N R E G	20
500	**Q** H T **N** V C **F** W Y I P P S L R	20
520	**E** E R **M** S R **L** S K V A P V I K	20
550	**L** G D **K** V N **F** F R M V I S N P	20
29	**W** C Q **V** A Q **K** F T G G I G N K	18
30	**C** Q V **A** Q K **F** T G G I G N K L	18
44	**L** C A **L** L Y **G** D A E K P A E S	18
88	**N** Y A **F** L H **A** T D L L P A C D	18
95	**T** D L **L** P A **C** D G E R P T L A	18
130	**T** K V **I** D F **H** Y P N E L L Q E	18
131	**K** V I **D** F H **Y** P N E L L Q E Y	18
132	**V** I D **F** H Y **P** N E L L Q E Y N	18
138	**P** N E **L** L Q **E** Y N W E L A D Q	18
157	**E** E I **L** M H **C** Q T T L K Y A I	18
172	**K** T G **H** P R **Y** F N Q L S T G L	18
185	**G** L D **M** V G **L** A A D W L T S T	18
189	**V** G L **A** A D **W** L T S T A N T N	18
193	**A** D W **L** T S **T** A N T N M F T Y	18
210	**A** P V **F** V L **L** E Y V T L K K M	18
221	**L** K K **M** R E **I** I G W P G G S G	18
233	**G** S G **D** G I **F** S P G G A I S N	18
235	**G** D G **I** F S **P** G G A I S N M Y	18
282	**H** F S **L** K K **G** A A A L G I G T	18
325	**K** G F **V** P F **L** V S A T A G T T	18
328	**V** P F **L** V S **A** T A G T T V Y G	18
329	**P** F L **V** S A **T** A G T T V Y G A	18
341	**Y** G A **F** D P **L** L A V A D I C K	18
358	**K** I W **M** H V **D** A A W G G G L L	18
360	**W** M H **V** D A **A** W G G G L L M S	18
361	**M** H V **D** A A **W** G G G L L M S R	18
386	**R** A N **S** V T **W** N P H K M M G V	18
397	**M** M G **V** P L **Q** C S A L L V R E	18
411	**E** E G **L** M Q **N** C N Q M H A S Y	18
431	**K** H Y **D** L S **Y** D T G D K A L Q	18
432	**H** Y D **L** S Y **D** T G D K A L Q C	18
453	**F** K L **W** L M **W** R A K G T T G F	18
455	**L** W L **M** W R **A** K G T T G F E A	18
482	**Y** N I **I** K N **R** E G Y E M V F D	18
489	**E** G Y **E** M V **F** D G K P Q H T N	18
491	**Y** E M **V** F D **G** K P Q H T N V C	18
502	**T** N V **C** F W **Y** I P P S L R T L	18
556	**F** F R **M** V I **S** N P A A T H Q D	18
557	**F** R M **V** I S **N** P A A T H Q D I	18
558	**R** M V **I** S N **P** A A T H Q D I D	18
33	**A** Q K **F** T G **G** I G N K L C A L	16
206	**T** Y E **I** A P **V** F V L L E Y V T	16
207	**Y** E I **A** P V **F** V L L E Y V T L	16
273	**L** I A **F** T S **E** H S H F S L K K	16
280	**H** S H **F** S L **K** K G A A A L G I	16
327	**F** V P **F** L V **S** A T A G T T V Y	16



468	**E** A H **V** D K **C** L E L A E Y L Y	16
485	**I** K N **R** E G **Y** E M V F D G K P	16
503	**N** V C **F** W Y **I** P P S L R T L E	16
529	**V** A P **V** I K **A** R M M E Y G T T	16
554	**V** N F **F** R M **V** I S N P A A T H	16

4.3 Results

4.3.1 Identification of proinsulin epitopes

Five proinsulin peptides have been identified (Figure 30).

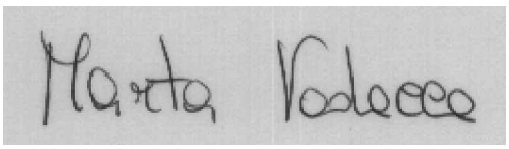
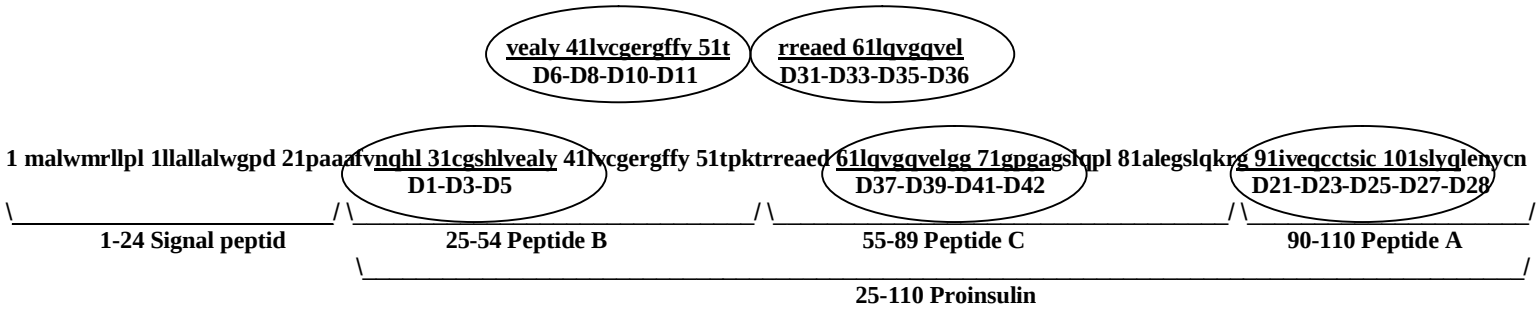
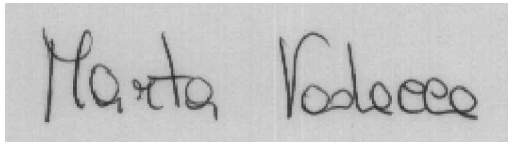


Figure 30. Peptides of proinsulin recognized by $CD4^+$ T cells of T1D patients





4.3.2 Identification of GAD epitopes

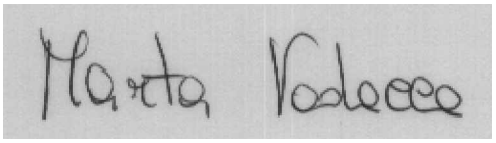
Five GAD 65 peptides have been identified (Figure 31).

```
      1          11         21         31         41         51
      |          |          |          |          |          |
    1 MASPGSGFWS FGSEDGSGDS ENPGTARAWC QVAQKFTGGI GNKLCALLYG DAEKPAESGG
60
   61 SQPPRAAARK AACACDQKPC SCSKVDVNYA FLHATDLLPA CDGERPTLAF LQDVMNILLQ
120
  121 YVVKSFDRST KVIDFHYPNE LLQEYNWELA DQPQNLEEIL MHCQTTLKYA IKTGHPRYFN
180
  181 QLSTGLDMVG LAADWLTSTA NTNMFTYEIA PVFVLLEYVT LKKMREIIGW PGGSGDGIFS
240
  241 PGGAISNMYA MMIARFKMFP EVKEKGMAAL PRLIAFTSEH SHFSLKKGAA ALGIGTDSVI
300
  301 LIKCDERGKM IPSDLERRIL EAKQKGFVPF LVSATAGTTV YGAFDPLLAV ADICKKYKIW
360
  361 MHVDAAWGGG LLMSRKHKWK LSGVERANSV TWNPHKMMGV PLQCSALLVR EEGLMQNCNQ
420
  421 MHASYLFQQD KHYDLSYDTG DKALQCGRHV DVFKLWLMWR AKGTTGFEAH VDKCLELAEY
480
  481 LYNIIKNREG YEMVFDGKPQ HTNVCFWYIP PSLRTLEDNE ERMSRLSKVA PVIKARMMEY
540
  541 GTTMVSYQPL GDKVNFFRMV ISNPAATHQD IDFLIEEIER LGQDL
```

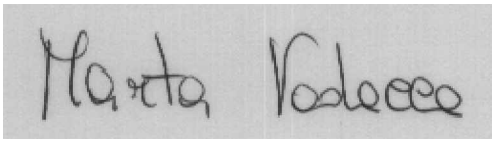
Figure 31. Peptides of GAD recognized by CD4+ T cells of T1D patients

4.4. Summary

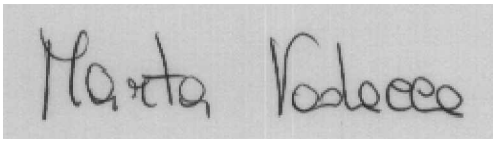
Epitopes which are entirely included within the sequences selected from the literature have been listed, and some of those (starting with highest scorers) are placed into Ii-Key/MHC class II hybrids. We expected that about 40% of the predicted epitopes should be recognized by humans. The remainder could be cryptic epitopes. Upon finding new epitopes for study, one might make nested deletions to identify presence of multiple slightly overlapping MHC II epitopes within peptides which were selected by the referenced authors for their promiscuity. Multiple epitopes are found within one homologous series, with presentation by multiple and differing suballeles. It is expected



that at least one MHC presented epitope will be found to be biologically active within one nested series of homologs. For example, two overlapping epitopes will be indicated, for example in longer hybrids of one series being presented by both DRB1*0303 and DRB1*0401 individuals while shorter hybrids of that same series are presented only by DRB1*0401 individuals. In that outcome, the DRB1*0303-presented epitope would be N-terminal to the DRB1*0401 epitope. Alternatively, only one biologically active epitope might be found within the homologous series. At that point we would consider making nested deletions from the C-terminus to narrow down on the exact epitope. Another substantial finding would be that an epitope-only peptide is presented, for example in only DRB1*0303 patients, while the hybrid of that epitope is presented in additional DRB1*03 suballeles, from example 0301, 0303, 0305, 0307, etc. If that finding were further supported in studies with 1:4 serial dilutions of peptides one might then claim that hybrids allow presentation by additional suballeles, thereby escaping in part the requirement for a basket of epitopes for a clinically useful peptide vaccines. The basket of epitopes hypothesis is that many peptides must be given in one injection, in order that as many patients in a general population, with many different MHC class II alleles, can be covered each by at least one or a few of the mixture of peptides. If a parallel finding of spreading presentation to other alleles occurred, for example from DRB1*0301 to 0401, 0501, 0701, etc., the significance of this effect would be greater. Because no one has reported the presence of $CD4^+$ T-cell stimulating antigenic epitopes in the longer peptides taken for our study, the precise 9-amino-acids-long sequences bound into antigenic epitope-binging trough (desotope) of the HLA-DR molecules is not



known. For this reason we expect that 2/3 of the predicted MHC class II epitopes are not recognized for several reasons. All of the predicted epitopes might contain specified amino acids in sequence positions, in a pattern highly correlating with those in either known presented peptides and/or peptides eluted from HLA-DR molecules and sequence-determined. But in practice, some of those potential epitopes are cleaved during processing of the antigen, or are competed in binding to MHC.



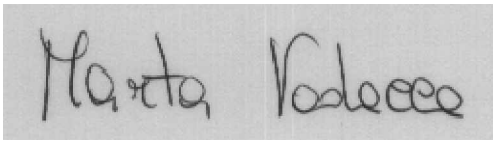
CHAPTER 5: Ii-KEY HYBRIDS PEPTIDES

5.4 Introduction

Hybrids consisting of antigenic peptide flexible linker Ii-Key peptide have up to 250 times the potency of the respective antigenic peptide in a T hybridoma response assay measured at half-maximal response. The linker e-amino-valeric acid, with four methylene (-CH2-) groups, is fully active and preferred over amino acids of the Ii protein sequence due to simplicity of synthesis. Ii-Key core peptides promote both the release of bound biotinylated antigenic peptides, and the exchange of a second peptide into the antigenic peptide binding site (Adams S, 1995; Adams S, 1997).

Covalent linkage of the Ii-Key core sequence with the antigenic peptide does not appear to lead to a competition between these two functions, but instead to an increased level of binding and recognition of antigenic determinants. This could occur due to increased representation of the antigenic epitope on the surface of the APC, or to some favorable structural change in the MHC antigenic peptide T cell receptor complex induced by the presence of the Ii-Key epitope. Such a favorable structural change could lead to either attraction or repulsion of various cell surface molecules interacting with the hybrid containing MHC class II complex. Such effects have been reported when antigenic epitopes are linked to antibodies reacting with T cell surface molecules other than T cell receptors (Rosenthal KS, 1999).

5.5 Materials and methods

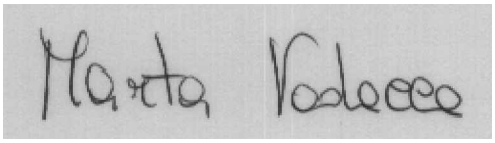


5.5.1 **Ii-key hybrids design**

Peptides of proinsulin and GAD65 shown to be recognized by CD4+ T cells of T1D patients have been selected from the literature. Sequences of the human proinsulin and GAD65 have been obtained from Gen Bank. Predicted DRB1*0301 and DRB1*0401 epitopes have been identified by application of the Rammensee SYFPEITH program (http://syfpeithi.bmi-heidelberg.com/Scripts/MHCServer.dll/EpPredict.html) to the sequences of the experimentally characterized peptides containing DR-presented sequences, as described in Chapter 4. In the case of relatively short peptides, the sequence for analysis by the program has been extended by 5 amino acids at both the N- and C-termini. The sequence of the top-scoring, epitopes predicted for DRB1*0301 and DRB1*0401 have been plotted against the primary amino acid sequence of the experimentally studied peptides.

Ii-Key hybrid peptides have been designed to contain:

a) constant LRMK-*ava*- (where *ava* = amino-valeric acid; 5-aminopentanoic acid), and
b) peptide sequences with a constant C-terminus, but varying at the N-terminus by one amino acid among members of the set, in a nested deletion pattern. N-termini have been acetylated and C-termini amidated to block catabolism by amino- and carboxy-peptidases, respectively.



Within a homologous series, the longest and shortest hybrids have been taken for initial synthesis plus the shortest peptide of 9 amino acids being considered to be a control. In addition, every other member of the series has been synthesized.

The peptides of proinsulin and GAD675 reported in tables 9 and 10 were synthesized by Commonwealth Biotechnologies Inc., 601 Biotech Drive, Richmond, VA 23225, USA.

Proinsulin epitopes: Five peptide series (5 epitope control and 15 proinsulin Ii-key hibrids) have been synthesized.

GAD epitopes: Five peptide series (5 epitope control and 14 GAD-65 Ii key hybrids) have been synthesized.

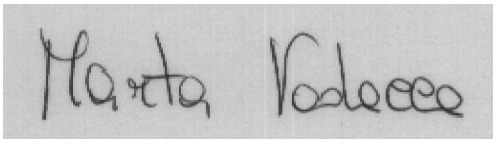
D1	Ac-LRMK-*ava*-	NQHLCGSHLVEALY	-NH_2
D3	Ac-LRMK-*ava*-	HLCGSHLVEALY	-NH_2
D5	**Ac-**	**NQHLCGSHLVEALY**	**-NH_2**

D6	Ac-LRMK-*ava*-	VEALYLVCGERGFFYT	-NH_2
D8	Ac-LRMK-*ava*-	ALYLVCGERGFFYT	-NH_2
D10	Ac-LRMK-*ava*-	YLVCGERGFFYT	-NH_2
D11	**Ac-**	**VEALYLVCGERGFFYT**	**-NH_2**

D21	Ac-LRMK-*ava*-	GIVEQCCTSICSLYQ	-NH_2
D23	Ac-LRMK-*ava*-	VEQCCTSICSLYQ	-NH_2
D25	Ac-LRMK-*ava*-	QCCTSICSLYQ	-NH_2
D27	Ac-LRMK-*ava*-	CTSICSLYQ	-NH_2
D28	**Ac-**	**GIVEQCCTSICSLYQ**	**-NH_2**

D31	Ac-LRMK-*ava*-	RREAEDLQVGGVEL	-NH_2
D33	Ac-LRMK-*ava*-	EAEDLQVGGVEL	-NH_2
D35	Ac-LRMK-*ava*-	EDLQVGGVEL	-NH_2
D36	**Ac-**	**RREAEDLQVGGVEL**	**-NH_2**

D37	Ac-LRMK-*ava*-	LQVGGVELGGGPGA	-NH_2
D39	Ac-LRMK-*ava*-	VGGVELGGGPGA	-NH_2
D41	Ac-LRMK-*ava*-	GVELGGGPGA	-NH_2



D42	**Ac-**	**LQVGGVELGGGPGA**	**-NH_2**

Table 9. Five peptide series (5 epitope control and 15 hybrids) of proinsulin Ii-key hybrids

	G1P1	**Ac-LRMK-*ava*-**	**LPRLIAFTSEHSHF**	**-NH_2**
	G1P2	**Ac-LRMK-*ava*-**	**ALPRLIAFTSEHSHF**	**-NH_2**
	G1P3	**Ac-LRMK-*ava*-**	**AALPRLIAFTSEHSHF**	**-NH_2**
⇒	**G1P4**	**Ac-**	**AALPRLIAFTSEHSHF**	**-NH_2**
	G2P1	**Ac-LRMK-*ava*-**	**NFFRMVISNPAAT**	**-NH_2**
	G2P2	**Ac-LRMK-*ava*-**	**VNFFRMVISNPAAT**	**-NH_2**
	G2P3	**Ac-LRMK-*ava*-**	**KVNFFRMVISNPAAT**	**-NH_2**
⇒	**G2P4**	**Ac-**	**KVNFFRMVISNPAAT**	**-NH_2**
	G3P1	**Ac-LRMK-*ava*-**	**SRLSKVAPVIKARMMEYGTT**	**-NH_2**
⇒	**G3P2**	**Ac-**	**SRLSKVAPVIKARMMEYGTT**	**-NH_2**
	G3aP1	**Ac-LRMK-*ava*-**	**LSKVAPVIKARMM**	**-NH_2**
	G3aP2	**Ac-LRMK-*ava*-**	**RLSKVAPVIKARMM**	**-NH_2**
	G3aP3	**Ac-LRMK-*ava*-**	**SRLSKVAPVIKARMM**	**-NH_2**
⇒	**G3aP4**	**Ac-**	**SRLSKVAPVIKARMM**	**-NH_2**
	G3bP1	**Ac-LRMK-*ava*-**	**PVIKARMMYGTT**	**-NH_2**
	G3bP2	**Ac-LRMK-*ava*-**	**VIKARMMYGTT**	**-NH_2**
	G3bP3	**Ac-LRMK-*ava*-**	**IKARMMYGTT**	**-NH_2**
	G3bP4	**Ac-LRMK-*ava*-**	**KARMMYGTT**	**-NH_2**
	G3bP5	**Ac-**	**PVIKARMMYGTT**	**-NH_2**

⇒

Table 10. Five peptide series (14 hybrids and 5 epitope-controls) of GAD65/Ii-key hybrids

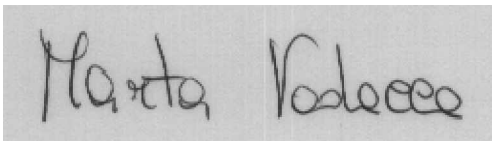
The purity and composition of each was confirmed by high performance liquid chromatography separation and mass spectrometry. All peptides were found to be > 98% pure by analytical HPLC and mass spectrometry (Commonwealth Biotechnologies, Richmond, VA). The peptides were dissolved in phosphate buffered saline (0.01 M sodium phosphate buffer, pH 7.2, 0.1 M NaCl).

Certificate of analysis and characteristics of the peptides, are reported in the Appendix.

5.5.2 Ii-key hybrids dilutions and long term storage

All peptides were dissolved in sterile distilled water (5 mg/ml) and stored at –80°C until used.

Properly solubilizing the petides is critical to its performance. It is desiderable to introduce charge to the peptide, through the protonation or deprotonation of the various basic or acidic residues present and/or the N and C-terminal amine and carboxyl, respectively (if present). In the case of a peptide with one or more Cys (C) residues, it has been avoided pH > 7, as this may promote the formation of intra-or inter-peptide disulfide bonds. The primary sequence of the peptide has been used to determine the overall net charge according to the following roles:

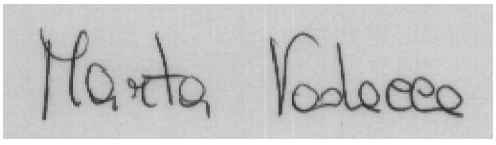


- assign a value of –1 to each acid amino acid. The acidic amino acids are Asp (D) and Glu (E). If the C-terminus is a free carboxyl (-COOH), add –1.
- Assign a value of +1 to each basic amino acid. The basic amino acids are Arg (R), Lys (K) and His (H). If the N terminus is a free-amine (-NH2), add +1.
- Calculate the overall net charge of the peptide.
- For very hydrophobic peptide sequences and neutral peptide, addition of DMSO has been used to increase the solubility of the peptide.

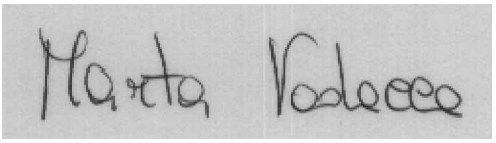
5.6 Summary

We have designs and compounds for antigen–nonspecific inhibition of MHC class II antigen presentation (Ii-Key-only ring homologs and Ii-Key-spacer-tetramer homologs) which might be tested as spillers or blockers of DM peptides presented to the T cell lines. With any of the above hybrids, one would make the corresponding terminally blocked epitope only peptides. It is no longer sufficient just to develop diagnostic reagents. They need to be tested in the context of understanding T cell subset biology. The single most important objective from the perspective of this work, was to determine whether some form of Ii-Key hybrids structure relate to selective activation of T cell subset responses.

Regarding GAD/hybrids, -Ac-LRMK-ava-270-283-NH2 (Ac-LRMK-ava-LPRLIAFTSEHSHF-NH2) and Ac-LRMK-ava-268-283-NH2 (Ac-LRMK-ava-AALPRLIAFTSEHSHF-NH2)-, we expected to establish the biological activity and



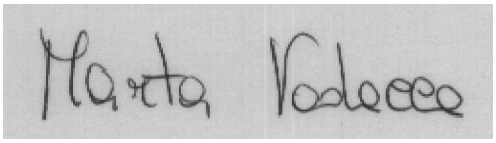
potency of the Ii-Key hybrid construct relative to control end-blocked epitope only peptides. Second in priority we would like to test Ac-LRMK-ava-555-567-NH2 and Ac-LRMK-ava-553-557-NH2. Then, would be testing Ac-LRMK-ava-524-543-NH2 and Ac-LRMK-ava-524-538-NH2. If those are active and more potent, then one could consider testing of N-Terminal nested deletions. Controls take many forms. The arbitrarily chosen most C-terminal 9-amino-acids-long peptide of a homologous series is an initial control to judge background non-responsiveness, or epitope-only response were that peptide to be presented. However, when a more N-terminal epitope has been discovered, a different epitope-only peptide needs to be synthesized for comparative purposes.



CHAPTER 6: II-KEY HYBRIDS TO DETECT T CELL RESPONSE IN TYPE 1 DIABETES

6.3 Introduction

T1D is the result of the immune mediated destruction of pancreatic β cells. Before the clinical onset of T1D, immune changes can be detected in peripheral blood including antibodies to insulin (IAA), glutamic acid decarboxylase (GAD), and insulinoma-associated antigen (IA-2). This immune response involves both cellular and humoral changes that persist over a prolonged period of time up to diagnosis. T cells recognition of islet antigen(s) via MHC class has been postulated as a key mechanism in the induction of such immune response. The specific epitopes recognized by pathogenic T cells in this autoimmune disease remain poorly defined although numerous relevant antigenic peptides (epitopes) have been identified after the synthesis of overlapping amino acid peptides of target antigenic proteins (Schlosser M, 2005). It is unclear which autoantigens and determinants are targets of the autoimmune attack towards β cells. In T1D it is widely assumed that the pathogenetic process is orchestrated by autoreactive T cells, however current studies in this respect are limited because in peripheral blood islet autoreactive T cells are likely to be rare and the epitopes remain poorly characterized. Recently, it was demonstrated that single cell resolution cytokine enzyme-linked immunosorbent spot (ELISPOT) assays allow the direct detection of individual antigen specific T cells even if these occur at very low frequencies in the peripheral blood (Otto PA, 2004). On the other hand, Ii -key hybrid peptides greatly enhance the potency of the



covalently tethered MHC class II epitope, and the induced response is specific to the epitope (Kallinteris NL, 2006).

The main objective of this study is to investigate the cellular immune response in T1D by characterising the cytokines secreted by peripheral blood mononuclear cells (PBMCs) in response to the exposure to Ii-key/MHC class II insulin/GAD epitope hybrids. To date, there are no studies using the recently introduced novel technique of Ii-key/MHC class II epitope hybrids in diabetes.

6.4 Materials and methods

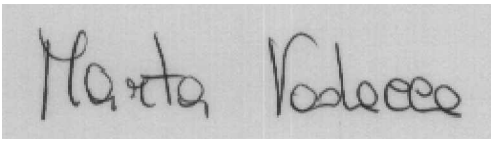
6.2.1 Selection of subjects

6.2.1.1 Selection of recent onset type 1 diabetic patients

Peripheral blood mononuclear cells for this study have been obtained from consecutive subjects affected by T1D who are diagnosed in Rome and other local hospitals within the IMDIAB group network. The IMDIAB Group, which was founded in 1989 by Professors Pozzilli and Ghirlanda have some of the largest experience worldwide in identifying and studying subjects with recent onset T1D whom are resident and diagnosed in continental Italy. This population has been investigated in a number of studies, mainly genetic and immunological**.**

T1D subjects for this study include those diagnosed both in paediatric and young age with the following characteristics:

1. 3-30 years of age
2. Time since T1D diagnosis <3 years



3. Caucasian ethnicity

Subjects with other concomitant autoimmune diseases will be excluded.

6.2.1.2 Selection of controls

We also studied as control group normal subjects age and sex and HLA matched with T1D subjects. They are enrolled in the University Campus Bio-medico, Rome. Subjects with autoimmune diseases or family history for T1D have been excluded.

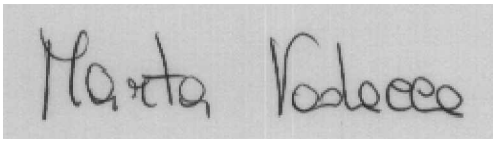
6.2.2 Time course of experiments

The Ii-Key-hybrids ELISPOT assays have been performed the first time as close as possible to the time of T1D diagnosis. When possible, the assays have been repeated in subjects who tested positive to at least one of the Ii-key/MHC class II epitope hybrids during a 2 year follow-up period.

6.2.3 Blood samples and blood volume

Blood samples from participating patients it has been collected by a nurse. Upon receiving the samples, they have been registered and given an identification number. Cells have been used fresh for the purpose of this study. All stored material is identified by its unique identification code only and can only be traced to the individual through secure computer and hard copy files.

Heparinized, freshly drawn blood samples from each subject were assayed within 6 hours. Usually,15 ml of blood was obtained, yielding theoretically $1x10^6$ PBMC/ml, and



10^5 cells per well are used, (after dividing into two portions for APC and responding cell) then up to 75 wells could be assayed. Because peptides have been tested in triplicate 25 peptides could be tested. When less blood volumes was available (very young children), proportionally fewer peptides have been assayed. One issue has been to use precisely 10^5 cells per well. More cells may consume medium more rapidly, release proteases or other factors which interfere with the assay.

6.2.4 HLA genotyping

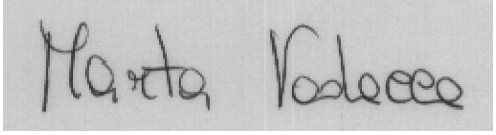
DR genotype has been obtained after assays. This was crucial to understand epitope specificities. For HLA typing genomic DNA will be extracted from peripheral EDTA - blood lymphocytes using the QIAmp DNA Blood Kit (QIAGEN Genomics Inc., Bothell, WA).

Further details are discussed in chapter 3.

6.2.5 Cell culture

6.2.5.1 PBMC isolation

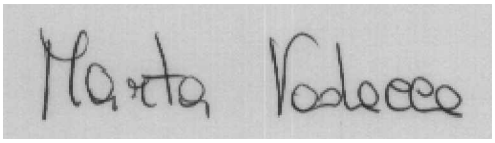
PBMC were isolated by Ficoll Hypaque density gradient centrifugation (ICN Biomedicals Inc., USA). We diluted whole blood 1:1 in complete medium (RPMI 10% FBS). Then, we overlaid 20 mL diluted blood very gently to 15 mL Ficoll without disturbing the layer. We centrifugated at 1200 rpm, 20 °C, for 35 minutes without brake. Then, we removed plasma layer and gently pipette lymphocyte fraction and transfer into clean 50 mL Falcon tube and added complete medium to wash. After that, we centrifugated for 15 minutes at 12000 rpm, removed supernatants, resuspended cells in



completed medium and wash again. We removed supernatant and resuspended cells in 1 mL of complete medium. We counted cells using Trypan blue.

6.2.5.2 Cell counting before and after irradiation to test cell viability

Cell counting has been done before and after irradiation to test cell viability. Trypan blue is the most common stain used to distinguish viable cells from nonviable cells; only non viable cells absorb the dye and appear blue and may also appear asymmetrical. Conversely, live, healthy cells appear round and retractile without absorbing the blue-colored dye. The use of this stain however is time sensitive. Viable cells absorb Trypan blu over time and can affect counting and viable results. We made dilutions just prior to counting to prevent viable cells from absorbing the stain, and thus appear no viable. In addition, Trypan blue has a greater affinity for serum proteins than for cellular proteins. For cells cultured in high serum conditions, the background in the haemocytometer may be too dark. For this reason, we isolated cells from the serum. We simply centrifuge the cells and resuspended the cell pellet in a serum free medium prior to counting. We diluted a small sample of the cell suspension 1:5 in 0.4% Trypan blue. We cantered a cover glass over the haemocytometer chambers. We filled one chamber with the cell dilutions using a Pasteur pipette. The solution passed under the cover glass by capillary action. We placed the haemocytometer on the stage of an inverted microscope and adjusted focus using 100X magnification. We used a hand-held counter to record cell counts in each of the four corner and central squares. A total of five squares are counted.



We determined the number of cells per millilitre and total number of cells using the following calculation:

Cells/mL= [# of cells counted/# squares counted] x 10^4 x dilutions factor

Total cells= cells/mL x vol. of original cell suspension

The percentage of viable cells can also be calculated using the following formula:

% viability= # viable cells counted/ total # cells counted x 100

After irradiation we found about 40% viable cells.

6.2.5.3 Optimisation of culture conditions for PBMC

1x10^6 PBMC/ml, and 10^5 cells per well are used, [after dividing into two portions for APC and responding cell]. We used precisely 10^5 cells per well. More cells may consume medium more rapidly, release proteases or other factors which interfere with the assay.

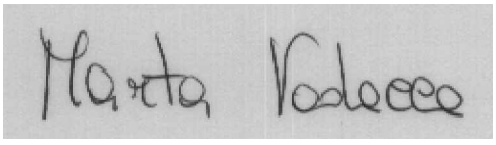
6.2.5.4 Irradiation of syngenic PBMC

One aliquot of PBMCs has been irradiated (3000 rad).

Autologous dendritic cells (DC) instead of irradiated total PBMC can be used as antigen presenting cells loaded with peptide (1 DC: 5 T cells or 1 DC: 10 T cells ratio).

6.2.6 T cell stimulation with cytokine

The manipulation of the cytokines in the culture medium is one technique that is rigorously being explored for specifically expanding certain T cell subsets (Knutson KL, 2000).



6.2.6.1 human rIL-12

IL-12 is a cytokine that is uniquely different from other commonly used cytokines such as IL-2, in that it only acts on activated T cells (Knutson KL, 2003).

6.2.6.2 IL-12 to augment secretion of Th1 cytokines

In a recent study has been reported that IL-12 is an important cytokine for ex vivo recovery and maintenance of antigen-specific CD4 T lymphocytes that would otherwise be lost by using IL-12 alone in combination with antigens (Knutson KL, 2003).

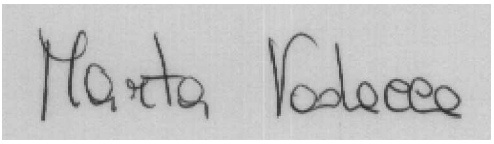
6.2.6.3 Optimal human rIL-12 dose

rIL-12 can generate background non-specific spots so it has been important defining the right dose. The 96 hr ELISPOT assay with rIL-12 has been working very well for cancer patients' PBMC stimulated with tumour associated epitopes.

To determine the optimal concentration of IL-12 (Sigma, Co. I2276) to add, different experiments have been performed with the following concentrations:

- 5 pg/ml/1x10^6 PBMC
- 10 pg/ml/1x10^6 PBMC
- 25 pg/ml/1x10^6 PBMC
- 50 pg/ml/1x10^6 PBMC

For the evaluation of the optimal dose spot size, spot number and spot intensity have been considered.



6.2.7 Human IFN-γ ELISPOT analysis (Direct *ex vivo* ELISPOT assay)

ELISPOT assay was performed with BD Pharmingen set for IFN-γ (BD™ ELISPOT Human INF-γ ELISPOT Set, BD Bioscences, San Diego, CA, USA) (Kallinteris NL, 2003; Kallinteris NL, 2006). ELISPOT assays were performed in triplicate.

6.2.7.1 Coating antibody

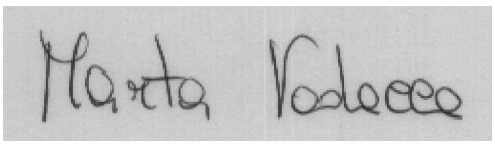
100 µl of purified anti-human INF-γ, diluted in sterile coating buffer (PBS, pH 7.2), were added to each well of the ELISPOT plate. Plate was coated overnight at 4 °C.

6.2.7.2 Blocking

On day +1, coating antibody was discarded. Wells were washed with 200 µl/well blocking solution [Cell culture medium (RPMI 1640) containing 10% human AB serum and 1% Penicillin-Streptomycin-Glutamine (Gibco-BRL)] and the plate was incubated for 2 hours at room temperature (RT) with blocking solution.

6.2.7.3 Cell activation

Irradiated autologous antigen presenting cells (APCs) pulsed (10^6 APCs/ml) were mixed with Ii-key hybrids (50 µg/ml), or epitope-controls (50 µg/ml), in complete medium and were dispensed in triplicate to the 96-well ELISPOT plate.



6.2.7.4 Experimental cell treatments:

Stimulated T cells in medium
Add 200 µl of cell plus IL-12.

Stimulated T cells with unpulsed PBMC
Add 100 µl cells plus 100 µl (RPMI Medium 2% FBS with Irradiate cells).

Stimulated T cells with PBMC pulsed with the designated peptide in the series
Add 100 µl cells plus 100 µl (RPMI Medium 2% FBS with Irradiate cells (APCs) and 50 µg/ml of each Ii-Key hybrid)

Final volume for each well was 200 µl.

6.2.7.5 Control cell treatments:

Cells without peptide or hybrid (previously propagated in the presence of IL-12) in medium
Add 200 µl of cell plus IL-12.

Cells without peptide or hybrid with unpulsed PBMC
Add 100 µl cells plus 100 µl (RPMI Medium 2% FBS with Irradiate cells).

Cells without hybrid with PBMC pulsed with the designated peptide in the series
Add 100 µl cells plus 100 Ml (RPMI Medium 2% FBS with Irradiate cells (APCs) and 50 mg/ml of each peptide)

Irradiated control PBMC medium
200 µl (RPMI Medium 2% FBS with Irradiate cells (APCs))

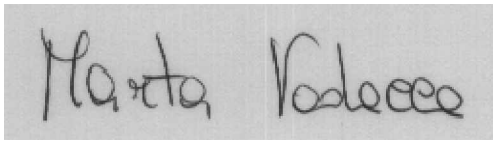
Final volume for each well 200 µl.

6.2.7.6 Positive control preparations

6.2.7.6.1 Phorbol myristate acetate (PMA)/Ionomycin

We used the following final concentration:

PMA 50 ng/ml



Ionomycin 1 microg/ml

We used three different concentrations to test positive controls:

1. Pos. Control wells: Add 50 µl Pos. Control cocktail + 150 µl Medium 2% FBS

2. Pos. Control wells: Add 100 µl Pos. Control cocktail + 100 µl Medium 2% FBS

3. Pos. Control wells: Add 200 µl Pos. Control cocktail.

In the figure 32 is reported an example of GAD experiment for one patient.

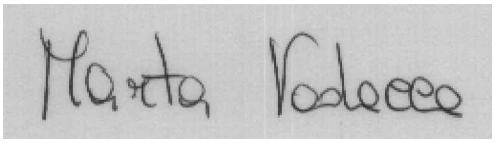
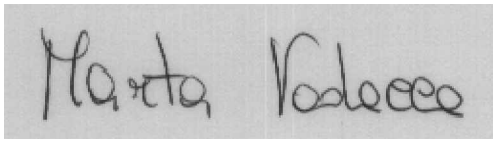


Figure 32. **An example of ELISPOT plate for testing all GAD epitopes, Ii-key hybrids and positive and negative controls.**

	1	2	3	4	5	6	7	8	9	10	11	12
A	**PBMC medium alone**	**PBMC medium alone**	**PBMC medium alone**	**Positive control 25 µl**	**Positive control 25 µl**	**Positive control 25 µl**	**Positive control 50 µl**	**Positive control 50 µl**	**Positive control 50 µl**	**IL-12 25 pg**	**IL-12 25 pg**	**IL-12 25 pg**
B	**GAD1 PEP1**	**GAD1 PEP1**	**GAD1 PEP1**	**GAD1 PEP2**	**GAD1 PEP2**	**GAD1 PEP2**	**GAD1 PEP3**	**GAD1 PEP3**	**GAD1 PEP3**	**GAD1 PEP4**	**GAD1 PEP4**	**GAD1 PEP4**
C	**GAD2 PEP1**	**GAD2 PEP1**	**GAD2 PEP1**	**GAD2 PEP2**	**GAD2 PEP2**	**GAD2 PEP2**	**GAD2 PEP3**	**GAD2 PEP3**	**GAD2 PEP3**	**GAD2 PEP4**	**GAD2 PEP4**	**GAD2 PEP4**
D	**GAD3 PEP1**	**GAD3 PEP1**	**GAD3 PEP1**	**GAD3 PEP2**	**GAD3 PEP2**	**GAD3 PEP2**	**GAD3a PEP1**	**GAD3a PEP1**	**GAD3a PEP1**	**GAD3a PEP2**	**GAD3a PEP2**	**GAD3a PEP2**
E	**GAD3a PEP3**	**GAD3a PEP3**	**GAD3a PEP3**	**GAD3a PEP4**	**GAD3a PEP4**	**GAD3a PEP4**	**GAD3b PEP1**	**GAD3b PEP1**	**GAD3b PEP1**	**GAD3b PEP2**	**GAD3b PEP2**	**GAD3b PEP2**
F	**GAD3b PEP3**	**GAD3b PEP3**	**GAD3b PEP3**	**GAD3b PEP4**	**GAD3b PEP4**	**GAD3b PEP4**	**GAD3b PEP5**	**GAD3b PEP5**	**GAD3b PEP5**			



6.2.7.7 Detection antibody

On day +5, cell suspension was aspired and wells were washed twice with deionized (DI) water first and with 200 µl/well PBS containing 0.05% Tween-20 then. 100 µl per well of biotinylated anti-human INF-γ antibody were added. Plate was incubated for 2 hours at RT.

6.2.7.8 Enzyme conjugate

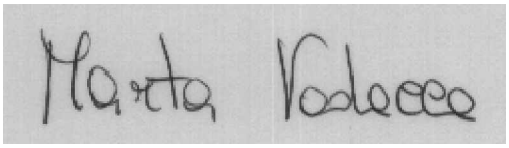
Wells were washed twice with 200 µl/well PBS. Streptavidin-HRP (100 µl/well) were dispensed into wells.

6.2.7.9 Substrate

After 1 hour at RT, 100 µl of final substrate solution (3-Amino-9-ethylcarbazole) were added to each well and incubated until the appearance of red spots in the wells (20-30 min). Plate was dried and stored in the dark, until it was analyzed.

6.2.8 ELISPOT software

Digitalized images of spots were analyzed with an *Eli.Analyse 4.0 software* (AEVS, Automated Elisa-Spot assay Video Analysis System), with the support of Dott. G. D'Agostino, Istituto Superiore di Sanità, Roma.

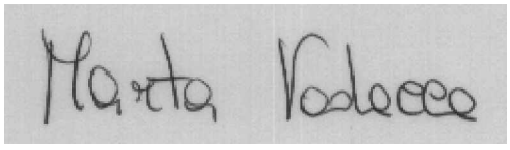


6.2.9 ELISPOT reader

Criteria for spot size, circularity and color density were determined by comparing control and experimental wells. Partially overlapping spots were separated and noise signals caused by substrate precipitation and non-specific antibody binding eliminated. Relative IFN-γ production was estimated by total spot areas. Total spot area (mm^2) equals the product of mean spot size x the number of spots. Counting results have been validated by the human eye to judge whether all spots have been counted accurately and artifacts have been excluded. The number of cytokine producing cells in antigen stimulated wells is measured in relation to the negative control wells (medium alone or epitope-controls). The difference between spot counts in the antigen-stimulated culture versus the control revealed how many antigen-specific T cells were present in the experimental wells. All data are expressed as means of triplicate determinations for each antigen.

The following parameters have been considered for the evaluation of the spots:

- Minimum spot size: defines as large a spot has to be to be counted. The unit is 500 μ^2.
- Minimum spot intensity: determines which intensity a spot much reach in order to be counted.
- Spot circularity: defines the circularity of spots to be counted. A value of 1 is a real circle, when the value becomes greater the spot shape can more differ from a circle.
- Separate spots: determines how connected spots will be separated. A lower value will only separate spots with a clear dividing line.



- These four parameters should be adjusted in such a manner, that only biologically meaningful spots are counted.

6.3 Sample size and statistical analysis

6.3.1 Sample size

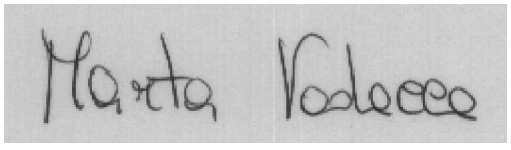
In a recent study performed to identify autoreactive T-cells in the peripheral blood of T1D subjects, 61% of them and 9.5% of control subjects were positive to GAD-65 and proinsulin tetramers (Oling V, 2005). A major issue of this project is the definition of the robustness of the immune responses to hybrids across T1D subjects with defined MHC class II genotypes. For this reason we compared T1D subjects with HLA matched control identified to be of similar for age and sex as well as recruited from the same geographical area. Based on the number of consecutive T1D subjects who are recruited each year in the Lazio region by the IMDIAB group, we expected that at least 20 T1D subjects with the requested HLA genotype can be enrolled in this study. The allele distribution of high risk HLA genes (DR3/DR4, DR3/DR3, and DR3/DRX) in the general population in Italy is estimated to be approximately 9% (Petrone A, 2002).

6.3.2 Statistical analysis

ELISPOT assays for IFN-γ production were performed in triplicate.

Statistical analysis will be performed using GraphPad statistical software, version 3.0.

Results will be expressed as mean (SD) or median (range) where appropriate. Changes in the results will be considered significant at $p<0.05$. The number of cytokine-producing cells in antigen stimulated wells and in negative control wells



(irrelevant peptide) will be compared using the one-way ANOVA test. Kruskall-Wallis test will be performed if data is not Gaussian distributed. Bonferroni or Dunns post tests, in Gaussian or non Gaussian distribution, respectively, will be used where appropriate. Distribution were tested with the Chi-square test, unless any expected value was less than five, when Fisher's exact test was used. The Mann-Whitney U-test was used to compare the results between patients and controls.

6.5 Results

6.5 Patients and control subjects

We performed the study in different steps:

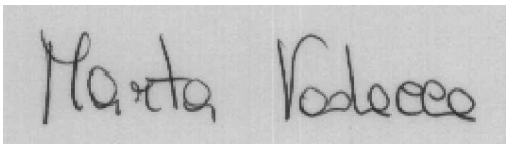
Proinsulin peptides

1) Cases 1-25. Controls 1-2.

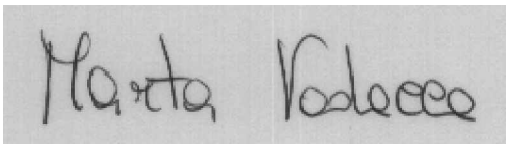
The first part of the study has been carried out in 23 patients affected by type 1 diabetes and in 2 controls. Diagnosis was defined according ADA classification; patients were of both sexes (18 males, 5 females), with an age comprised between 7 and 41 years. All but 3 patients had a recent onset of diabetes. We used these patients to optimize the test.

2) Cases 26-35. Controls 3-12.

Secondly, a total of 15 Caucasian type 1 diabetic patients (diagnosed according to the criteria of the American Diabetes Association) were studied (10 male patients aged 8-22 and 5 female patients aged 7-22; mean ± SD 13.7 ± 5.4 years). The clinical characteristics of this diabetic subjects are summarized in Table 11. A total of 10 healthy subjects without diabetes or any other autoimmune disease (6

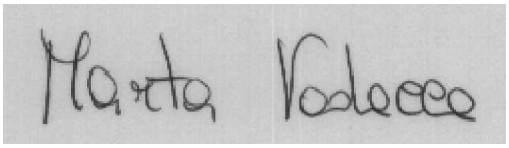


men aged 24-54 and 4 women aged 25-45; mean ± SD 33.9 ± 10.6 years) were included in the study.



					Autoantibody		
Diabetic subjects	**Sex**	**Age (years)**	**Duration of diabetes (months)**	**DRB1 genotype**	**IA-2 (U/ml) (< 1.1)**	**GAD (U/ml) (< 1)**	**Anti Insulin**
1	M	21	22	DR3/DR3 0201	------	------	------
2	F	22	30	DR4/DR4 0302	------	------	------
3	M	21	29	DR3/DR4 0302	------	------	------
4	M	8	37	DR3/DR4 0302	**0,3**	**1,3**	**Pos**
5	M	22	28	DR3/DR4 0302	------	------	------
6	M	16	37	DR3/DR4 0302	------	------	**Neg**
7	F	13	34	DR3/DR4 0302	**4,0**	**2,4**	**Neg**
8	F	10	36	DR4/DR4 0302	------	------	------
9	F	7	26	DR3/DR4 0302	**0,1**	**1,6**	**Pos**
10	M	9	36	DR3/DR3 0201	**3,7**	**7,0**	**Neg**
11	M	12	38	DR3/DR4 0302	------	**0,5**	------
12	M	14	30	DR3/DR4 0302	**0,6**	**0,5**	**Pos**
13	M	12	37	DR4/DR4 0302	**11,0**	**2,2**	**Neg**
14	M	8	33	DR3/DR4 0302	**43,0**	**0,6**	**Neg**
15	F	11	34	DR3/DR3 0201	**19,0**	**12,0**	**Pos**
Healthy subjects							
1	M	39	------	DR1/DR4	------	------	------
2	M	44	------	DR8/DR4	------	------	------
3	M	54	------	DR3/DR6	------	------	------
4	F	27	------	------	------	------	------
5	F	45	------	DR3/DR5	------	------	------
6	M	26	------	------	------	------	------
7	M	27	------	------	------	------	------
8	F	28	------	------	------	------	------
9	M	24	------	------	------	------	------
10	F	25	------	------	------	------	------

Table 11. Profile of type 1 diabetic subjects and healthy control subjects



GAD-65 peptides

3) Cases 36-43. Controls 13-19.

The third part of the study was carried out in 8 recent onset T1D subjects (3 males) affected by T1D and in 7 normal subjects (Table 12). Diagnosis was defined according ADA classification.

					Autoantibody		
Diabetic subjects	**Sex**	**Age (years)**	**Duration of diabetes (months)**	**DRB1 genotype**	**IA-2 (U/ml) (< 1.1)**	**GAD (U/ml) (< 1)**	**Anti Insulin**
1	F	11	11		------	------	------
2	M	14	12		------	------	------
3	M	16	1		------	------	------
4	F	4	11		**Neg**	**39**	**Neg**
5	F	21	24		**22**	**4,7**	**Pos**
6	F	26	31		------	------	**Neg**
7	F	33	4		**Pos**	**------**	**Pos**
8	M	21	12		**------**	**------**	**-------**
Healthy subjects							
1	M	28	------	DR8/6/DR8/6	------	------	------
2	M	29	------	DR8/6/DR8/6	------	------	------
3	M	29	------	DR7/DR7	------	------	------
4	F	32	------	------	------	------	------
5	F	29	------	------	------	------	------
6	M	27	------	------	------	------	------
7	M	29	------	------	------	------	------

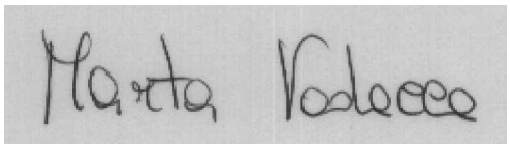
Table 12. Profile of type 1 diabetic subjects and healthy control subjects

6.6.1 PBMC isolation

$1x10^6$ PBMC/ml, and 10^5 cells per well were used.

6.6.2 T cell viability after irradiation

The percentage of viable cells has been calculated using the following formula:



% viability= # viable cells counted/ total # cells counted x 100

After irradiation we found about 40% viable cells.

6.6.2.1 Optimal human rIl-12 dose

IL-12 (25 pg/ml final concentration) was added to wells with medium alone and with hybrids.

6.6.3 Detection of cytokines secreting cells in the presence of negative control preparations

Spontaneous production of INF-gamma was present at similar, very low levels in both patients and healthy controls, (median number of spots 0, mean number 4.1, range 0-22/100,000 cells in type 1 diabetic patients, P = NS).

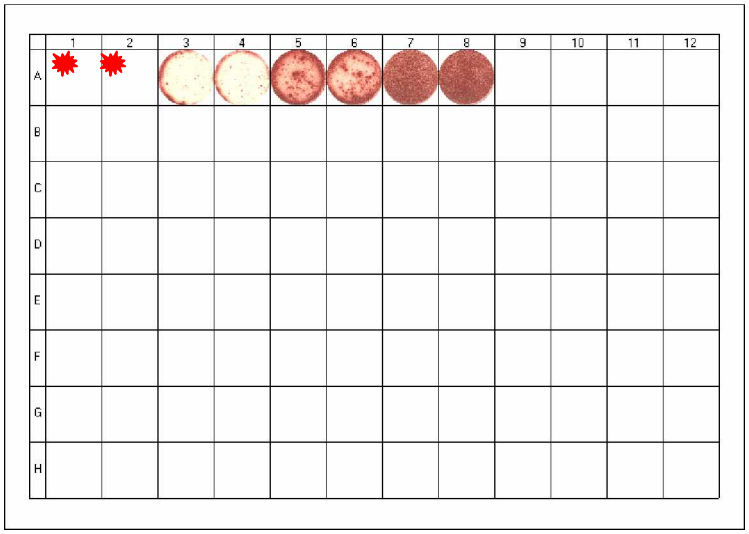
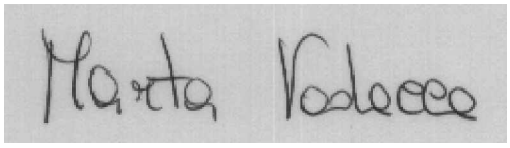


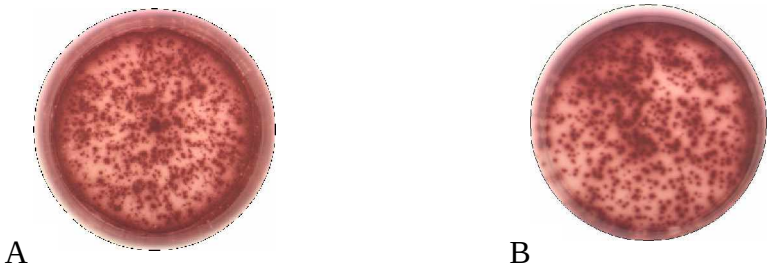
Figure 33. Spontaneous production of INF-γ.



6.6.3.1 Detection of cytokine secretion in the presence of Phytohemagglutinin (PHA) and Phorbol myristate acetate (PMA)/Ionomycin

When we used PHA there was a strong positive response to specific T cell antigen in most but not all samples.

There was a strong positive response to specific T cell antigen (PMA/Ionomycin) using the proliferation assay in all samples. All cases and control subjects showed a detectable and significant INF-γ response to stimulation with the polyclonal T cell stimulus PMA/Ionomycin, and the frequency and the magnitude of these responses were similar in all groups (Figure 34).



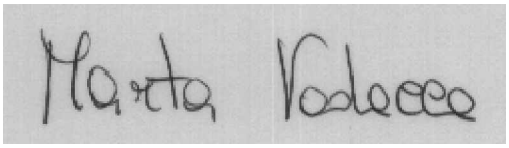
A B

Figure 34. Representative cytokine ELISPOT responses in patients (A) and controls (B).

6.6.4 IFN-γ production

6.6.4.1 Cases

1) To assess the test, we firstly analyze only few peptides. When sufficient cells were available, all 5 epitopes series were analyzed; otherwise they were analyzed in the order of D1-D5, D6-D11, D21-D28, D31-D36 and D37-D42. Data are summarized in graphs.



Relative IFN-γ productions were estimated by total spot areas. Total spot area (mm^2) equals the product of mean spot size times the number of spots.

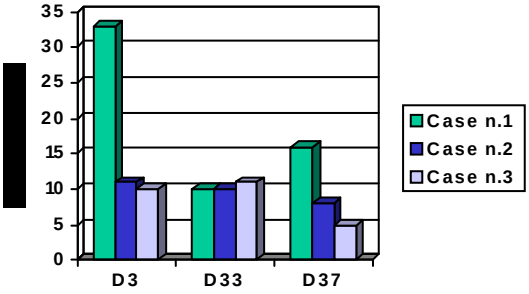


Figure 35. Three DM 1 patients: lymphocytes were tested against different epitopes (hybrids) of three different series.

Figure 36a

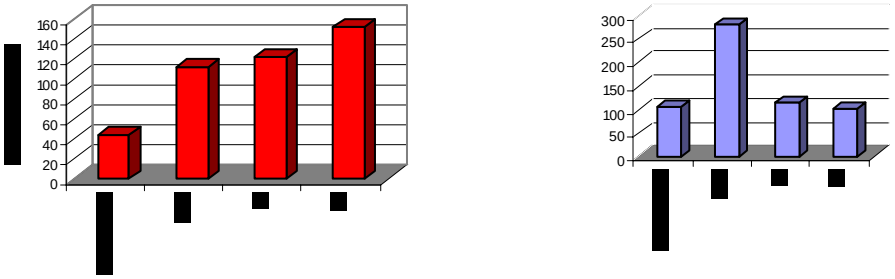


Figure 36b

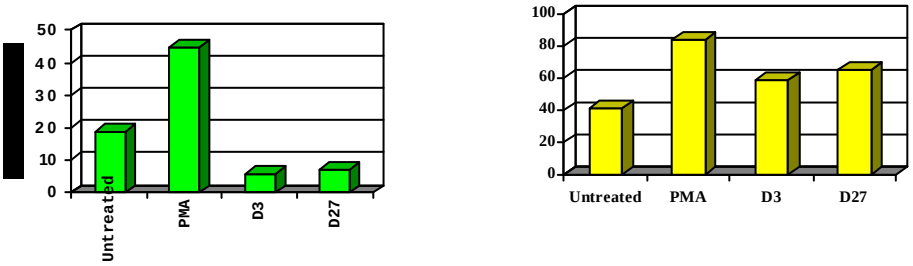
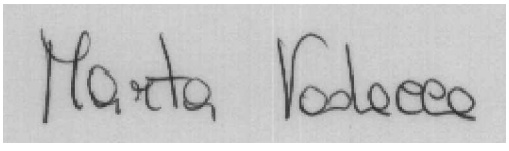


Figure 36a. Two DM 1 patients (4 and 5) show different response to same epitopes (D1-D3). PHA represents positive control.
Figure 36b. Two DM 1 patients (6 and 7) show different response to same epitopes (D3-D37). PMA represent positive control.



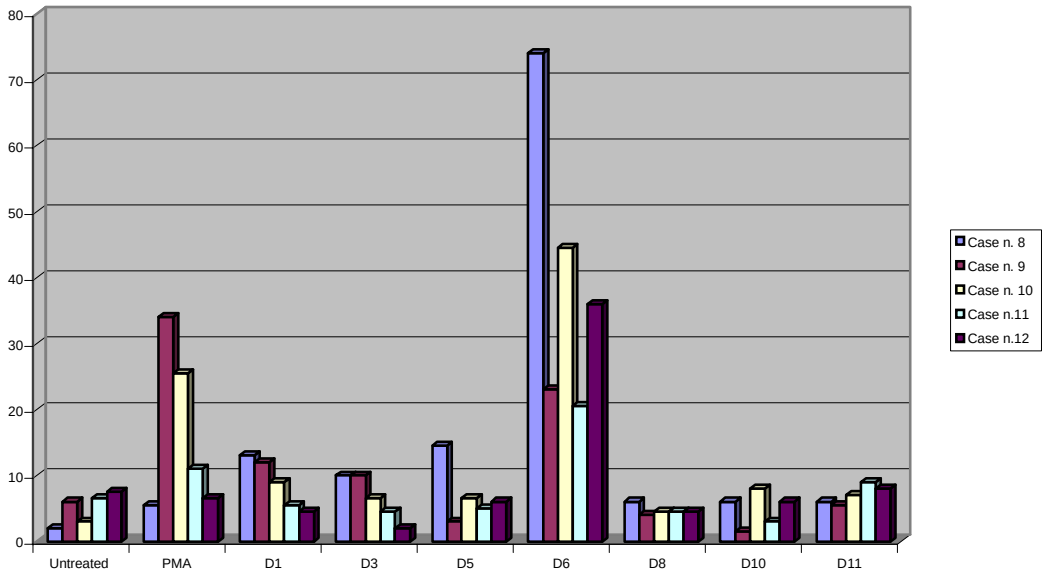
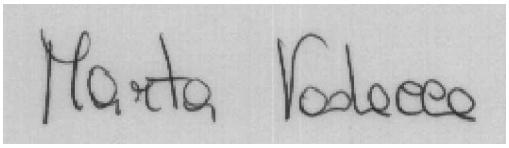


Figure 37. PBMC were mixed with hybrids and epitope-only peptides of the first two series (D1-D5, D6-D11). Case n. 12 represents an HLA-matched control.



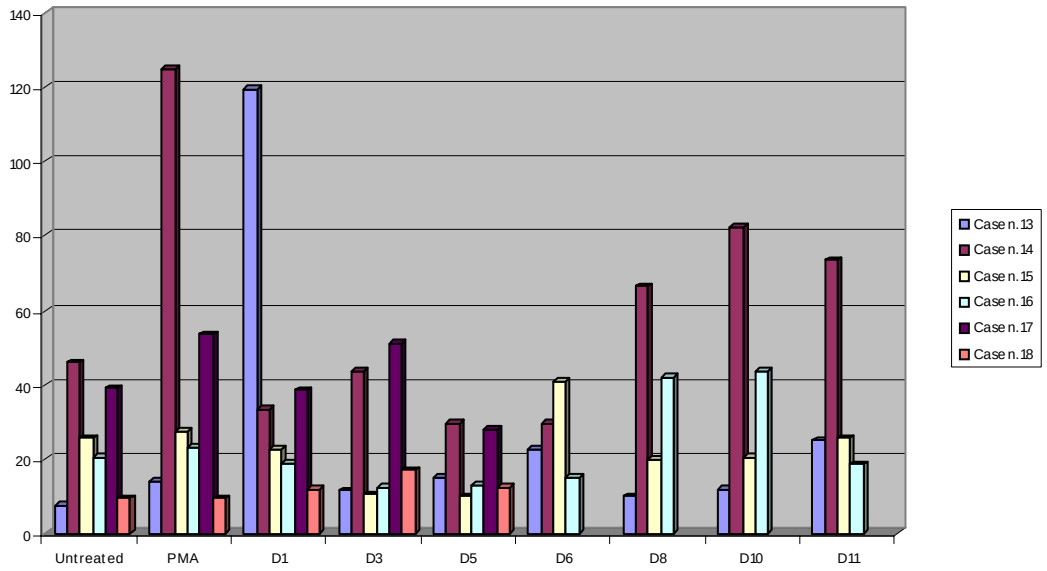


Figure 38. Case n.13 had strong activity with the longest hybrid (D1) of the first series.

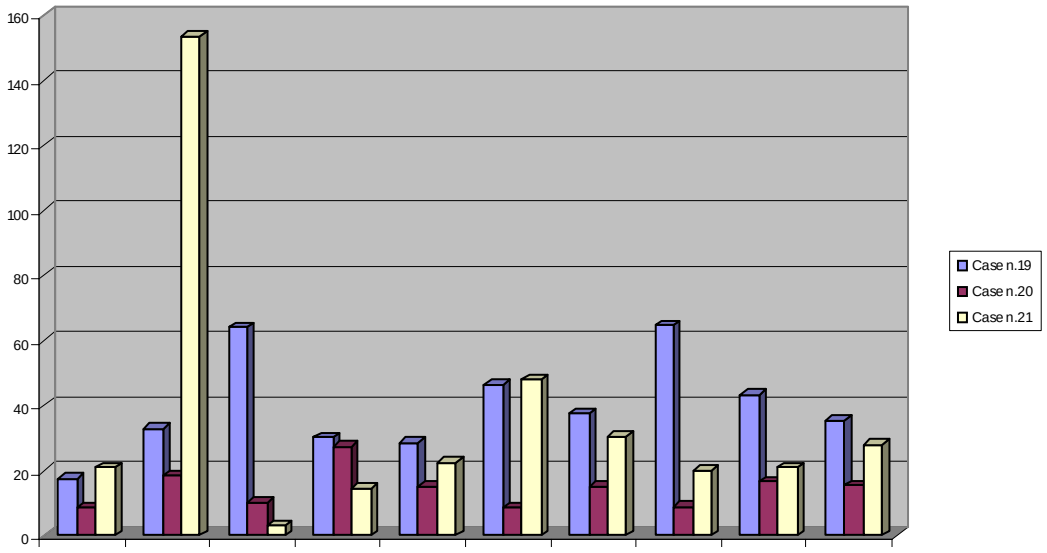
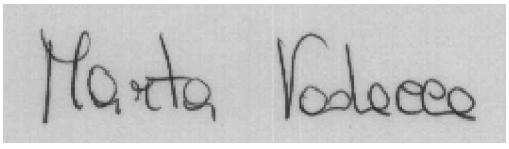


Figure 39. PBMC were mixed with hybrids and epitope-only peptides of series D6-D11 and D37-D42. Case n.20 represents a control.



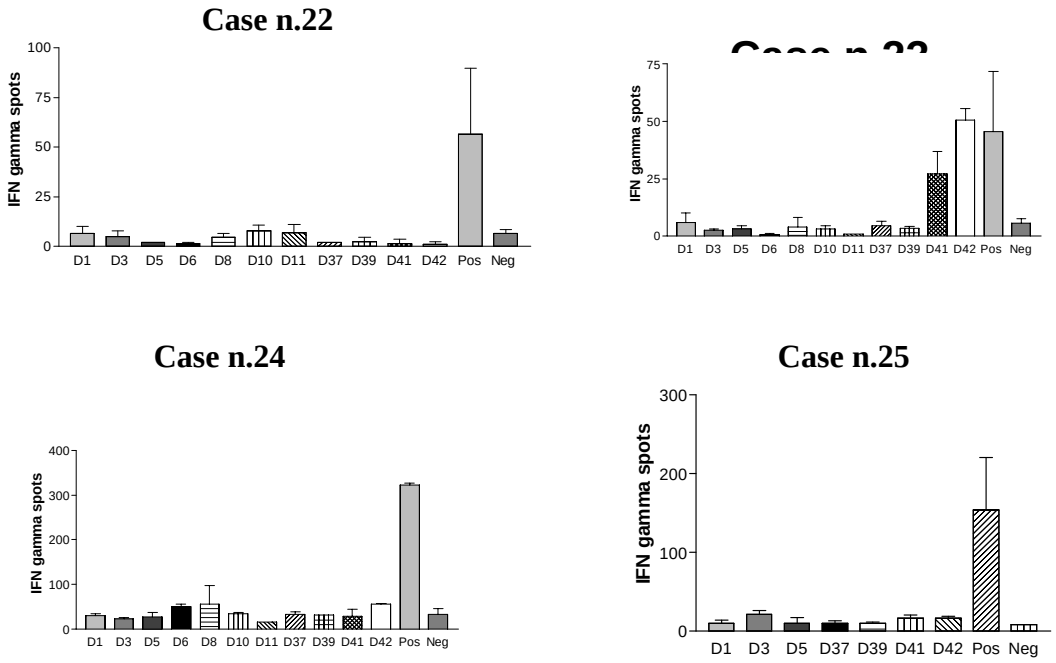
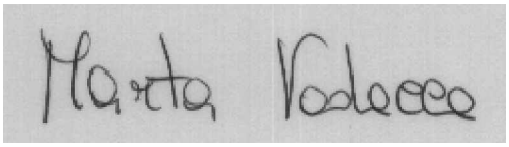
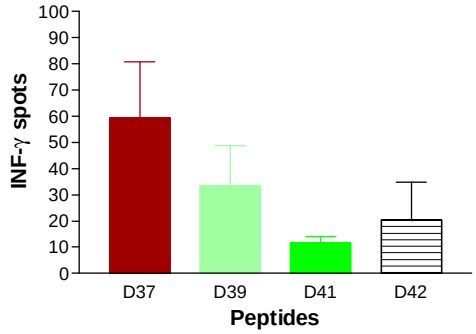


Figure 40. Frozen lymphocytes were utilized in case n.25.



2) After optimization of the assay, we tested other 15 patients and 10 controls.

Positive results are summarized in graphs.

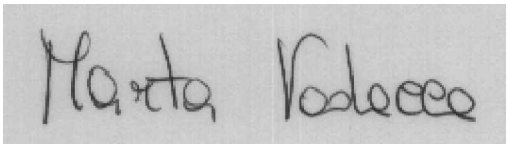


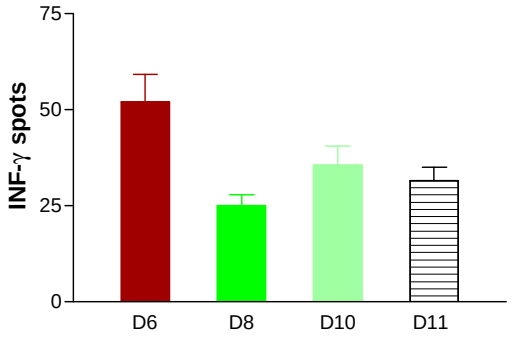
D37
D39
D41
D42 (epitope only)

	INF-γ spots
D37	59.3 ± 21.5
D42	20 ± 14.5
Medium alone	22 ± 0

D37	Ac-LRMK-*ava*-	LQVGGVELGGGPGA	-NH_2
D39	Ac-LRMK-*ava*-	VGGVELGGGPGA	-NH_2
D41	Ac-LRMK-*ava*-	GVELGGGPGA	-NH_2
D42	Ac-	LQVGGVELGGGPGA	-NH_2

Graph 1. Pt. n. 4, Male, 8 ys, HLA DR3/DR4 0302

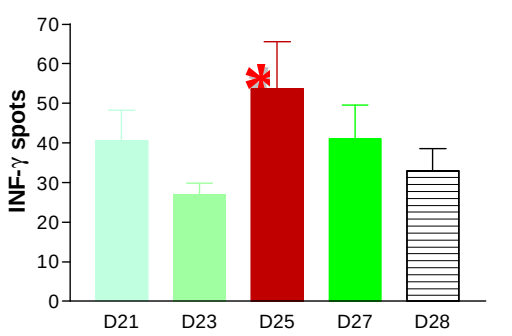




	INF-γ spots
D6	52 + 7
D11	31.5 ± 3.5
IL-12	27.5 + 13.4
Medium alone	5.5 ± 0.7

D6	Ac-LRMK-*ava*-	VEALYLVCGERGFFYT	-NH_2
D8	Ac-LRMK-*ava*-	ALYLVCGERGFFYT	-NH_2
D10	Ac-LRMK-*ava*-	YLVCGERGFFYT	-NH_2
D	Ac-	VEALYLVCGERGFFYT	-NH_2

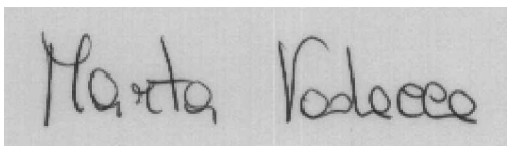
Graph 2. Pt.n. 5, Male, 22 ys, HLA DR3/DR4 0302



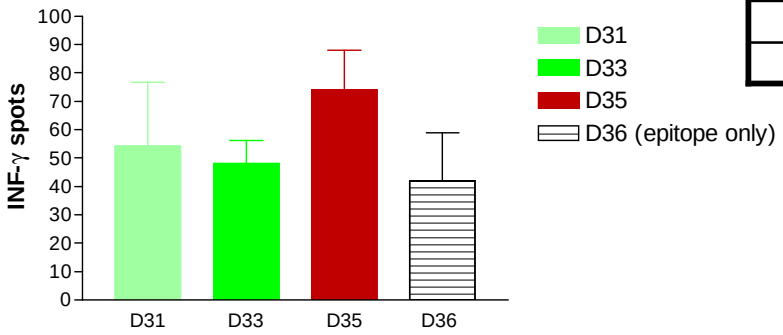
	INF-γ spots
D25	53.5 ± 12
D28	33 ± 5.6
IL-12	27.5 ± 13.4
Medium alone	6.6 ± 2.0

D21	Ac-LRMK-*ava*-	GIVEQCCTSICSLYQ	-NH_2
D23	Ac-LRMK-*ava*-	VEQCCTSICSLYQ	-NH_2
D25	Ac-LRMK-*ava*-	QCCTSICSLYQ	-NH_2
D27	Ac-LRMK-*ava*-	CTSICSLYQ	-NH_2
D28	Ac-	GIVEQCCTSICSLYQ	-NH_2

Graph 3. Pt. n. 5, Male, 22 ys, HLA DR3/DR4 0302

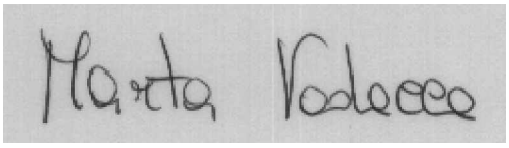


	INF-γ spots
D35	74 ± 14.1
D36	42 ± 16.9
IL-12	48 ± 12.7
Medium alone	9 ± 1.4



D31	Ac-LRMK-*ava*-	RREAEDLQVGGVEL	-NH_2
D33	Ac-LRMK-*ava*-	EAEDLQVGGVEL	-NH_2
D35	Ac-LRMK-*ava*-	EDLQVGGVEL	-NH_2
D36	Ac-	RREAEDLQVGGVEL	-NH_2

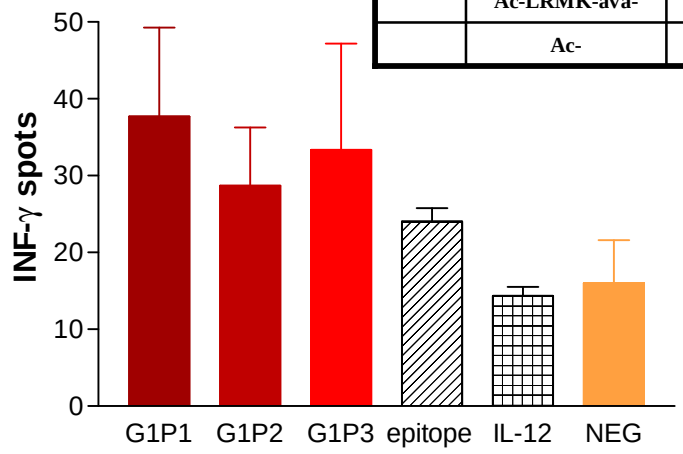
Graph 4. Pt.n.7, Female, 13 ys, HLA DR3/DR4 0302



3) Cases 36-43. Controls 13-19.

The third part of the study was carried out to test GAD peptides in 8 patients and 7 controls. Positive results are summarized in graphs.

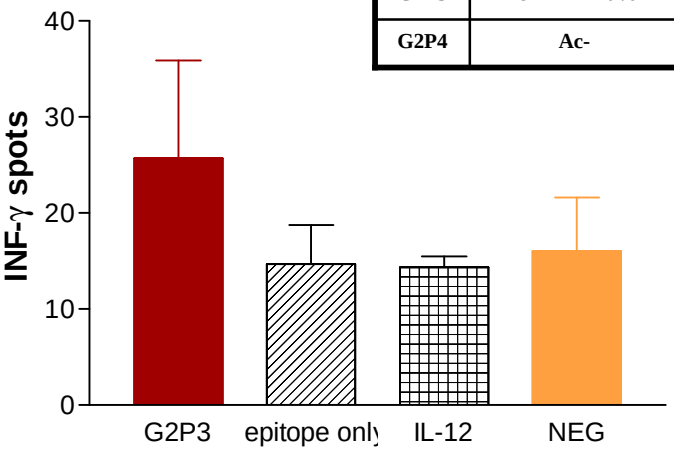
G1	Ac-LRMK-*ava*-		-NH2
		ALPRLIAFTSEHSHF	-NH2
	Ac-LRMK-ava-	AALPRLIAFTSEHSHF	-NH2
	Ac-	AALPRLIAFTSEHSHF	-NH2



Graph 5. Pt. n. 7, Female, 32 ys

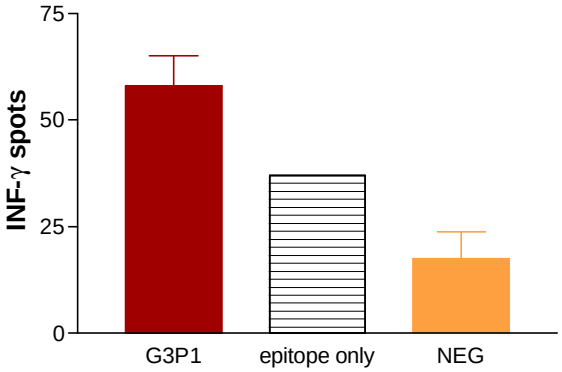
Graph 6. Pt. n. 7, Female, 32 ys

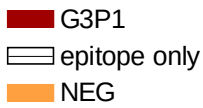
G2P3	Ac-LRMK-ava-		-NH2
G2P4	Ac-	KVNFFRMVISNPAAT	-NH2



Marta Vadacca

G3P1	Ac-LRMK-*ava*-		-NH2
G3P2	Ac-	SRLSKVAPVIKARMMEYGTT	-NH2





G3P1	58 ± 7.07
G3P2	37 ± 0
Negative	17.5 ± 6.3

Graph 7. Pt. n. 2, Male, 14 ys

6.6.4.2 Controls

Control subjects showed no detectable response to stimulation with Ii-key hybrids or peptides, not for proinsulin nor for GAD.

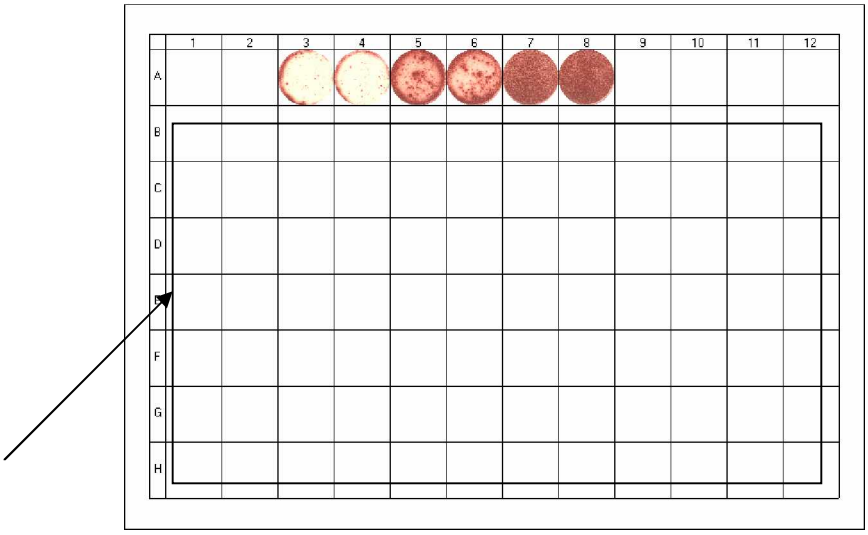
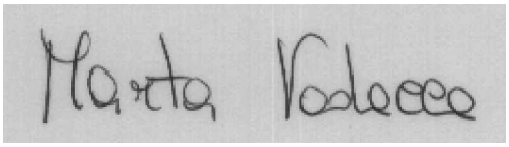


Figure 41. Key-hybrids and peptides in a control subject.



6.5.5 Activity of proinsulin hybrids

Initial data showed that some hybrids stimulate reactions in a few patients. Three out 15 patients (20%) demonstrated a positive response to one or more peptides compared with none of 10 nondiabetic control subjects tested. There is a high degree of variability among patients and a lack of systematically uniform responses to a series of homologues around any one epitope.

6.5.6 Activity of GAD hybrids

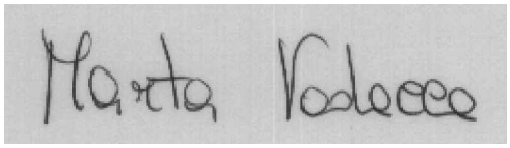
Two out 8 patients (25%) demonstrated a positive response to one or more Ii-Key/GAD65 hybrids compared to none of 7 nondiabetic control subjects tested.

6.5.7 Short term and long term *in vitro* stimulation

The practical problem in our data is the fact that these patients have at present only memory T cell responses, when assayed for 48 hr. Basically there is restimulation for 12-14 days, with IL-12 expansion, and a need for APCs for the final ELISPOT assay.

6.5.8 Interpretation of spot size and intensity

Criteria for spot size, circularity and color density were determined by comparing control and experimental wells. *Minimum spot size* defines how large a spot has to be in order to be counted. The unit is 500 μm^2. *Spot circularity* defines the circularity of spots to be counted. A value of 1 is a real circle, when the value becomes greater the spot shape can more differ from a circle. The value of *minimum spot intensity* determines which intensity a spot must reach in order to be counted. Partially

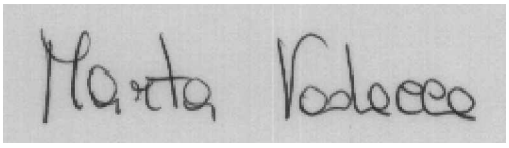


overlapping spots were separated and noise signals caused by substrate precipitation and non-specific antibody binding eliminated. Relative IFN-γ production was estimated by total spot areas. Total spot area (mm^2) equals the product of mean spot size x the number of spots. Counting results have been validated by the human eye to judge whether all spots have been counted accurately and artifacts (such as crystalline reagent deposition or pollutions) have been excluded. The number of cytokine-producing cells in antigen stimulated wells are measured in relation to the negative control wells (medium alone or irrelevant peptide). The difference between spot counts in the antigen stimulated culture versus the control reveals how many antigen specific T cells were present in the experimental wells.

6.5.9 Intraindividual variability of INF-γ release

It has been reported the phenomena that of low INF-γ response against insulin in both high-risk individuals and newly diagnosed diabetic children, in comparison with other antigens e.g. the GAD65 peptide, as observed by ELISPOT technique (Karlsson Faresjo MGE, 2000). At the onset of the disease, the spontaneous secretion of cytokines (INF-γ, IL-10, Il-13) is described to be low, which could be a sign of the metabolic disturbance. In subsequent samples spontaneous cytokines secretion increased with duration of T1D (Alleva DG 2001; Karlsson Faresjo MGE, 2004). Duration after diagnosis, as well as metabolic state, should be carefully considered both in studies of the pathogenesis of T1D and in immune intervention studies at the onset.

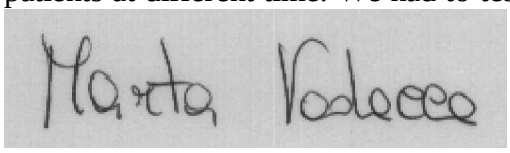
6.5.10 Frozen versus fresh lymphocytes



The PBMC are usually used fresh. In one case, PBMC were cryopreserved within 4 hours of isolation and had been kept for about one month. After that, sample was thawed and assayed. The reactivity to control antigen (PMA/Ionomycin) did not differ from fresh PBMC. This suggests that this ELISPOT assay is a reproducible method with frozen PBMC.

6.6 Summary

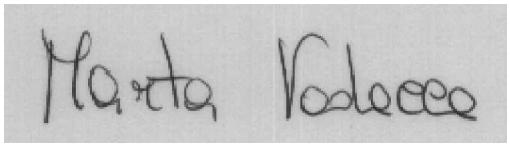
In the early phase of this study, energy has been prioritized to defining the robustness of responses to hybrids across more DM patients with defined MHC class II genotypes. For this reason, we have focused on MHC class-II presented epitopes in insulin. Initial data showed that some hybrids stimulate reactions in a few patients. The insulin study has been carried out in 35 patients (20 males) affected by T1D, and in 12 normal subjects. Twenty percent of patients demonstrated a positive response to one or more peptides compared with none of 12 nondiabetic control subjects tested. Initial data showed that some hybrids, and not the epitope-only peptide, stimulate some reactions in some patients. In order to increase the sensitivity of the test we decided to extend to *human glutamic acid decarboxylase (GAD)*. One region of GAD 65 (residues 509-543) is immunodominant and, strikingly, also comprises an immunodominant region (473-555) recognized by T cells obtained from peripheral blood of patients with type 1 diabetes. In recent papers using an ELISPOT assay, GAD reactive T helper 1 cells in PBMC from type 1 diabetic patients have been identified at a higher frequency than by other assays. Several reports indicate in GAD the major antigen for type 1 diabetes especially in young adolescents and adults. Using GAD hybrids we found a positive response to Ii-key hybrids in 2 out 8 patients (25%). We performed proinsulin and GAD65 ELISPOT assay in different patients at different time. We had to test the same patient for proinsulin and GAD Ii-



key hybrids at the same time, in order to increase the sensitivity of the test. The combined use of insulin and GAD key-hybrids might therefore assure a more selective and sensitive cytokine detection in response to antigen stimulation in T1D offering a new highly specific marker of cell mediated immunity in T1D.

Tested peptides have been shown to be recognized in DR3/DR4 0302+ve diabetic patients, but not in other patients (DR3 or DR4+ve) or normal subjects.

The fact that our peptides were not targeted in the HLA-matched healthy controls, suggests a disease related priming. The diversity of peptides recognized in the patients is not surprising considering that in addition to the accounted DRB1*04 molecule, all the other DR, DQ, and DP molecules expressed in the patients are attractive candidates for restricting specific responses.

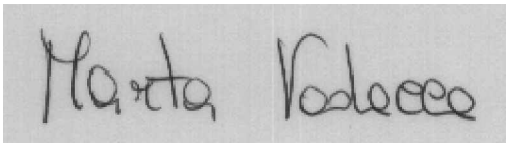


CHAPTER 7: DISCUSSION

7.13 General discussion

Susceptibility to T1D is genetically controlled, and in humans particular alleles of the major histocompatibility complex DR and DQ loci confer high disease risk. During normal immune responses, molecules encoded by DR and DQ genes bind and present peptide fragments of protein antigens to lymphocytes of the CD4 subset. These class II molecules could play a pivotal role in the development of T1D through presentation of islet cell-specific peptides to autoimmune CD4 T lymphocytes. Several β cell specific proteins have been identified as disease targets using patient autoantibodies; more than 80% of prediabetic and most recent onset diabetics have autoantibodies directed against the enzyme glutamic acid decarboxylase. The destruction of pancreatic beta cells, however, is thought to be mediated by T cells. Peripheral blood mononuclear cells from approximately one-half of new-onset T1D patients were found to respond to GAD. Recently, using bulk culture stimulation assays with synthetic peptides of GAD, lymphocyte proliferation was detected in patients as well as in normal persons, albeit in the majority of subjects the stimulation indices were quite low. Cytotoxic T cells recognizing an HLA-A2–restricted peptide of GAD have been found in prediabetics and patients with recent onset disease, but not in normal control donors.

A number of antigens have been associated with T1D, insulin and GAD peptides being probably the most relevant ones, however a T cell assay for insulin/GAD peptides with high specificity and sensitivity is still missing.



To date, the full characterization of naturally processed T cell epitopes of insulin and GAD65, including definition of the minimal peptides and identification of the class II molecules required for peptide presentation to CD4 T cells, has not been available.

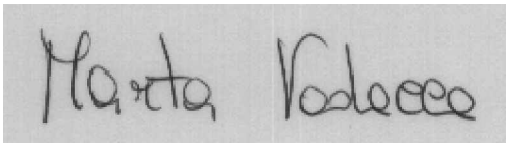
7.14 Rationale of the project

The aim of the study was to apply the novel Ii-key hybrids technique to establish the validity of Ii-key/MHC class II insulin/GAD epitopes hybrids for detection of anti insulin and GAD response in T1D patients. It could lead to define a new method to detect T cells response in T1D.

The hypothesis is based on previously described and novel observation in T cell detection of T1D patients and the use of Ii-key hybrids in different diseases.

In a recent study type 1 diabetes patients displayed T-cell response to both GAD65 and proinsulin, but only one patient had reactivity to both autoantigens. However, only 11 patients were analyzed with multiple tetramers due to the limited sample size. T cell response to the multiple autoantigens and potential epitope spreading during the preclinical phase of the disease process needs to be investigated in an extended study in a larger cohort of type 1 diabetes patients and at-risk subjects.

A novel approach to study antigen presentation via HLA class II is represented by Ii protein. The central hypothesis is that Ii-key peptide homologous can modulate the binding of antigenic peptides to MHC class II molecules by targeted interactions at the allosteric site. This ability offers the potential for a new generation of immunotherapeutics. In fact, key-hybrids could induce either an active immune

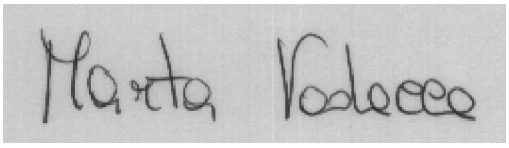


response or antigenic-specific tolerance. Because Ii-key hybridized vaccine peptide hybrids can be introduced to the immune system in very low dose without inflammation, such hybrids might effectively induce tolerance. In an HIV study, gag-hybrids consistently elicitated IFN-γ responses. In breast cancer, the Ii-key modified HER-2/neu hybrid greatly augments proliferative responses in human PBMC and lymphocytes from metastatic lymphonodes. Lower amounts of the Ii proteins were sufficient to break self-tolerance and induce autoimmunity in mice with experimental allergic encephalomyelitis (EAE). These data consistently demonstrated the capacity of Ii proteins to modulate immune responses in vivo and suggest their potential use in the antigen-specific treatment of autoimmunity. Peptide vaccination due to the antigenic specificity of the immune process underlying type 1 diabetes, by aiming at inactivating selectively the T cells involved in this process, has the advantage of preserving the function of the immune system in general. However, since in human type 1 diabetes the initiating autoantigen is unknown, the only possible approach for inducing a state of immune regulatory cells is in subjects who already have an ongoing autoimmune process. With this approach antigen-induced regulatory responses can downregulate effector T cells in the target organism, a mechanism known as "bystander suppression" mediated locally by the release of Th2 cytokines.

7.15 Analysis of the results

7.15.1 Proinsulin and GAD epitopes identification

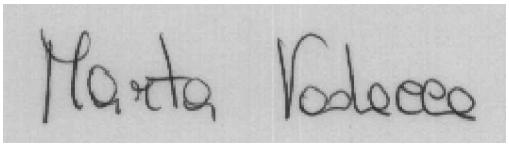
We have designs and compounds for antigen–nonspecific inhibition of MHC class II antigen presentation (Ii-Key-only ring homologs and Ii-Key-spacer-tetramer homologs) which might be tested as spillers or blockers of DM peptides presented to



the T cell lines. More importantly, one can also make Ii-key/epitope hybrids, which allows one to make the corresponding terminally blocked epitope only peptides. It is no longer sufficient just to develop diagnostic reagents. They need to be tested in the context of understanding T cell subset biology. The single most important objective from the perspective of this work, was to determine whether some form of Ii-Key hybrids structure relate to selective activation of T cell subset responses. Epitopes which are entirely included within the sequences selected from the literature have been listed, and some of those (starting with highest scores) are placed into Ii-Key/MHC class II hybrids. On the basis of the reported data, we selected 5 proinsulin epitopes and 5 GAD epitopes.

7.15.2 Cytokine production

Different in cytokines secretion and detection in T1D could be due to several factors. In our study, we have recent onset disease as well as long standing disease patients. It has been reported the phenomena that of low INF-γ response against insulin in both high-risk individuals and newly diagnosed diabetic children, in comparison with other antigens e.g. the GAD65 peptide, as observed by ELISPOT technique (Karlsson Faresjo MGE, 2000). At the onset of the disease, the spontaneous secretion of cytokines (INF-γ, IL-10, and IL-13) is described to be low, which could be a sign of the metabolic disturbance. In subsequent samples spontaneous cytokines secretion increased with duration of T1D (Alleva DG 2001; Karlsson Faresjo MGE, 2004). Duration after diagnosis, as well as metabolic state, should be carefully considered both in studies of the pathogenesis of T1D and in immune intervention studies at the onset.



7.15.3 Positive and negative controls

There was a strong positive response to specific T cell antigen (PHA or PMA/Ionomycin) using the proliferation assay in all samples.

7.15.4 Spot size and intensity

The plates were counted by a blinder investigator to avoid any influence of knowledge of the contents on the outcome. Even though the spots varied greatly in size and density, homogeneously stained spots were seen in positive wells, whereas the small dense spots that were occasionally seen both in wells with cells and in control wells without cells were distinguished as artefacts.

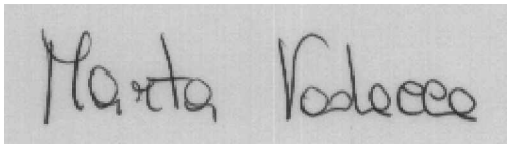
7.15.5 Effective responses in "low responder" to the epitope-only peptide

Initial data show possibly that some hybrids, and not the epitope-only peptide, stimulate some reactions in some patients. Possibly, the increased potency of MHC class II epitope presentation created by the Ii-key moiety in a hybrid, will allow effective responses in "low responder" to the epitope.

7.15.6 Background non-specific spots

Partially overlapping spots were separated and noise signals caused by substrate precipitation and non-specific antibody binding eliminated. Counting results have been validated by the human eye to judge whether all spots have been counted accurately and artefacts have been excluded. The number of cytokine producing cells in antigen stimulated wells is measured in relation to the negative control wells (medium alone or epitope-controls). The difference between spot counts in the antigen-stimulated culture versus the control revealed how many antigen-specific T cells were present in the experimental wells.

7.15.7 Specificity and sensitivity of the assay



Although the rationally behind the use of this technology relates to more selective and sensitive detection of lymphocyte subpopulations in peripheral blood, preliminary data presented demonstrated that positive results were demonstrated only in 20% (3/15) patients screened with insulin Ii-key MHC Class II-hybrids and that two out 8 patients (25%) demonstrated a positive response to one or more Ii-Key/GAD65 hybrids compared to none of 7 nondiabetic control subjects tested. Our test offers a specificity of 100%, but lack of sensitivity.

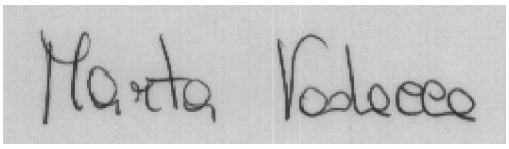
We propose to use the insulin and GAD65 Ii-key MHC Class II-hybrids to increase the sensitivity of the assay compared to insulin and GAD65 Ii-key MHC Class II-hybrid alone.

7.15.8 Duplicate and triplicate reading

First we noted substantial variation in the responses with wide standard deviation. We believe that duplicates are unacceptable given the results obtained initially. Where SD was greater than 10% assay the data have been discarded. Triplicates should be the minimum number of plates studied. We have only reported triplicate readings.

7.15.9 HLA genotype and cytokine production

DR genotype should be obtained after assays. This is crucial to understand epitope specificities. Concept for control is testing MHC class II-matched healthy individuals for responses to epitopes, which are identified with PBMCs of newly diagnosed type I diabetics. Such a comparison is needed to conclude diabetes-specific responsiveness to these epitopes.



The following considerations can be made for proinsulin peptides:

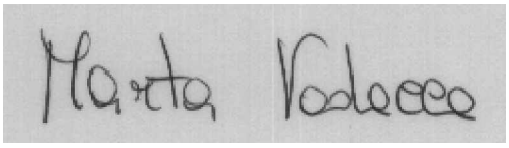
1. HLA seems to be crucial and this can help to understand epitope specificity.
2. No one of the 10 normal subjects studied (4/10 subjects were DR3 or DR4) responded.

Tested peptides have been shown to be recognized in DR3/DR4 0302+ve diabetic patients, but not in other patients (DR3 or DR4+ve) or normal subjects.
The fact that our peptides were not targeted in the HLA-matched healthy controls, suggests a disease related priming. The diversity of peptides recognized in the patients is not surprising considering that in addition to the accounted DRB1*04 molecule, all the other DR, DQ, and DP molecules expressed in the patients are attractive candidates for restricting specific responses.

7.15.10Relationship between autoantibodies and cytokine production

Two out three patients that demonstrated a T cell response for insulin Ii-key hybrids have been tested for autoantibodies; patient n.4 showed positivity for anti-insulin and antiGAD autoantibodies and patient n.7 for anti IA-2 and antiGAD autoantibodies. One out two patients that demonstrated a T cell response for GAD Ii-key hybrids has been tested for autoantibodies and showed the presence of anti IA-2 and anti-insulin autoantibodies. T cell responses to defined autoantigens can be used to differentiate responses in patients with type 1 diabetes and normal control subjects, and, when combined with measurements of standard biochemical autoantibodies, they can discriminate responses with a high degree of sensitivity and specificity (Seyfert-Margolis V, 2006).

7.15.11Difference between recent onset and longstanding diabetic patients



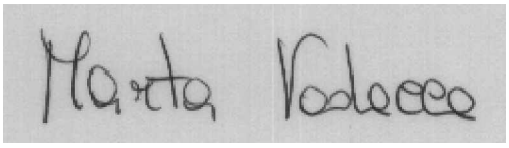
It has been noted that the responder rate is lower in type 1 diabetes of long-standing than in newly-diagnosed patients. However, we noted that patients did respond to the key hybrids and that there is not therefore a response unique to recently-diagnosed patients. In fact, using peptides we measured the frequencies of peptide specific INF-γ producing memory T cells in long-standing diabetic patients: memory cells are very long lived. We did not found a particular difference with patients with a long number of years after onset of TID. For the aim of our study, a cooperative, informed, helpful, genotyped young adult drawn on one or more occasions has been much more valuable, than non-genotyped children at the early stage of the disease.

7.15.12Structure-activity relationship among series of hybrid peptides and homologous series

We showed some structure-activity relationship among series of hybrid peptides in the 4 or so homologous series around insulin MHC II epitopes. This type of pattern we have seen with several cancer and HIV epitopes with other investigators studying PBMCs. One patient had strong activity with the longest hybrid and zero activity with one of the shorter hybrids and all hybrids shorter than that one. Wonderfully clear definition of the epitope in that patient. In cancer studies greater potency *in vitro* was associated with shorter spacers. In our case, the longer the distance between the Ii-key moiety and the MHC class II epitope, the more potent the activity. In fact, in this patient hybrids containing shorter spacer sequences were all less potent than hybrid that had the longer spacer sequence.

7.15.13Reproducibility with positive patients

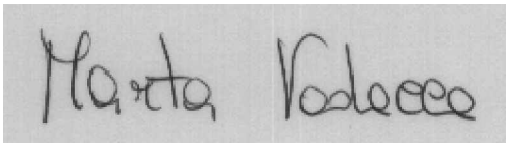
Early in this study, one might prefer to assay all peptides of all 5 epitope series. If there are not enough cells to do all peptides, then all peptides of one of the 5 series should be done when a response is seen with one of the epitopes of the series,



because some hybrid structures (which one might not predict) should be stronger than others. The long-term pattern has been that one *ava* is best (or 3 amino acids spacer of 3 amino acids instead of one *ava*), but we do not want to pre-judge experimental results. One does want to see a measurable epitope-only response. Later when one knows which of the 5 epitopes stimulate certain DR genotypes, if blood is limiting one could prioritize such sets. It is important to pick patients who are accessible for redrawing samples, when they have been identified to have significant responses to some epitopes.

7.16 Comparison of the present study to published observations

In a recent study (Oling V, 2005) it has been demonstrated that GAD65 and proinsulin tetramers binding CD4 T cells are detectable more often in peripheral blood of type 1 diabetes patients and at-risk subjects than healthy controls. Peakman and colleagues (Peakman M, 2004) have identified a panel of naturally processed islet epitopes by direct elution from APC bearing HLA-DR4. The IA-2 peptides they synthesized from this information were 29, 28, 24, 25, 20 and 22 amino acids in length. These are long sequences, which might each contain one or more MHC II epitopes. In any event precise MHC class II epitopes were not determined experimentally. In this work there are presented IFN-γ ELISPOT counts of cells responding to 6 IA-2 peptides and 3 PI (proinsulin) peptides. The lack of discrimination of responses among those peptides mimics our data. There appears not to be strong differences in responses among a) T1D patients with DR4, b) T1D patients without DR4, and c) nondiabetic controls using their methods. Our test showed instead a very high specificity for T1D subjects.

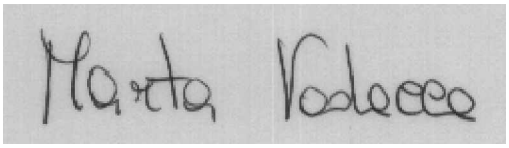


7.17 Validity of using Ii-key/MHC class II proinsulin and GAD epitope hybrids I type 1 diabetes

In the present study we were looking for evidence of the T cell hypothesis, according to the specific INF-gamma producing cells of patients with T1D occur in higher frequencies than the cells of healthy HLA-matched controls, because they were primed in vivo during the spontaneous autoimmune response. In order to detect the specific repertoire, we used peptide antigens that can bind directly to MHC molecules and be presented. The peptide length chosen is ideal for class II binding and hence favours the detection of CD4 cells. To further enhance antigen presentation by the resting APC in the test system, we used Ii-key hybrids. We showed that the number of reactive INF-gamma producing cells was higher in patients than in controls, in which secretion was not detectable.

7.18 Availability of PBMC from young patients

Theoretically, if 30 ml of blood is obtained, yielding $1x10^6$ PBMC/ml, and 10^5 cells per well are used, [after dividing into two portions for APC and responding cell] then up to 150 wells can be assayed. If peptides are tested in duplicate at one concentration, 75 peptides can be tested. In triplicate 50 peptides can be tested. With less blood volumes, proportionally fewer peptides can be assayed. The problem was to collect 20-30 mL from very young subjects; in some cases, we had to test a limited number of peptides. We preferred to test older subject, willing to offer two or more blood donations, so that reproducibility of findings and statistical analysis of variance of triplet ELISPOT IFN-γ responses could be obtained.



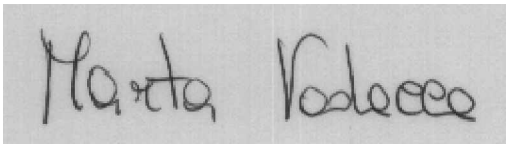
7.19 Limitation on finding age-matched controls

The choice of recent onset T1D subjects for this study is based on the concept that they still have an active ongoing autoimmune process towards beta cells and residual beta cell function. However, studying young people became difficult because for ethical reasons it has been not easy to obtain samples from age-matched control. For this reason, we decided to apply our study at long standing patients, too.

7.20 Use of cytokines to stimulate T cell proliferation

The manipulation of the cytokines in the culture medium is one technique that is rigorously being explored for specifically expanding certain T cell subsets. The most common cytokine to be added to T cell culture is IL-2. However antigen specificity is often lost when using IL-2 alone for the generation of antigen-specific T CD4 helper cells, particularly when antigen specific T cell precursor are low (Knutson KL, 2000). Another cytokine that has been extensively characterized over the past decade and which could be useful for ex vivo expansion of human antigen specific T cells is IL-12 (Knutson KL, 2003). In a recent study has been reported that IL-12 is an important cytokine for ex vivo recovery and maintenance of antigen-specific CD4 T lymphocytes that would otherwise be lost by using IL-12 alone in combination with antigens (Knutson KL, 2003).

In the first part of the study, we add IL-2; we performed different test without cytokine stimulation; at the end, we added IL-12 for are tested patients and controls. In our tests we added IL-12, because it's a uniquely different cytokine that only acts on activated T cells. The addition of IL-12 did not change the overall recovery of



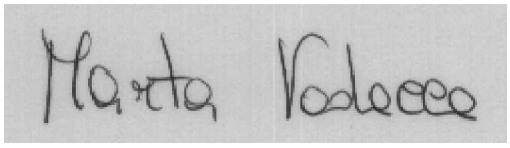
viable cells and resulted in augmentation of antigen specific proliferation in a dose-dependent manner. In a recent study has been demonstrated that IL-12, when added to cell cultures with peptides promotes increased recovery of $CD4^+$ T cells specific for the peptide. T cell lines incubated with antigen and IL-12 showed augmented function and secretion of Th1 type cytokines.

7.21 Large numbers of tested peptides

We focused first on MHC class-II presented epitopes in insulin because computer based analysis revealed a plethora of theoretical epitopes in the sequence of the insulin, which have been reported to contain active epitopes that stimulate responses by $CD4^+$ T cells of type 1 diabetics. Which of the theoretically predicted epitopes are actually recognized by a patient's immune response should be determined through experiments with panels of Ii-Key hybrids with systematic variations in N-terminal lengths of the epitope-containing segment. The first goal was to get assays of every peptide (hybrids and epitope only) for one of the 4 or 5 epitopes in insulin, in order to demonstrate structure-activity relationships among the series (presumably with data mimicking the overall pattern seen in recent melanoma gp100 paper). The genotype correlation can be indicated, but there were not strong enough data to make proved correlations.

7.22 High degree of variability among clinical samples

There is a lack of systematically uniform responses of patients to series of homologs around any one epitope. There are a series of reasons for this variance. We are seeing a comparable story in the Her-2/neu epitopes study with patients who have

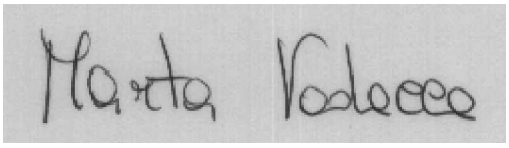


Her-2/neu-positive tumours. Probably we are scratching at a biological phenomenon of potentially very great clinical significance. It has been noted that the responder rate is lower in type 1 diabetes of long-standing than in newly-diagnosed patients. However, we noted that patients did respond to the key hybrids and that there is not therefore a response unique to recently-diagnosed patients.

Recognition of several of the peptides was shared among the patients, consistent with their shared HLA haplotype. However, none of those determinants was immune dominant. The data reproduce findings reported by different authors (Peakman M, 1999) who defined naturally processed epitopes of IA-2 eluted from HLA-DRB1*0401 molecules of IA-2 pulsed B cells. These peptides have been shown to be 12-24 amino acids long and to be recognized in DR4 positive T1D patients, but not in DR4 negative patients or healthy controls.

7.23 Conclusions

Although the number of individuals studied here is too limited to make general conclusions, our findings are in line with a recent report demonstrating with bulk culture assays that there is a higher incidence of GAD reactivity in recent onset T1D patients, especially in prediabetic, as compared to normal controls. In addition, cytotoxic T lymphocytes specific for a GAD peptide could only be detected in the peripheral blood of subjects with recent onset T1D and in prediabetic, but not in normal persons. Expression of IFN-γ under the control of the insulin promoter in transgenic mice was shown to cause inflammation of the islets, and progression to diabetes. IFN-γ has biological effects on the insulin production of the beta cells. Given the important immunoregulatory role of IFN-γ for enhancing the cytolytic activity of natural killer cells and monocytes and macrophages, GAD reactive CD4 T

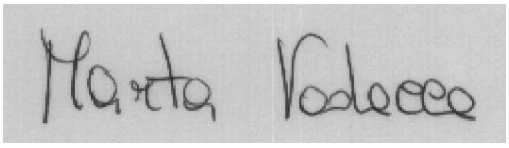


cells that secrete this cytokine may have an important role in vivo by promoting the development of T1D in genetically susceptible individuals.

It should be noted that our patients are composed by adults and children. Type 1 diabetes is heterogeneous with respect to the age of disease onset and also with respect to progression. The major of our patients are of later onset-type, however the clinical criteria for T1D (extensive weight loss, low body mass index, hyperketonuria, primary insulin dependency) were fulfilled. Although our results are not likely representative of the early events of the disease, T cells reacting to GAD at the time of clinical diagnosis could represent a valid component of the process of β cell killing associated with the development of hyperglycemias. It has been shown that at the time of onset, the pancreas contains islets in all stages of destruction, and the destructive process continues until all β cells are destroyed. The analysis of T cell response profiles in these T1D patients indicates that at the final stage of the disease different patterns of T cell responses to proinsulin/GAD can exist: response can be confined to a single immunodominant epitope, as identified in DRB1*0301/0401 patients, or can be spread over three clustered autoreactive peptides.

Taken together, these data suggest that at the end point of a chronic disease like T1D, the T cell response profiles can vary from an effector repertoire restricted to only a single epitope, to one with a rather broad autoimmune specificity based on recognition of multiple epitopes.

Precise definition of the MHC/peptide specificity and functional properties of CD4 T cells populations is required for mechanistic understanding of the disease process of T1D. In order to design prevention therapies and for proper immunomonitoring we

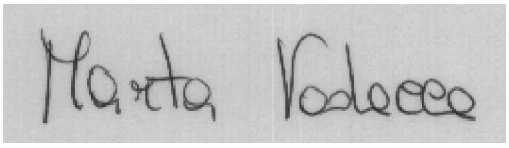


need to learn the specificity and phenotype of the T cells that are prerequisite for the disease outcome and sufficient for the clinical disease.

7.24 Future works

The obvious diversity of T helper cell epitopes recognized by T1D patients makes it unlikely that an epitope-specific immune intervention will be easily developed for T1D. It is possible, however, that the initial response is directed against particular determinants, as is the case in the NOD mouse model. Investigation of the T cell response in different disease stages may resolve this issue, and perhaps allow a specific immunotherapy to be initiated at a stage when sufficient numbers of beta cells are still available to maintain metabolic homeostasis.

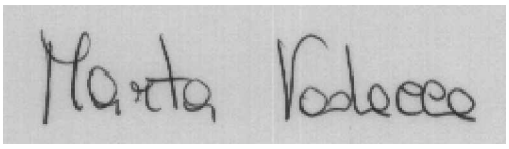
We have designs and compounds for antigen–nonspecific inhibition of MHC class II antigen presentation (Ii-Key-only ring homologs and Ii-Key-spacer-tetramer homologs) which might be tested as spillers or blockers of DM peptides presented to the T cell lines. After the initial studies are fully done, we found that notably, the patients responded with considerable heterogeneity. In face of this heterogeneity of peptide recognition suggested by this study, and to cover a wide spectrum of determinants, after testing in total about 40 peptides (20 for insulin and 20 for GAD) we can now select the peptides recognized from a higher number of patients. Reassaying these positive patients, in triplicates with making DCs for restimulation with these specific peptides after insulin in vitro feeding it is very likely we will get a strong evidence of Ii-key hybrids potential. Another hypothesis might be getting two or three T cell lines, each to a different epitope. Those lines might come from different patients. T cell lines (and later, possibly clones obtained by limiting



dilution) will allow cell number-unlimited assays, probably triplicates with all peptides in a series and the one-*ava* homologs from other no stimulating series. Dose-response curves can be done with epitope-only hybrids. The epitope-only peptide response proves the T cell line is directed to the epitope part of a hybrid and does not represent disparate cross-reaction to another epitope involving Ii-Key; i.e., wherein the L or M residues of LRMK in the hybrid occupy the P1 site of the MHC class II molecule.

Major issue now is pre-selecting patients according to genotypes for assays with certain epitopes. If you have evidence that D31-37 contains an epitope presented by DR3, then in subsequent assays, whenever we have a DR3 person, we can include D31-D37. If we found that DR3 presents an epitope in D38-D42, then whenever we have a DR4 person, we have to test that epitope.

This is an ambitious study. The goal is to associate a specific Ii-key hybrids response with a clinical phenotype (disease or asymptomatic state). On the basis of the above, we do not proceed to design and synthesize other peptides at this time, but comparing respective series of homologous Ii-Key hybrids within a limited number of patients and healthy subjects, in order to establish the increased potency of hybrids for such assays.



CHAPTER 8: REFERENCES

Abbas AK, Lichtman AH. Cellular and molecular immunology. Update edition, 2005

Achenbach P, Koczwara K, Knopff A, Naserke H, Ziegler AG, Bonifacio E. Mature high-affinity immune responses to (pro)insulin anticipate the autoimmune cascade that leads to type 1 diabetes. J Clin Invest. 2004 Aug;114(4):589-97.

Adams S, Humphreys RE. Invariant chain peptides enhancing or inhibiting the presentation of antigenic peptides by major histocompatibility complex class II molecules. Eur J Immunol 1995;25:1963-702.

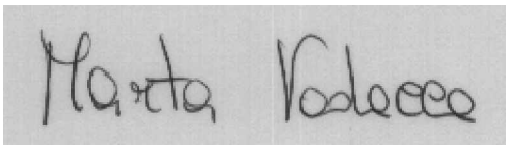
Adams S, Albericio F, Alsina J, Smith ER, Humphreys RE. Biological activity and therapeutic potential of homologs of an Ii peptide which regulates antigenic peptide binding to cell surface MHC class II molecules. Arzneimittelforschung 1997 47;1069-77.

Adeghate E, Schattner P, Dunn E. An update on the etiology and epidemiology of diabetes mellitus. Ann N Y Acad Sci. 2006 Nov;1084:1-29.

Al-Bukhari TA, Radford,P.M., Bouras,G., Davenport,C., Trigwell,S.M., Bottazzo,G.F., Lai,M., Schwartz,H.L., Tighe,P.J. and Todd I. Distinct antigenic features of linear epitopes at the N-terminus and C-terminus of 65 kDa glutamic acid decarboxylase (GAD65): implications for autoantigen modification during pathogenesis. Clin Exp Immunol, 2002: 130 (1), 131-139.

Alleva DG, Crowe PD. A disease associated cellular immune response in T1D to an immunodominant epitope of insulin. J Clin Invest 2001;107:173.80.

Aly T, Devendra D, Eisenbarth GS. Immunotherapeutic approaches to prevent, ameliorate, and cure type 1 diabetes. Am J Ther. 2005 Nov-Dec;12(6):481-90.



Arif S, Tree TI, Astill TP, et al. Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. J Clin Investig 2004;113:451-63.

Atkinson MA, Honeymoon N, et al. The ISD T cell workshop Committee. T cells marker in type 1 diabetes. Diabetologia, 2000; 43: 819-20.

Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet 2002;358:221-9.

Bach JF, B.J. Francois, Regulatory T cells under scrutiny, Nat Rev. Immunol, 2003: 189–198.

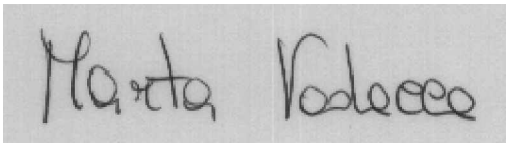
Bach JF. Infections and autoimmune diseases. J Autoimmun. 2005;25 Suppl:74-80.

Baecher-Allan C, Viglietta V, Hafler DA. Inhibition of human $CD4^{+}CD25^{+}$ regulatory T cell function. J Immunnol 2002;169:6210-7.

Baecher-Allan C, Viglietta V, Hafler DA. Human $CD4^{+}CD25^{+}$ regulatory T cells. Seminars Immunol 2004;16:89-97.

Banga JP, Moore JK, Duhindan N, Madec AM, van Endert PM, Orgiazzi J, Endl J. Modulation of antigen presentation by autoreactive B cell clones specific for GAD65 from a type I diabetic patient.Clin Exp Immunol. 2004 Jan;135(1):74-84.

Barker JM, Barriga KJ, Yu L, Miao D, Erlich HA, Norris JM, Eisenbarth GS, Rewers M; Diabetes autoimmunity Study in the Young. Prediction of autoantibody positivity and progression to type 1 diabetes: Diabetes Autoimmunity Study in the Young (DAISY). J Clin Endocrinol Metab. 2004 Aug;89(8):3896-902.



Barrat BJ, Payne F, Lowe CE, Hermann R, Healy BC, Harold D, et al. Remapping the Insulin Gene/IDDM2 Locus in type 1 Diabetes. Diabetes 2004, 53;1884-9.

Baxevanis CN, Sotiropoulou PA, Sotiriadou NN, et al. Immunobiology of HER-2/neu oncoprotein and its potential application in cancer immunotherapy. Cancer Immunol Immunother 2004;53:166-175.

Beyan H, Buckley LR, Yousaf N, Londei M, Leslie RD. A role for innate immunity in type 1 diabetes? Diabetes Metab Res Rev. 2003 Mar-Apr;19(2):89-100.

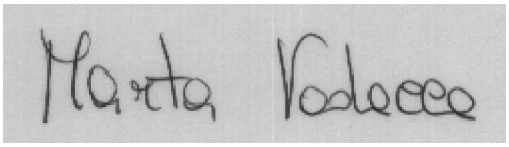
Bennet ST, Lucassen AM, Gough SCL. Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellilte locus. Nat Genet 1995;9:284-92.

Bielekova B, Catalfamo M, Reichert-Scrivner S, Packer A, Cerna M, Waldmann TA, McFarland H, Henkart PA, Martin R. Regulatory CD56(bright) natural killer cells mediate immunomodulatory effects of IL-2Ralpha-targeted therapy (daclizumab) in multiple sclerosis. Proc Natl Acad Sci U S A. 2006 Apr 11;103(15):5941-6. Epub 2006 Apr 3.

Bingley PJ, Bonificio E, Mueller PW. Diabetes Antibody Standardization Program: first assay proficiency evaluation. Diabetes 2003; 52:1128-36.

Bischof F, Wienhold W, Wirblich C, et al. Specific treatment of autoimmunity with recombinant invariant chains in which CLIP is replaced by self-epitopes. Proc Natl Acad Sci U S A 2001;98(21):12168-73. Epub 2001 Oct 02

Borg H, Marcus C, Sjoblad S, Fernlund P, Sundkvist G. Insulin autoantibodies are of less value compared with islet antibodies in the clinical diagnosis of autoimmune type 1 diabetes in children older than 3 yr of age. Pediatr Diabetes. 2002 Sep;3(3):149-54.



Bottazzo GF, Florin-Christensen A, Doniach D. Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. Lancet. 1974 Nov 30;2(7892):1279-83.

Bour-Jordan H, Bluestone JA. B cell depletion: a novel therapy for autoimmune diabetes? J Clin Invest. 2007 Dec;117(12):3642-5.

Bresson D, von Herrath M. Moving towards efficient therapies in type 1 diabetes: to combine or not to combine? Autoimmun Rev. 2007 Apr;6(5):315-22. Epub 2006 Oct 18.

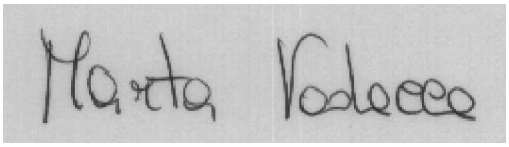
Buckner JH, U. Holzer, E.J. Novak, H. Reijonen, W.W. Kwok and G.T. Nepom, Defining antigen-specific responses with human MHC class II tetramers, *J Allergy Clin Immunol* 110**;** 2002, 199–208.

Burrows SR, Kienzle N, Winterhalter A, Bharadwaj M, Altman JD, Brooks A. Peptide-MHC class I tetrameric complexes display exquisite ligand specificity. J Immunol 2000;165:6229-34.

Buzzetti R, Galgani A, Petrone A, et al. Genetic prediction of type 1 diabetes in a population with low frequency of HLA risk genotypes and low incidence of the disease (the DIABFIN study). Diabetes Metab Res Rev 2004; 20: 137-143.

Buzzetti R, Galgani A, Petrone A, Del Buono ML, Erlich HA, Bugawan TL, Lorini R, Meschi F, Multari G, Pozzilli P, Locatelli M, Bottazzo G, Di Mario U. Genetic prediction of type 1 diabetes in a population with low frequency of HLA risk genotypes and low incidence of the disease (the DIABFIN study). Diabetes Metab Res Rev. 2004 Mar-Apr;20(2):137-43.

Cadario F, Vercellotti A, Trada M, Zaffaroni M, Rapa A, Iafusco D, Salardi S, Baldelli R, Bona G; Diabetes Study Group of the Italian Society for Pediatric Endocrinology and Diabetology. Younger age at diagnosis of type 1 diabetes



mellitus in children of immigrated families born in Italy. J Endocrinol Invest. 2004 Nov;27(10):913-8.

Capitani G, De Biase D, Gut H, Ahmed S, Grutter MG.Structural model of human GAD65: prediction and interpretation of biochemical and immunogenic features. Proteins. 2005 Apr 1;59(1):7-14.

Cardwell CR, Carson DJ, Patterson CC. Secular trends, disease maps and ecological analyses of the incidence of childhood onset Type 1 diabetes in Northern Ireland, 1989-2003. Diabet Med. 2007 Mar;24(3):289-95.

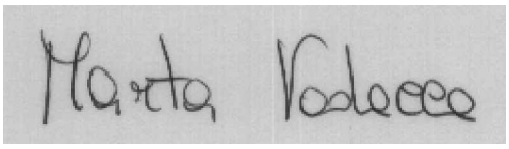
Carle F, Gesuita R, Bruno G, Coppa GV, Falorni A, Lorini R, Martinucci ME, Pozzilli P, Prisco F, Songini M, Tenconi MT, Cherubini V; RIDI Study Group. Diabetes incidence in 0- to 14-year age-group in Italy: a 10-year prospective study. Diabetes Care. 2004 Dec;27(12):2790-6.

Cesares S, Hurtado A, McEvoy RC, et al. Down-regulation of diabetogenic CD4+ T cells by a soluble dimeric peptide-MHC class II chimera. Nature Immunol 2002;3:383-91.

Chen YG, Silveira PA, Osborne MA, Chapman HD, Serreze DV. Cellular expression requirements for inhibition of type 1 diabetes by a dominantly protective major histocompatibility complex haplotype. Diabetes. 2007 Feb;56(2):424-30.

Chessler SD and Lernmark A. Alternative splicing of GAD67 results in the synthesis of a third form of glutamic-acid decarboxylase in human islets and other non-neural tissues. J Biol Chem, 2000; 275 (7), 5188-5192.

Christie MR, Tun RY, Lo SS, Cassidy D, Brown TJ, Hollands J, Shattock M, Bottazzo GF, Leslie RD. Antibodies to GAD and tryptic fragments of islet 64K antigen as distinct markers for development of IDDM. Studies with identical twins. Diabetes. 1992 Jul;41(7):782-7.



Christie MR, Brown TJ, Cassidy D. Binding of antibodies in sera from Type 1 (insulin-dependent) diabetic patients to glutamate decarboxylase from rat tissues. Evidence for antigenic and non-antigenic forms of the enzyme. Diabetologia. 1992 Apr;35(4):380-4.

Costa M, Saiz A, Casamitjana,R., Castaner,M.F., Sanmarti,A., Graus,F. and Jaraquemada,D. T-cell reactivity to glutamic acid decarboxylase in stiff-man syndrome and cerebellar ataxia associated with polyendocrine autoimmunity. Clin Exp Immunol 2002; 129 (3), 471-478.

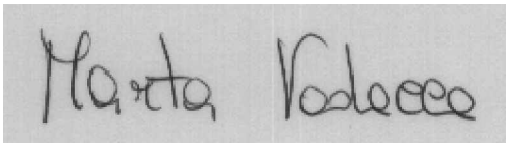
Dai YD, Jensen KP, Lehuen A, Masteller EL, Bluestone JA, Wilson DB, Sercarz EE. A peptide of glutamic acid decarboxylase 65 can recruit and expand a diabetogenic T cell clone, BDC2.5, in the pancreas. J Immunol 2005;175(6):3621-7.

Denkberg G, Cohen CJ, Segal D, Kirkin AF, Reiter Y. Recombinant human single-chain MHC-peptide complexes made from E. coli by in vitro refolding: functional single-chain MHC-peptide complexes and tetramers with tumor associated antigens. Eur J Immunol 2000;30:3522-32.

Disis ML, Knutson KL, Schiffman K, et al. Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. Breast Cancer Res Treat. 2000;62:245-52.

Dretzke J, Cummins C, Sandercock J, Fry-Smith A, Barrett T, Burls A. Autoantibody testing in children with newly diagnosed type 1 diabetes mellitus. Health Technol Assess. 2004 Jun;8(22):iii-xi, 1-183.

Durinovic-Bello I, Boehm BO, Ziegler AG. Predominantly recognized proinsulin T helper cell epitopes in individuals with and without islet cell autoimmunity. J Autoimmun 2002;18(1):55-66.



Endl J, Rosinger S, Schwarz B, Friedrich SO, Rothe G, Karges W, Scholsser M, Eiermann T, Schendel DJ, Boehm BO. Co-expression of CD25 and Ox40 (CD134) receptors delineates autoreative T cells in type 1 diabetes mellitus. Diabetes 2006;55(1):50-60.

Ejrnaes M, Videbaek N, Christen U, Cooke A, Michelsen BK, von Herrath M. Different diabetogenic potential of autoaggressive CD8+ clones associated with IFN-gamma-inducible protein 10 (CXC chemokine ligand 10) production but not cytokine expression, cytolytic activity, or homing characteristics. J Immunol. 2005;174(5):2746-55.

Eisenbarth GS. Prediction of type 1 diabetes: the natural history of the prediabetic period. Adv Exp Med Biol. 2004;552:268-90.

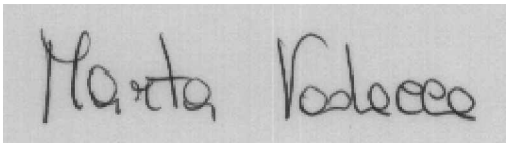
Eisenbarth G, Nepom GT. Class II peptide multimers: promise for type 1 diabetes? Nature Immunology 2002;3:344-5.

Eskola V, Vahasalo P, Akerblom HK, Knip M; Finnish ENDIT Study Group. Increased frequency of islet cell antibodies in unaffected brothers of children with type 1 diabetes. Horm Res. 2003;59(4):195-200.

EURODIAB GROUP. Vitamin D supplement in early childhood and risk for Type I (insulin-dependent) diabetes mellitus. The EURODIAB Substudy 2 Study Group. Diabetologia. 1999 Jan;42(1):51-4.

Filippi C, von Herrath M. How viral infections affect the autoimmune process leading to type 1 diabetes. Cell Immunol. 2005 Feb;233(2):125-32.

Fleckenstein B. Quantitative analysis of peptide MHC class II interactions. Semin Immunol 1999; 11:405-16.



Frisk G. and Tuvemo T. Enterovirus infections with beta-cell tropic strains are frequent in siblings of children diagnosed with type 1 diabetes children and in association with elevated levels of GAD65 antibodies. J. Med. Virol. 2004;73 (3), 450-459.

Galgani A, Petrone A, Spoletini M, Hodge A, Del Buono ML, Locatelli M, Buzzetti R, Diabfin Study Group. HLA class II typing in newborns reveals a low frequency of the DRB1*04 allele and a high frequency of DRB1*11 allele in three regions of Continental Italy. Hum Immunology 2004;65:366-72.

Gillogly ME, Kallinteris NL, Xu M, et al. Ii-key/HER-2/neu MHC class-II antigenic epitope vaccine peptide for breast cancer. Cancer Immunol Immunother 2004;53:39-44.

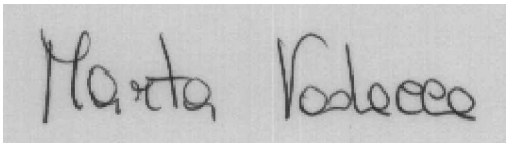
Gebe JA, Masewicz SA, Kochik SA, Reijonen H, Nepom GT. Inhibition of altered peptide ligand-mediated antagonism of human GAD65-responsive CD4+ T cells by non-antagonizable T cells. Eur J Immunol. 2004 Dec;34(12):3337-45.

Gezinat G, Sallusto F. Cytokine driven proliferation of human CD4 cells. J Exp Med 2001; 194;1711-19.

Ghosh P, Amaya M, Mellins E, Wiley DC. The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. Nature 378:457-62, 1995.

Greening JE, Tree,T.I., Kotowicz,K.T., van Halteren,A.G., Roep,B.O., Klein,N.J. and Peakman,M. Processing and presentation of the islet autoantigen GAD by vascular endothelial cells promotes transmigration of autoreactive T-cells. Diabetes 2003;52(3),717-725.

Han G, Li Y, Wang J, Wang R, Chen G, Song L, Xu R, Yu M, Wu X, Qian J, Shen B. Active tolerance induction and prevention of autoimmune diabetes by



immunogene therapy using recombinant adenoassociated virus expressing glutamic acid decarboxylase 65 peptide GAD(500-585). J Immunol. 2005:15;174(8):4516-24**.**

Hanninen A, Jalkanen S, Salmi M, Toikkanen S, Nikolakaros G, Simell O. Macrophages, T cell receptor usage, and endothelial cell activation in the pancreas at the onset of insulin-dependent diabetes mellitus. J Clin Invest. 1992 Nov;90(5):1901-10.

Hawa MI, Beyan H, Buckley LR, Leslie RDG. Impact of genetic and non-genetic factors in type 1 diabetes. Am J Med Gen 2002;115:8 -17.

Helgason T, Danielsen R, Thorsson AV. Incidence and prevalence of type 1 (insulin-dependent) diabetes mellitus in Icelandic children 1970-1989. Diabetologia. 1992 Sep;35(9):880-3.

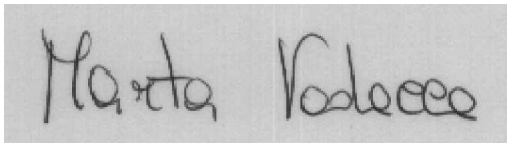
Helms T, Boehm BO, Asaad RJ. Direct visulazation of cytokine producing recall antigen specific CD4 memory T cells. J Immunol 2000; 1641353-61.

Hill NJ, Hultcrantz M, Sarvetnick N, FlodstrÃm-Tullberg M. The target tissue in autoimmunity--an influential niche. Eur J Immunol. 2007 Mar;37(3):589-97.

Holmberg H, Vaarala O, Sadauskaite-Kuehne V, Ilonen J, Padaiga Z, Ludvigsson J. Higher prevalence of autoantibodies to insulin and GAD65 in Swedish compared to Lithuanian children with type 1 diabetes. Diabetes Res Clin Pract. 2006 Jun;72(3):308-14. Epub 2006 Jan 26.

Homann D, M. von Herrath, Regulatory T cells and type 1 diabetes, Clin. Immunol, 2004: 202–209.

Honeyman M. How robust is the evidence for viruses in the induction of type 1 diabetes? Curr Opin Immunol. 2005 Dec;17(6):616-23.



Hoppu S, Harkonen T, Ronkainen MS, Simell S, Hekkala A, Toivonen A, Ilonen J, Simell O, Knip M. IA-2 antibody isotypes and epitope specificity during the prediabetic process in children with HLA-conferred susceptibility to type I diabetes. Clin Exp Immunol. 2006 Apr;144(1):59-66.

Hummel M, Bonifacio E, Schmid S, Walter M, Knopff A, Ziegler AG. Brief communication: early appearance of islet autoantibodies predicts childhood type 1 diabetes in offspring of diabetic parents. Ann Intern Med. 2004 Jun 1;140(11):882-6.

Hummel S, Hummel M, Banholzer J, Hanak D, Mollenhauer U, Bonifacio E, Ziegler AG. Development of autoimmunity to transglutaminase C in children of patients with type 1 diabetes: relationship to islet autoantibodies and infant feeding. Diabetologia. 2007 Feb;50(2):390-4.

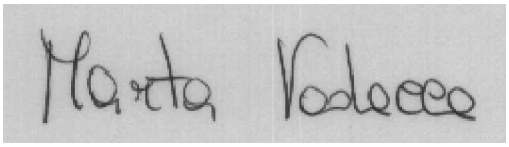
Humphreys RE, Adams S, Ko ldzic G, et al. Increasing the potency of MHC class II-presented peptides by linkage to Ii-peptide. Vaccine 2000;18:2693-97.

Ilonen J, Sjoroos M, Knip M, Venijola R, Simell O, Akerblom HK, et al. Estimation of genetic risk for type 1 diabetes. Am J Med Genet 2002; 115:30-6.

Jaume JC, Parry SL, Madec,A.M., Sonderstrup G, Baekkeskov S. Suppressive effect of glutamic acid decarboxylase 65-specific autoimmune B lymphocytes on processing of T cell determinants located within the antibody epitope. J Immunol, 2002; 169 (2), 665-672.

Johnson JA, Pohar SL, Majumdar SR. Health care use and costs in the decade after identification of type 1 and type2 diabetes: a population-based study. Diabetes Care. 2006 Nov;29(11):2403-8.

Johnson GC, Payne,F., Nutland,S., Stevens,H., Tuomilehto-Wolf,E.,Tuomilehto,J. and Todd,J.A. A comprehensive, statistically powered analysis of GAD2 in type 1 diabetes.Diabetes 2002;51(9),2866-2870.



Jonuleit H, E. Schmitt, The regulatory T cell family: distinct subsets and their interrelations, J. Immunol. 171 (2003) 6323–6327.

Kallinteris NL, Lu X, Wu S, et al. Ii-key/MHC class II epitope hybrid peptide vaccines for HIV. Vaccine 2003;21:4128-32.

Kallinteris NL, S Wu, X Lu, E von Hofe, RE Humphreys, M Xu. Linkage of Ii-Key segment to gp100(46–58) epitope enhances the production of epitope-specific antibodies. Vaccine 2005;23: 2336–8.

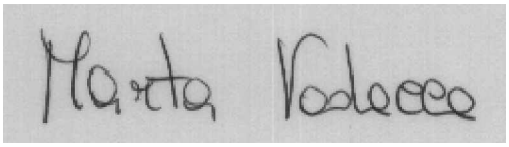
Kallinteris NL, Wu S, Lu X, Humphreys RE, von Hofe E, Xu M. Enhanced CD4+ T-cell response in DR4-transgenic mice to a hybrid peptide linking the Ii-Key segment of the invariant chain to the melanoma gp100(48-58) MHC class II epitope. J Immunother. 2005 Jul-Aug;28(4):352-8.

Kallinteris N, Powell D, Blackwell C, et al. Ii-key/MHC class II epitope peptides as helper T cell vaccines for cancer and infectious disease. Frontiers in Bioscience 2006;11:46-58.

Kallinteris NL, Lu X, von Hofe E, Humphreys RE, Xu M. Ii-Key/MHC class II epitope hybrids: a strategy that enhances MHC class II epitope loading to create more potent peptide vaccines. Expert Opin Biol Ther. 2006 Dec;6(12):1311-21.

Kanaani J, el-Husseini,Ael.-D., Aguilera-Moreno,A., Diacovo,J.M., Bredt DS and Baekkeskov S. A combination of three distinct trafficking signals mediates axonal targeting and presynaptic clustering of GAD65. J. Cell Biol, 2002;158 (7), 1229-1238.

Karlsson Faresjo MGE, Ludvigsson J. Th1 like dominance in high risk first degree relatives of patients with T1D. Diabetologia 2000; 43:742-9.



Karlsson Faresjo MGE, Ludvigsson J. Cytokine profile in children during the first 3 months after the diagnosis of type 1 diabetes. S J Immun 2004; 59:517-526.

Karlsson Faresjo MGE, Ludvigsson J. Diminished Th1-like response to autoantigens in children with a high risk of developing type 1 diabetes. Scan J Immunol 2005; 61:173-9.

Karulin AY, Hesse MD, Tary-Lehmann M, et al. Single-cytokine-producing CD4 memory cells predominate in type 1 and type 2 immunity. J Immunol 2000;15:164:1862-72.

Karvonen M, Viik-Kajander M, Moltchanova E, Libman I, LaPorte R, Tuomilehto J. Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group. Diabetes Care, 2000 Oct;23(10):1516-26.

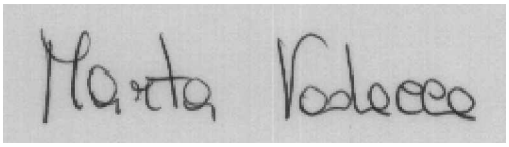
Kent SC, Y. Chen, L. Bregoli, S.M. Clemmings, N.S. Kenyon and C. Ricordi *et al.*, Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope, Nature 435 2005, 224–228.

Knip M. Natural course of preclinical type 1 diabetes. Horm Res. 2002;57 Suppl 1:6-11.

Knip M, Veijola R, Virtanen SM, Hyoty H, Vaarala O, Akerblom HK. Evironmental triggers and determinants of type 1 diabetes. Diabetes. 2005 Dec;54 Suppl 2:S125-6.

Knutson KL, Disis ML. Expansion of HER-2/neu specific T cells following immunization witha peptide based vaccine. Clin Breast Can 2000,2:73-9.

Knutson KL, Disis ML. IL-12 enhances the generation of tumour antigen-specific T cells during ex vivo expansion. Clin Exp Immunol 2004; 135:322-329.



Knutson KL, Schiffman K, Cheever MA, et al. Immunization of cancer patients with a HER-2/neu, HLA-A2 peptide, p369-377, results in short-lived peptide-specific immunity. Clin Cancer Res 2002;8:1014-18.

Kobayashi T, Tanaka,S., Okubo,M., Nakanishi,K., Murase,T. And Lernmark,A. Unique epitopes of glutamic acid decarboxylase autoantibodies in slowly progressive type 1 diabetes. J. Clin. Endocrinol. Metab 2003;88(10),4768-4775.

Kotani R, Nagata M, Moriyama H, et al. Detection of GAD65-reactive T cells in type 1 diabetes by immunoglobulin-free ELISPOT assays. Diabetes Care 2002;25:1390-7.

Kretowski A, Mysliwiec A. In vitro I-13 production by peripheral blood in patients with T1D. Scand J Immunol 2000; 51; 321-5.

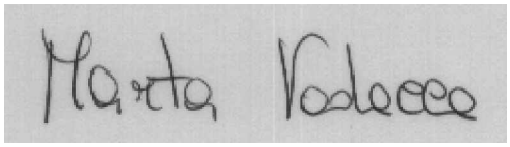
Kropshofer H, Vogt AB, Stern LJ, Hammerling GJ. Self-release of CLIP in peptide loading of HLA-DR molecules. Science. 1995 Nov 24;270(5240):1357-9.

Kropshofer H, Vogt AB, Hammerling GJ. Structural features of the invariant chain fragment CLIP controlling rapid release from HLA-DR molecules and inhibition of peptide binding. Proc Natl Acad Sci U S A 92(18):8313-7, 1995.

Lalvani A, Brookes R. Rapid effector function in CD8 memory cells. J Exp Med 1997; 186:859-865.

Lampeter EF, McCann SR, Kolb H. Transfer of diabetes type 1 by bone-marrow transplantation. Lancet. 1998 Feb 21;351(9102):568-9.

Laron-Kenet T, Shamis I, Weitzman S, Rosen S, Laron ZV. Mortality of patients with childhood onset (0-17 years) Type I diabetes in Israel: a population-based study. Diabetologia. 2001 Oct;44 Suppl 3:B81-6.



Larsson K, Elding-Larsson H, Cederwall E, Kockum K, Neiderud J, Sjoblad S, Lindberg B, Lernmark B, Cilio C, Ivarsson SA, Lernmark A. Genetic and perinatal factors as risk for childhood type 1 diabetes. Diabetes Metab Res Rev. 2004 Nov-Dec;20(6):429-37.

Lazar L, Ofan R, Weintrob N, Avron A, Tamir M, Elias D, Phillip M, Josefsberg Z. Heat-shock protein peptide DiaPep277 treatment in children with newly diagnosed type 1 diabetes: a randomised, double-blind phase II study. Diabetes Metab Res Rev. 2007 May;23(4):286-91.

Lee HJ, Kim KJ, Park MH, Kimm K, Park C, Oh B, Lee JY. Single-nucleotide polymorphisms and haplotype LD analysis of the 29-kb IGF2 region on chromosome 11p15.5 in the Korean population. Hum Hered. 2005;60(2):73-80.

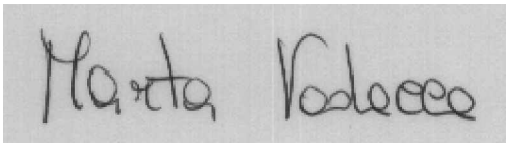
Leslie D, Lipsky P, Notkins AL. Autoantibodies as predictors of disease. J Clin Invest 2001 Nov;108(10):1417-22.

Leslie RD. Preventing type 1 diabetes mellitus: hype and hope. Clin Med. 2004 Sep-Oct;4(5):442-6.

Leslie RD, Williams R, Pozzilli P. Clinical review: Type 1 diabetes and latent autoimmune diabetes in adults: one end of the rainbow. J Clin Endocrinol Metab. 2006 May;91(5):1654-9.

Levy-Marchal C, Patterson CC, Green A; EURODIAB ACE Study Group. Europe and Diabetes. Geographical variation of presentation at diagnosis of type I diabetes in children: the EURODIAB study. European and Dibetes. Diabetologia. 2001 Oct;44 Suppl 3:B75-80.

Lieberman S.M. and T.P. DiLorenzo, A comprehensive guide to antibody and T-cell responses in type 1 diabetes, Tissue Antigens 62; 2003, 359–377.



Linsen L, Somers V, Stinissen P. Immunoregulation of autoimmunity by natural killer T cells. Hum Immunol. 2005 Dec;66(12):1193-202. Epub 2006 Mar 31.

Liu N, Ohnishi N, Ni L, Akira S, Bacon KB. CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. Nat Immunol 2003;4:687–93.

Lo J, Clare-Salzler MJ. Dendritic cell subsets and type I diabetes: focus upon DC-based therapy. Autoimmun Rev. 2006 Jul;5(6):419-23. Epub 2005 Dec 29.

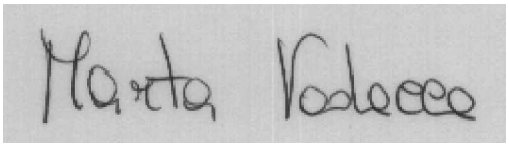
Lohmann T, Leslie RD, Hawa M, Geysen M, Rodda S, Londei M. Immunodominant epitopes of glutamic acid decarboxylase 65 and 67 in insulin-dependent diabetes mellitus. Lancet. 1994 Jun 25;343(8913):1607-8.

Lorini R, Minicucci L, Napoli F, Padovani P, Bazzigaluppi E, Tortoioli C, Cherubini V, Bottazzo G, Pozzilli P, Falorni A, Buzzetti R. Screening for type 1 diabetes genetic risk in newborns of continental Italy. Primary prevention (Prevefin Italy)--preliminary data. Acta Biomed. 2005;76 Suppl 3:31-5.

Luo D, Gilliam,L.K., Greenbaum,C., Bekris,L., Hampe,C.S., Daniels,T., Richter,W., Marcovina,S.M., Rolandsson,O., Landin-Olsson,M., Kockum,I. and Lernmark,A. Conformation-dependent GAD65 autoantibodies in diabetes. Diabetologia 2004;47(9),1581-1591.

Lu S, Reyes VE, Lew RA, Anderson J, Mole J, Humphreys RE, Ciardelli T. Role of recurrent hydrophobic residues in catalysis of helix formation by T cell-presented peptides in the presence of lipid vescicles. J Immunol 1990;145:899-904.

Mayr A, Schlosser M, Grober N, Kenk H, Ziegler AG, Bonifacio E, Achenbach P. GAD autoantibody affinity and epitope specificity identify distinct immunization profiles in children at risk for type 1 diabetes. Diabetes. 2007 Jun;56(6):1527-33.



Mannering SI, Morris JS, Stone NL, Jensen KP, VAN Endert PM, Harrison LC. CD4+ T cell proliferation in response to GAD and proinsulin in healthy, pre-diabetic, and diabetic donors. Ann N Y Acad Sci. 2004 Dec;1037:16-21.

Mannering SI, Morris JS, Stone NL, Jensen KP, Harrison LC. An efficient method for cloning human autoantigen specific T cells. J Immunol Meth 2005; 298:83-92.

Martin S, Wolf-Eichbaum D, Duinkerken G, Scherbaum WA, Kolb H, Noordzij JG, Roep BO. Development of type 1 diabetes despite severe hereditary B-lymphocyte deficiency. N Engl J Med. 2001 Oct 4;345(14):1036-40.

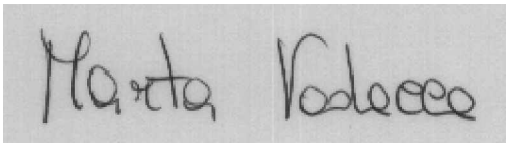
Mayans S, Lackovic K, Nyholm C, Lindgren P, Ruikka K, Eliasson M, Cilio CM, Holmberg D. CT60 genotype does not affect CTLA-4 isoform expression despite association to T1D and AITD in northern Sweden. BMC Med Genet. 2007 Feb 6;8:3.

McHugh RS, M.J. Whitters, C.A. Piccirillo, D.A. Young, E.M. Shevach, M. Collins, M.C. Byrne, CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor, Immunity. 16:2002; 311–323.

Medici F, Hawa MI, Giorgini A, Panelo A, Solfelix CM, Leslie RD, Pozzilli P. Antibodies to GAD65 and a tyrosine phosphatase-like molecole IA-2ic in Filippino type 1 diabetic patients. Diabetes Care 1999;22:1458-61.

Meierhoff G, Ott PA, Lehmann PV, et al. Cytokine detection by ELISPOT: relevance for immunological studies in type 1 diabetes. Diabetes Metab Res Rev 2002;18:367-80.

Miyake S, Yamamura T. NKT cells and autoimmune diseases: unraveling the complexity. Curr Top Microbiol Immunol. 2007;314:251-67.



Milgrom F, Witebsky E. Autoantibodies and autoimmune diseases. JAMA. 1962, 25;181:706-16.

Miller GG, Pollack MS. Insulin-specific human T cells. J Immunol 1987, 139: 3622-29.

Molina V, Shoenfeld Y. Infection, vaccines and other environmental triggers of autoimmunity. Autoimmunity. 2005 May;38(3):235-45.

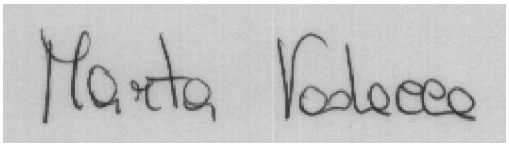
Monetini L, Barone F, Stefanini L, Petrone A, Walk T, Jung G, Thorpe R, Pozzilli P, Cavallo MG. Establishment of T cell lines to bovine beta-casein and beta-casein-derived epitopes in patients with type 1 diabetes. J Endocrinol. 2003 Jan;176(1):143-50.

Nagata M, Kotani R, Moriyama H, Yokono K, Roep BO, Peakman M. Detection of autoreactive T cells in type 1 diabetes using coded autoantigens and an immunoglobulin-free cytokine ELISPOT assay: report from the fourth immunology of diabetes society T cell workshop. Ann N Y Acad Sci 2004;1037:10-5.

Nakayama M, N. Abiru, H. Moriyama, N. Babaya, E. Liu and D. Miao et al., Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice, Nature 435 2005, 220–223.

Neighbors M, Hartley SB, Xu X, Castro AG, Bouley DM, O'Garra A. Breakpoints in immunoregulation required for Th1 cells to induce diabetes. Eur J Immunol. 2006 Sep;36(9):2315-23.

Nepom GT, Lippolis JD, White FM, et al. Identification and modulation of a naturally processed T cell epitope from the diabetes-associated autoantigen human glutamic acid decarboxilase 65 (hGAD65). Proc Natl Acad Sci 2001;98:1763-8.



Nerup J, Andersen OO, Christy M, Platz P, Ryder L, Thomsen M, Svejgaard A. HLA, autoimmunity, virus and the pathogenesis of juvenile diabetes mellitus. Acta Endocrinol Suppl (Copenh). 1976;205:167-75.

Noble JA, Valdes AM, Cook M, Klitz W, Thomson G, Elrich HA. The role of HLA class II genes in insulindependent diabetes mellitus: molecular analysis of 180 Caucasian multiplex families. Am J Hum Genet 1996, 59;11:1134-48.

Novak EJ, Liu AW, Nepom GT, Kwok WW. MHC class II tetramers identify peptide-specific human CD4+T cells proloferating in response to influenza A antigen. J Clin Invest 1999;104:R63-7.

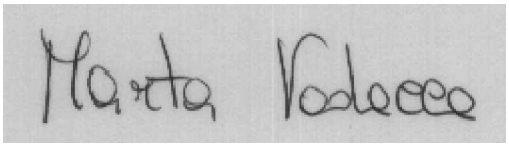
Novak J, Griseri T, Beaudoin L, Lehuen A. Regulation of type 1 diabetes by NKT cells. Int Rev Immunol. 2007 Jan-Apr;26(1-2):49-72.

Oling V, Marttila J, Ilonen J, Kwok WW, Nepom G, Knip M, Simell O, Reijonen H. GAD65- and proinsulin-specific CD4+ T-cells detected by MHC class II tetramers in peripheral blood of type 1 diabetes patients and at-risk subjects. J Autoimmun. 2005 Nov;25(3):235-43.

Ott PA, Dittrich MT, Herzog BA, Guerkov R, Gottlieb PA, Putnam AL, Durinovic-Bello I, Boehm BO, Tary-Lehmann M, Lehmann PV. T cells recognize multiple GAD65 and proinsulin epitopes in human type 1 diabetes, suggesting determinant spreading. J Clin Immunol. 2004 Jul;24(4):327-39.

Otto PA, Herzog BA, Quast S, et al. Islet-cell antigen-reactive T cells show different expansion rates and Th1/Th2 differentiation in type 1 diabetic patients and healthy controls. Clin Immunology 2005;115:102-14.

Ott PA, Anderson MR, Tary-Lehmann M, Lehmann PV. CD4+CD25+ regulatory T cells control the progression from periinsulitis to destructive insulitis in murine autoimmune diabetes. Cell Immunol. 2005 May;235(1):1-11.



Padoa CJ, Crowther NJ, Thomas JW, Hall TR, Bekris LM, Torn C, Landin-Olsson M, Ortqvist E, Palmer JP, Lernmark A, Hampe CS. Epitope analysis of insulin autoantibodies using recombinant Fab. Clin Exp Immunol. 2005 Jun;140(3):564-71.

Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, Paquette TL. Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science. 1983 Dec 23;222(4630):1337-9.

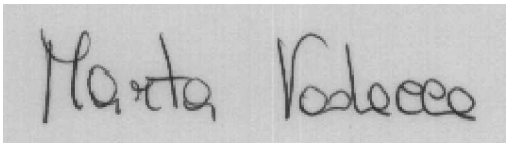
Palmer JP. Immunomodulatory therapy of human type 1 diabetes: lessons from the mouse. J Clin Invest. 2001 Jul;108(1):31-3.

Park Y, Kim S, Park S, Kim T, Yu L, Rewers M, Eisenbarth GS. Differential expression of nonislet autoimmunity: comparison of Korean and U.S. patients with type 1 diabetes. Ann N Y Acad Sci. 2004 Dec;1037:69-73.

Parving HH, Tarnow L, Nielsen FS, Rossing P, Mandrup-Poulsen T, Osterby R, Nerup J. Cyclosporine nephrotoxicity in type 1 diabetic patients. A 7-year follow-up study. Diabetes Care. 1999 Mar;22(3):478-83.

Peakman M, T.I. Tree, J. Endl, P. van Endert, M.A. Atkinson and B.O. Roep, Characterization of preparations of GAD65, proinsulin, and the islet tyrosine phosphatase IA-2 for use in detection of autoreactive T-cells in type 1 diabetes: report of phase II of the Second International Immunology of Diabetes Society Workshop for Standardization of T-cell assays in type 1 diabetes, Diabetes 50;2001, 1749–1754.

Perez SA, Sotiropoulou PA, Sotiriadou NN. et al. HER-2/neu-derived peptide 884-899 is expressed by human breast, colorectal and pancreatic adenocarcinomas an dis recognized by in vitro-induced specific CD4+ T cell clones. Cancer Immmunol Immunother. 2002;50:615-24.



Perone MJ, Bertera S, Tawadrous ZS, Shufesky WJ, Piganelli JD, Baum LG, Trucco M, Morelli AE. Dendritic cells expressing transgenic galectin-1 delay onset of autoimmune diabetes in mice. J Immunol. 2006 Oct 15;177(8):5278-89.

Petrone A, Battelino T, Krzisnik C, Bugawan T, Erlich H, Di Mario U, Pozzilli P, Buzzetti R. Similar incidence of type 1 diabetes in two ethnically different populations (Italy and Slovenia) is sustained by similar HLA susceptible/protective haplotype frequencies. Tissue Antigens. 2002 Sep;60(3):244-53.

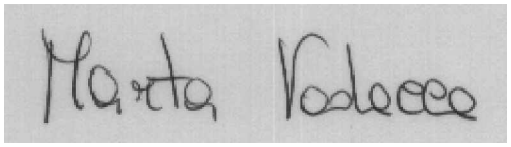
Piccirillo CA, J.J. Letterio, A.M. Thornton, R.S. McHugh, M. Mamura, H. Mizuhara, E.M. Shevach, CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness, J. Exp. Med. 196 (2002) 237–246.

Pietropaolo M, Yu S, Libman IM, Pietropaolo SL, Riley K, LaPorte RE, Drash AL, Mazumdar S, Trucco M, Becker DJ. Cytoplasmic islet cell antibodies remain valuable in defining risk of progression to type 1 diabetes in subjects with other islet autoantibodies. Pediatr Diabetes. 2005 Dec;6(4):184-92.

Pitocco D, Crino A, Di Stasio E, Manfrini S, Guglielmi C, Spera S, Anguissola GB, Visalli N, Suraci C, Matteoli MC, Patera IP, Cavallo MG, Bizzarri C, Pozzilli P; IMDIAB Group. The effects of calcitriol and nicotinamide on residual pancreatic beta-cell function in patients with recent-onset Type 1 diabetes (IMDIAB XI). Diabet Med. 2006 Aug;23(8):920-3.

Pociot F, McDermott MF. Genetics of type 1 diabetes mellitus. Genes Immun. 2002 Aug;3(5):235-49.

Pozzilli P, Manfrini S, Crino A, Picardi A, Leomanni C, Cherubini V, Valente L, Khazrai M, Visalli N; IMDIAB group. Low levels of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 in patients with newly diagnosed type 1 diabetes. Horm Metab Res. 2005 Nov;37(11):680-3.



Pozzilli P, Manfrini S, Picardi A. Cow's milk and trials for prevention of Type 1 diabetes. Diabet Med. 2003 Nov;20(11):871-2.

Pozzilli P. Immuno-intervention and preservation of beta-cell function in type 1 diabetes. Diabetes Metab Res Rev. 2007 May;23(4):255-6.

Ranheim EA, Tarbell KV, Krogsgaard M, Mallet-Designe V, Teyton L, McDevitt HO, Weissman IL. Selection of aberrant class II restricted CD8+ T cells in NOD mice expressing a glutamic acid decarboxylase (GAD)65-specific T cell receptor transgene. Autoimmunity 2004;37(8):555-67.

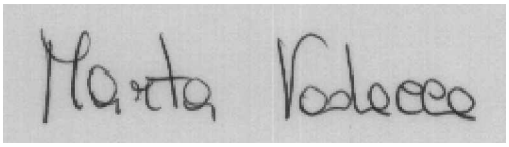
Read S, V. Malmstrom, F. Powrie, Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inXammation, J. Exp. Med. 192 (2000) 295–302.

Redondo MJ, Yu L, Hawa M, Mackenzie T, Pyke DA, Eisenbarth GS, Leslie RD. Heterogeneity of type I diabetes: analysis of monozygotic twins in Great Britain and the United States. Diabetologia. 2001 Mar;44(3):354-62.

Reichstetter S, R.A. Ettinger, A.W. Liu, J.A. Gebe, G.T. Nepom and W.W. Kwok, Distinct T cell interactions with HLA class II tetramers characterize a spectrum of TCR affinities in the human antigen-specific T cell response, J Immunol ,165;2000, 6994–6998.

Reijonen H, EJ Novak, S. Kochik, A. Heninger, A.W. Liu and W.W. Kwok *et al.*, Detection of GAD65-specific T-cells by major histocompatibility complex class II tetramers in type 1 diabetic patients and at-risk subjects, Diabetes 51;2002, 1375–1382.

Reijonen H, Mallone,R., Heninger,A.K., Laughlin,E.M., Kochik,S.A., Falk,B., Kwok,W.W., Greenbaum,C. and Nepom GT. GAD65-specific CD4+ T-cells with



high antigen avidity are prevalent in peripheral blood of patients with type 1 diabetes. Diabetes 2004;53(8),1987-1994.

Reijonen H, Kwok WW, Nepom GT. Detection of CD4+ autoreactive T cells in T1D using HLA class II tetramers. Ann N Y Acad Sci 2003;1005:82-7.

Rinaldi L, Gallo P, Calabrese M, Ranzato F, Luise D, Colavito D, Motta M, Guglielmo A, Del Giudice E, Romualdi C, Ragazzi E, D'Arrigo A, Dalle Carbonare M, Leontino B, Leon A. Longitudinal analysis of immune cell phenotypes in early stage multiple sclerosis: distinctive patterns characterize MRI-active patients. Brain. 2006 Aug;129(Pt 8):1993-2007.

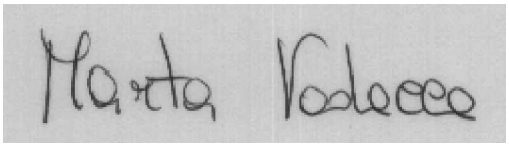
Richter W, Shi Y, Baekkeskov S. Autoreactive epitopes defined by diabetes-associated human monoclonal antibodies are localized in the middle and C-terminal domains of the smaller form of glutamate decarboxylase. Proc Natl Acad Sci U S A. 1993 Apr 1;90(7):2832-6.

Rodacki M, Svoren B, Butty V, Besse W, Laffel L, Benoist C, Mathis D. Altered natural killer cells in type 1 diabetic patients. Diabetes. 2007 Jan;56(1):177-85.

Roep BO, Atkinson MA. Autoreactive T cell responses in insulin-dependent diabetes. Report of the first international workshop. J Autoimmun 1999; 13:267-282.

Roep BO, Hiemstra,H.S., Schloot,N.C., De Vries,R.R., Chaudhuri,A., Behan,P.O. and Drijfhout,J.W. Molecular mimicry in type 1 diabetes: immune cross-reactivity between islet autoantigen and human cytomegalovirus but not Coxsackie virus. Ann N Y Acad Sci, 2002; 958, 163-165.

Ronkainen MS, Hoppu S, Korhonen S, Simell S, Veijola R, Ilonen J, Simell O, Knip M. Early epitope- and isotype-specific humoral immune responses to GAD65 in young children with genetic susceptibility to type 1 diabetes. Eur J Endocrinol. 2006 Oct;155(4):633-42.



Rose NR, Bona C. Defining criteria for autoimmune diseases (Witebsky's postulates revisited). Immunol Today. 1993;14(9):426-30.

Rosenthal KS, Mao H, Horne WI, Wright C, Zimmerman D. Immunization with a LEAPS heteroconjugate containing a CTL epitope and a peptide from beta-2-microglobulin elicits a protective and DTH response to herpes simplex virus type 1. Vaccine 1999;17:535±42.

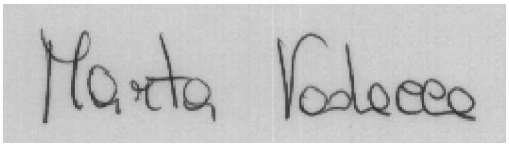
Saeki K, Zhu M, Kubosaki A, Xie J, Lan MS, Notkins AL.Targeted disruption of the protein tyrosine phosphatase-like molecule IA-2 results in alterations in glucose tolerance tests and insulin secretion. Diabetes. 2002 Jun;51(6):1842-50.

Sakaguchi S, N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, T. Takahashi, Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance, Immunol. Rev. 182, 2001; 18–32.

Salomon B, D.J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, J.A. Bluestone, B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes, Immunity 12, 2000; 431–440.

Sanjeevi CB, Lybrand TP, Stevanovic S, Rammensee HG. Molecular modeling of eluted peptides from DQ6 molecules (DQB1*0602 and DQB1*0604) negatively and positively associated with type 1 diabetes. Ann N Y Acad Sci. 2002 Apr;958:317-20.

Schloot NC. Comparison of cytokine ELISPOT assay formats for the detection of islet antigen autoreactive T cells. Report of the Third Immunology of Diabetes Society T-cell Workshop. J Autoimmun 2003;21:365-76.



Schloot NC, Guido Meierhoff, Csaba Lengyel, Gyozo Vandorfi, Jozsef Takacs, Pal Panczel, Laszlo Barkai, Laszlo Madacsy, Tamas Oroszlan, Peter Kovacs, Gabor Suto, Tadej Battelino, Nora Hosszufalusi, Gyorgy Jermendy. Effect of heat shock protein peptide DiaPep277 on beta-cell function in paediatric and adult patients with recent-onset diabetes mellitus type 1: two prospective, randomized, double-blind phase II trials Diabetes/Metabolism Research and Reviews 2006.
Diabetes Metab Res Rev. 2007 Feb 22

Schlosser M, Banga,J.P., Madec,A.M., Binder,K.A., Strebelow,M., Rjasanowski,I., Wassmuth,R., Gilliam,L.K., Luo,D. and Hampe,C.S. Dynamic changes of GAD65 autoantibody epitope specificities in individuals at risk of developing type 1 diabetes. Diabetologia 2005;48(5),922-930.

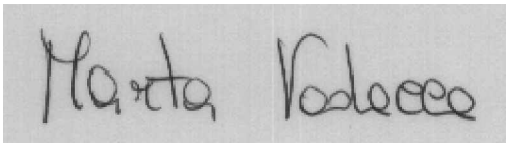
Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers, Nat. Rev. Immunol. 2 (2002) 389–400.

Shimizu J, S. Yamazaki, T. Takahashi, Y. Ishida, S. Sakaguchi, Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance, Nat. Immunol. 3;2002: 135–142.

Schlosser M, Banga JP, Madec AM, Binder KA, Strebelow M, Rjasanowski I, Wassmuth R, Gilliam LK, Luo D, Hampe CS. Dynamic changes of GAD65 autoantibody epitope specificities in individuals at risk of developing type 1 diabetes. Diabetologia 2005;48:922-30.

Skyler JS. Prediction and prevention of type 1 diabetes: progress, problems, and prospects. Clin Pharmacol Ther. 2007 May;81(5):768-71.

Skinner PJ, Daniels MA, Schmidt CS, Jameson SC, Haase AT. Cutting edge: in situ tetramer staining of antigen-specific T cells in tissues. J Immunol 2000;165:613-7.



Sotiriadou R, Perez SA, Gritzapis AD, et al. Peptide HER2(776-788) represents a naturally processed broad MHC class II-restricted T cell epitope. Br J Cancer 2001; 85: 1527-3.

Sotiriadou NN, Kallinteris NL, Gritzapis AD, Voutsas IF, Papamichail M, von Hofe E, Humphreys RE, Pavlis T, Perez SA, Baxevanis CN. Ii-Key/HER-2/neu(776-790) hybrid peptides induce more effective immunological responses over the native peptide in lymphocyte cultures from patients with HER-2/neu+ tumors. Cancer Immunol Immunother. 2007 May;56(5):601-13. Epub 2006 Sep 8.

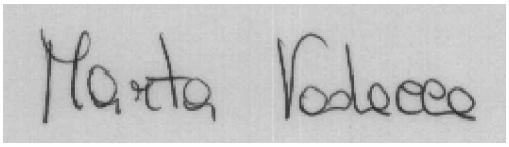
Stern LJ, Brown JH, Jardetzky TS, Gorga JC, Urban RG, Strominger JL, Wiley DC. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. Nature 368:215-21, 1994.

Tang Q, K.J. Henriksen, M. Bi, E.B. Finger, G. Szot, J. Ye, E.L. Masteller, H. McDevitt, M. Bonyhadi, J.A. Bluestone, In vitroexpanded antigen-speciWc regulatory T cells suppress autoimmune diabetes. J. Exp. Med. 199; 2004: 1455–1465.

Tong JC, Myers,M.A., Mackay,I.R., Zimmet,P.Z. and Rowley,M.J. The PEVKEK region of the pyridoxal phosphate binding domain of GAD65 expresses a dominant B cell epitope for type 1 diabetes sera. Ann. N. Y. Acad. Sci. 2002;958,182-189.

Traggiai C, Stanhope R, Nussey S, Leiper AD. Diabetes mellitus after bone marrow transplantation during childhood. Med Pediatr Oncol. 2003 Feb;40(2):128-9.

Trudeau JD, C. Kelly-Smith, C.B. Verchere, J.F. Elliott, J.P. Dutz and D.T. Finegood et al., Prediction of spontaneous autoimmune diabetes in NOD mice by quantification of autoreactive T cells in peripheral blood, J Clin Invest; 111**,** 2003, 217–223.



Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature. 2003 May 29;423(6939):506-11.

Viglietta V, Kent S, Orban T, et al. GAD65 reactive T cells are activated in patients with autoimmune type 1 diabetes. J Clin Invest 2002; 109:895-903.

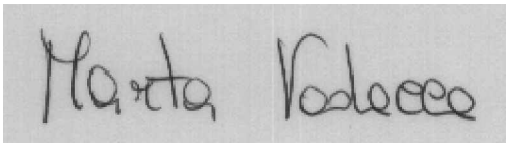
Viskari H, Kondrashova A, Koskela P, Knip M, Hyoty H. Circulating vitamin D concentrations in two neighboring populations with markedly different incidence of type 1 diabetes. Diabetes Care. 2006 Jun;29(6):1458-9.

Vandewalle CL, Decraene T, Schuit FC, De Leeuw IH, pipeleers DG, Gorus FK. Insulin autoantibodies and high titre islet cell antibodies are preferentially associated with the HLA DQA1*0301-DQB1*0302 haplotype at clinical type 1 (insulin-dependent) diabetes mellitus before age 10 years, but not at onset between 10 and 40 years. The Belgian Diabetes Registry. Diabetologia 1993, 36:1555-62.

Voutsas IF, Gritzapis AD, Mahaira LG, Salagianni M, Hofe EV, Kallinteris NL, Baxevanis CN. Induction of potent CD4(+) T cell-mediated antitumor responses by a helper HER-2/neu peptide linked to the Ii-Key moiety of the invariant chain. Int J Cancer. 2007 Jul 16.

Wambre E, Van Overtvelt L, Maillere B, Humphreys R, von Hofe E, Ferhat L, Ebo D, Moingeon P. Single cell assessment of allergen specific T cell responses with MHC class II peptide tetramers: methodological aspects. Int Arch Allergy Immunol 2008:14699-112.

Wang J, Miao D, Babu S, Yu J, Barker J, Klingensmith G, Rewers M, Eisenbarth GS, Yu L. Prevalence of autoantibody-negative diabetes is not rare at all ages and increases with older age and obesity. J Clin Endocrinol Metab. 2007 Jan;92(1):88-92. Epub 2006 Oct 24.



Wei J, Jin Y, Wu,H., Sha,D. and Wu,J.Y. Identification and functional analysis of truncated human glutamic acid decarboxylase 65. J Biomed Sci 2003, 10 (6 PT 1), 617-624.

Wei J and Wu J.Y. Structural and functional analysis of cysteine residues in human glutamate decarboxylase 65 (GAD65) and GAD67. J Neurochem, 2005 93 (3), 624-633.

Westerlund A, Ankelo M, Ilonen J, Knip M, Simell O, Hinkkanen AE. Absence of avidity maturation of autoantibodies to the protein tyrosine phosphatase-like IA-2 molecule and glutamic acid decarboxylase (GAD65) during progression to type 1 diabetes. J Autoimmun. 2005 Mar;24(2):153-67.

Xu M, Capraro GA, Daibata M, Reyes VE, Humphreys RE. Cathepsin B cleavage and release of invariant chain from MHC class II molecules follow a staged pattern Mol Immunol 1994;31:723-31.

Xu M, Jackson R, Adams S, Humphreys RE. Studies on activities of invariant chain peptides on releasing or exchanging of antigenic peptides at human leukocyte antigen-DR1. Arzneimittelforschung 49(9):791-9, 1999.

Xu M, Li J, Gulfo JV, et al. MHC class II allosteric site drugs: new immunotherapeutics for malignant, infectious and autoimmune diseases. Scand J Immunol 2001;54:39-44.

Yoon JW, Jun HS. Viruses cause type 1 diabetes in animals. Ann N Y Acad Sci. 2006 Oct;1079:138-46.

You S, Chen C, Lee WH, Brusko T, Atkinson M, Liu CP. Presence of diabetes-inhibiting, glutamic acid decarboxylase-specific, IL-10-dependent, regulatory T cells in naive nonobese diabetic mice. J Immunol. 2004;173(11):6777-85.